Stochastic Modelling of Tumorigenesis in p53 Deficient Transgenic Mice

Jian-Hua Mao

Department of Radiation Oncology, CRC Beatson Laboratories, Glasgow

Thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy.

January 1997.

To Mum and Dad

Contents

List of Tables

List of Figures

Acknowledgements

I wish to express my deep thanks to my supervisors, Dr Tom Wheldon and Professor Alan Balmain, for their guidance and helpful comments through my research. They have not only given me invaluable technical supervision, but also have been so patient in reading my thesis draft and other papers several times to correct the mistakes, and this has helped me a great deal in improving my English. Many of the mistakes (both technical and linguistic) and vagueness in the earlier drafts of this thesis have been eliminated due to their meticulous and critical reading. I am very grateful to them for all their help.

I also wish to thank Sheila Bryson for teaching me the experimental methods (such as x-gal staining) and supporting me in animal experiments. Many other people in both Department of Radiation Oncology and Department of Medical Oncology have also helped in many ways, for which I am indeed grateful.

Special thanks go to Dr Kenneth A. Lindsay (Department of Mathematics) for giving a lot of helpful comments during my study, and also to Keith Vass and Peter McHardy for their help to solve my computer problems.

This work was supported by a University Scholarship and an ORS award. I am grateful to both funding bodies for giving me this opportunity.

Abstract

The aim of this project was to develop the stochastic models of tumorigenesis to investigate the implications of experimental data on tumour induction in wild type and p53 deficient mice for tumorigenesis mechanisms. These studies have focused on the development of stochastic process models for p53 mediated spontaneous and radiation-induced tumorigenesis in mice, in which up to 3 stages are assumed to be required for malignant transformation. The stages are conceived as the inactivation of one and both p53 alleles with a third, genetically unspecified stage which may be composite. The model has been used to explore the influence of mutation rate, stage number, and the number of stem cells at risk on the kinetics of spontaneous appearance of tumours and tumour multiplicity. As expected, tumours tended to occur earlier and the more tumours per mouse tend to be acquired with lesser stage number, higher mutation rate and higher stem cell number. However, a striking observation was that each of these dependencies was more pronounced at lower than higher mutation rate. At high mutation rates, the rate of growth of the earliest transformed cell exerted a dominant influence on tumour latency independently of the other variables. With the incorporation of radiation effects, i.e. cell killing and mutation induction into these models, the studies showed that the dose-tumour-latency and dose-tumour-multiplicity relationships strongly depend on the spontaneous mutation rate, stage number, and age at exposure. The stage number is most influential at low spontaneous mutation rate. The optimal time (age) of irradiation for maximum yield of tumours is around the time of birth. The exploration of p53 mediated spontaneous and radiation-induced tumorigenesis using these models revealed a fundamental problem in that all such models predicted excessively large numbers of tumours in both p53 deficient genotypes. Analysis showed that this prediction applied for up to five stages being required for

malignant transformation in wild type mice. These observations have allowed this category of simple multistage model to be rejected.

However, we have found that it is possible to accommodate the experimental data in p53 deficient mice on a multipath/multistage model in which a p53 mediated pathway coexists with one or more p53 independent pathways. The analysis favours at least a 5-stage model for the p53 mediated pathways with the p53 independent pathway being the majority route of tumorigenesis in wild type mice but the minority route in p53 deficient genotypes. However, this model requires the assumption of one or more unknown pathways and does not enable age-specific tumour incidence curves to be computed. An alternative version is a 'multi-gate' model in which tumorigenesis results from a small number of gate-pass (enabling) events independently of p53 status. The role of p53 inactivation is as a rate modifier which accelerates the gate-pass events. Age-incidence curves can be computed and are consistent with experimental observations, for as few as 2 or 3 gate-pass events. The model more easily accommodates the data if the p53 second allele experiences a higher spontaneous mutation rate than the gate-pass genes. This model implies that wild type p53 acts to maintain genetic stability, in accordance with the 'guardian of the genome' concept, and that p53 inactivation amplifies the rate of tumorigenic mutation by a factor which we estimate to be about ten-fold. Experimental studies on genetic stability, and on the proportions of tumours in different genotypes whose development is p53 mediated, should help to decide between these models.

Competitive development of tumours in two mouse tissues showing different growth patterns was studied using both the multistage and multigate/multistage model. Significant difference in tumour spectra in $p53^{+/-}$ and $p53^{-/-}$ mice were found (eg different proportion of lymphomas or sarcomas). This finding is in accord with Donehower's hypothesis on the

existence of 'windows of opportunity' for tumour development dependent on stem cell kinetics and stages required for tumorigenesis.

In future, the multigate/multistage model will be extended to simulate the effects of irradiation. It is hoped that some of the methods developed here will be applicable to tumour development in other mouse models, and ultimately to human cancer.

Chapter 1

The Biology of Cancer

1.1 Introduction

Despite considerable progress, cancer is still a major cause of human mortality. In developed countries, cancer is the second most common cause of death (after cardiovascular diseases) and its importance is rapidly growing in developing countries. Although there many different types of cancer (more than 100), they share a common fundamental basis: abnormal growth and division of cells, which eventually spread through the body, invading and interfering with the function of normal tissues and organs. Cancer is thus fundamentally a disease at the cellular level, in which the cancer cell fails to respond to the controls that regulate normal cell growth and division. Such loss of growth control usually requires the accumulation of damage to several different cellular regulatory mechanisms.

The interaction of positive and negative signals to maintain cellular homeostasis is compatible with the basic yin-yang principle for the universe. In cancer, where homeostasis is impaired, mutations in genes responsible for sending or receiving the positive or negative signals accumulate and eventually lead to the disruption of homeostasis in growth regulation. An unknown number of mutations or pathways are needed for accomplishing this disruption; the number can vary depending on cell of origin and cancer type.

Moreover, cancers usually arise from the transformation of a single precursor cell, which proliferates to form a clone, i.e., tumours are most often monoclonal in origin. Despite their common origin, the cell populations in most tumours are quite heterogeneous with respect to their properties, suggesting that cancer cells continually modify their properties during the growth of the tumour.

1.2 Multistep nature of tumorigenesis

A body of evidence exists to show that tumorigenesis is a multistage process (Table 1.1), which involves sequences of discrete genetic or epigenetic events probably differing from one tumour type to another (Barrett, 1987B and 1993; Farber, 1984; Fearon and Vogelstein, 1990; Vogelstein and Kinzler, 1993; Weinberg, 1989). Elucidation of these processes in terms of number and order of stages, and ultimately the identification of obligatory events in molecular terms, is a major goal of basic cancer research.

Table 1.1 Evidence for multistage models of carcinogenesis

• Mathematical models based on age-specific tumour incidence curves are consistent with a multistage model of neoplastic development involving 1 to 10 independent alterations (see section 1.2.1)
• Chemical carcinogenesis studies in mouse skin and other tissues reveal that carcinogenesis involves stages of initiation, promotion, and progression (see section 1.2.2)
• Cell culture studies with chemical carcinogen-induced neoplastic transformation indicate that multiple changes must occur in the transformation of a normal cell to a tumour cell (see section 1.2.3)
• Inherited cancer (such as familial retinoblastoma, Wilms' tumour) requires a second, somatic mutation (see section 1.3.4)
• Tumorigenesis in transgenic and knock-out mice is consistent with a multistage process (see section 1.3.5)

1.2.1 Age—incidence relationship

Cancer can occur at all ages, but in most tumour types it becomes much more common with advancing age excluding the distinctive group of childhood tumours (Figure 1.1). The increasing incidence of cancer with age reflects a fundamental feature of the biology of

cancer cells. The conversion of a normal cell into a cancer cell does not occur as a single one-step event, because if a single mutation were responsible, occurring with a fixed probability per year, the chance of developing cancer in any given year should be independent of age. Rather, development of cancer involves a series of progressive changes that gradually convert a normal cell into a cancer cell. Usually, many years are required to accumulate the multiple abnormalities needed to generate most cancer cells, so the majority of cancers develop late in life.

For most types of cancer the incidence goes up very steeply with age, typically as the third, fourth, or fifth power. From such statistics it has been estimated that somewhere between three and seven independent events, each of low probability, are typically required to change a normal cell into a cancer cell (Renan, 1993).

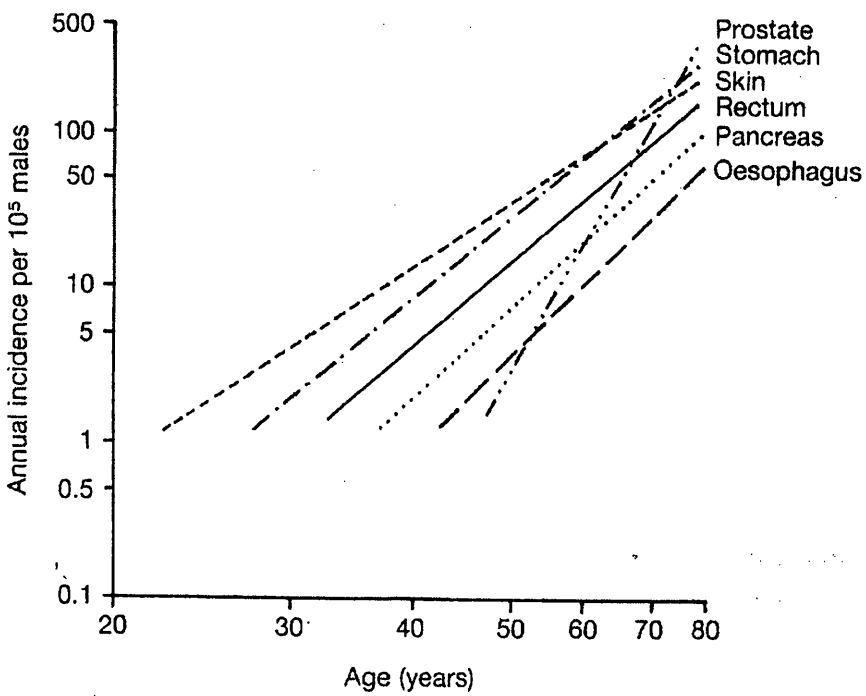


Figure 1.1 The age-incidence relationship (Vogelstein and Kinzler, 1993).

1.2.2 Animal experiments: initiation, promotion and progression

Animal experiments (mouse or rat) involving different tumour sites, such as skin, liver, breast and bladder, have identified and characterized three stages of the tumorigenic process: initiation, promotion and progression.

The studies of experimental tumorigenesis in skin, first started in the 1940's (Rous and Kidd, 1941; Berenblum, 1941; Friedewald and Rous, 1944), have led to the paradigm of "two-stage carcinogenesis", initiation and promotion. The multistage nature of the tumorigenic process was investigated further by Boutwell (1964) who proposed the concept that tumour promotion involves two stages. In this model, there are at least three stages in the tumorigenic process, i.e., initiation and two promotional stages. Consistent with the multistage theory, multiple types of lesions were identified by Burns et al (1976) during the course of mouse skin initiation-promotion studies.

Apart from skin, other animal organs in which reproducible initiation-promotion systems have been developed are the rat liver (Peraino et al., 1971; Farber, 1987; Farber and Sarma, 1987), and the urinary bladder (Fukushima et al, 1983). An extensive review of the initiation-promotion literature prior to the nineteen eighties has been given by Farber and Cameron (1980) and by Farber (1984).

It is important to recognize that the later stages of neoplastic development involve distinctively different stages of promotion and progression. As reported by Hennings et al (1983), papillomas might be further induced to form carcinomas by initiators but not by promoters. By using the induction of hepatocelluar carcinoma (rat liver cancer) as a model, Farber (1984, 1987) and Farber and Sarma (1987) have demonstrated that each of three

phases (initiation, promotion and progression) may involve many steps.

1.2.3 Transformation *in vitro*

Studies using cell culture models of carcinogenesis have clearly demonstrated the progressive, multistep nature of neoplastic transformation. Berward and Sachs (1963 and 1965) were first to demonstrate the induction by chemical carcinogens of neoplastic transformation of mammalian cells in culture. Following exposure to 3-methylcholanthrene or benzo(a)pyrene (BaP), Syrian hamster embryo fibroblasts (SHE) were observed to escape 'cellular senescence', the process which limits the proliferation of normal cells in culture to a certain number of cell divisions. The carcinogen-treated cells had an indefinite life span in culture, which has since been termed immortalization (Newbold et al, 1982), and were morphologically transformed. The morphologically altered cell lines ultimately produced malignant fibrosarcomas when injected into syngeneic hamsters (Berward and Sachs, 1963), whereas untreated SHE cells were nontumorigenic. Tumours can be produced by injections of less than 10 transformed cells while 10^7 to 10^9 normal SHE cells are nontumorigenic even after several passages in culture (Huberman and Sachs, 1966; Benedict et al, 1975; Barrett et al, 1979).

Morphological transformation is the earliest phenotypic marker that can be detected in SHE cells following carcinogen treatment (Barrett and Ts'O, 1978). Other phenotypic changes occur following carcinogen exposure with a different temporal sequence. Cells with an enhanced fibrinolytic activity are observed at 2 or 3 weeks, whereas cells with the ability to grow when suspended in a semisolid medium (i.e., soft agar) or able to form tumours when injected into syngeneic animals are not observed until 6 to 15 weeks after carcinogen treatment (Barrett and Ts'O, 1978). In this cellular system the ability to grow in agar

correlates very well with tumorigenicity for chemical carcinogen-induced transformation (Barrett et al, 1979). The inability to detect cells which grow in agar or as tumors in animals is not due to insensitivity of the assays to detect cells with these phenotypes. Rather, cells expressing the early phenotypic changes are preneoplastic and require additional changes to become tumorigenic. This hypothesis is supported by observations of a number of laboratories working with this system (Berward and Sachs, 1963, 1965; Borek and Sachs, 1966; DiPaolo and Donovan, 1967). Kuroki and Sato (1968) extensively studied the neoplastic transformation *in vitro* of SHE cells by 4-nitroquinoline-1-oxide (4-NQO). The earliest time at which cells capable of producing progressively growing tumours were obtained was 49 days after initial carcinogen treatment. In contrast, early changes in morphology were seen 3 to 4 days after treatment, and histological evidence of transformation was observed as early as 23 days after treatment. Furthermore, Kuroki and Sato identified three different stages in the neoplastic development of the transformed cultures, which they cite as evidence for progression *in vitro*. A diagram of the most common pathway for neoplastic transformation is shown in Figure 1.2.

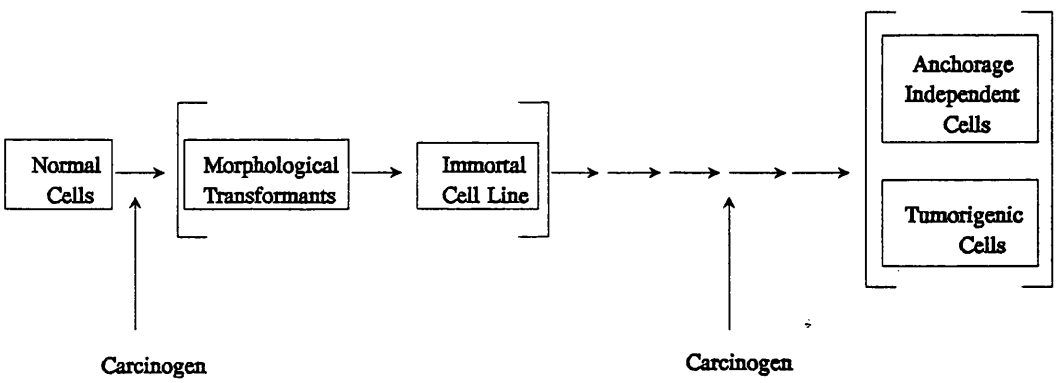


Figure 1.2 Neoplastic progression of Syrian hamster embryo cells (Barrett and Fletcher, 1987)

Similar stages of neoplastic progression of other cell types in culture has also been described. For example, a number of stages in the transformation of guinea pig cells has been identified (Evans and DiPaolo, 1975 and 1982).

1.3 Genes and cancer

Neoplasms arise and progress through the accumulation of various genetic changes (Barrett, 1987A; Bishop, 1987, 1991 and 1995; Hall, 1993). These changes involve alterations of specific "cancer genes", generally divided into two major categories. One category is that of proto-oncogenes which, when activated by mutation, translocation or amplification, drives the process. The second broad category includes tumour suppressor genes whose function is lost (usually by inactivation) during tumorigenesis (Bishop, 1991). Thus, the continued acquisition of clonal alterations in proto-oncogenes and tumour suppressor genes drives the development of cancers.

1.3.1 Genetic alterations in tumour development

Cancer can be considered a genetic disorder of somatic cells (Cohen and Ellmein, 1991; Harris, 1986; Knudson, 1973, 1986 and 1995; Weinberg, 1988). Strong evidence from several areas of investigation validate this statement:

(1) chromosomal analysis reveals that cancer cells usually have abnormal karyotypes;

(2) some inherited syndromes are associated with an increased risk of cancer and for others, cancer itself occurs as an inherited trait (see section 1.3.4);

(3) cells can become malignant as a result of exposure to a variety of agents that damage

DNA;

(4) some types of viruses can induce tumours (Bishop, 1987; Stoler, 1991; Varmus, 1984 and 1988).

Each of the steps in tumorigenesis is thought to be the result of a genetic alteration, i.e., mutation, leading to increased cell growth. In this context, mutations are broadly defined to include any change in the genome (Table 1.2).

Table 1.2 Types of genetic alterations in tumorigenesis

• Gene mutations (base substitution, frameshift mutations, deletions and insertions)
• Gene duplication or amplification (increased number of copies of a gene)
• Chromosome aberrations (translocations, inversions, and deletions)
• Aneuploidy (abnormal numbers of chromosomes)

1.3.2 Proto-oncogenes

Proto-oncogenes are normal cellular genes that are responsible for positive growth signals (Cooper, 1990; Druker et al, 1989; Hesketh, 1994 and 1995). Activation of proto-oncogenes as oncogenes causes dysregulation of growth and differentiation pathways and enhances the probability of neoplastic transformation (Buckley, 1988; Cooper, 1990; Miller and Dmitrovsky, 1991).

The critical feature of proto-oncogenes is that they act in a dominant fashion, which means

that the presence of a single copy of the gene in the cell is sufficient to produce the transformed phenotype, even in the presence of normal copies of the same proto-oncogene (see Table 1.3; Cooper, 1982).

Table 1.3 Properties of proto-oncogenes and tumour suppressor genes

Property	Proto-oncogenes	tumour suppressor gene
1. Number of mutational events required to contribute to the cancer	one	two
2. Function of the mutant allele	gain of function, acts in a dominant fashion	loss of function, acts in a recessive fashion
3. Mutant allele may be inherited through the germ line	no examples at this time	frequently has an inherited form
4. Somatic mutation contributes to cancer	yes	yes
5. Tissue specificity of mutational event	some, but can act in many tissues	the inherited form commonly has a tissue preference

To date about 70-80 oncogenes have been identified (Hesketh, 1994 and 1995), but very few are known to be important to human tumorigenesis (Table 1.4). Members of the *ras* family are found most frequently: H-*ras*, K-*ras*, and N-*ras*. Activated *ras* oncogenes have been identified in most forms of human cancer (Bos, 1988 and 1989; Kiaris and Spandidos, 1995).

There are three principal mechanisms by which proto-oncogenes can be activated to produce a malignant cell: point mutation, chromosomal rearrangement or translocation, and gene amplification (Table 1.4; Cooper, 1990; Hesketh, 1994 and 1995). For example, a point mutation in N-*ras* is found in the cancer cells of most patients suffering from acute leukaemia (Bos, 1988). A translocation between chromosomes 2 and 8 is responsible for *myc* activation in Burkitt's lymphoma (Dalla-Favera et al, 1983). Gene amplification of N-*myc* is characteristic of many neuroblastoma (Brodeur et al, 1984).

Table 1.4 Oncogenes and human cancer

Oncogene	Type of cancer	Activation Mechanism
abl	Chronic myelogenous leukaemia, acute lymphocytic lecukaemia	Translocation
bcl-2	Follicular B-cell lymphoma	Translocation
E2A	Acute lymphocytic lecukaemia	Translocation
erbB-2	Breast and ovarian carcinoma	Amplification
gip	Adrenal cortical and ovarian carcinomas	Point mutation
gsp	Pituitary tumours	Point mutation
c-myc	Burkitt's and other B-cell lymphoma	Translocation
	Breast and lung carcinomas	Amplification
L-myc	Lung carcinoma	Amplification
N-myc	Neuroblastoma, lung carcinoma, rhabdomyosarcomas	Amplification
RAR	Acute promyelocytic leukaemia	Translocation
H-ras	Thyroid carcinoma	Point mutation
K-ras	Colon, lung, pancreatic, and thyroid carcinoma	Point mutation
N-ras	Acute myelocytic and lymphocytic leukaemia, thyroid carcinoma	Point mutation
ret	Thyroid carcinoma	DNA rearrangement
trk	Thyroid carcinoma	DNA rearrangement

1.3.3 Tumour suppressor genes

In contrast to proto-oncogenes, tumour suppressor genes are normal cellular genes that are responsible for negative growth signals (Hesketh, 1994 and 1995; Hinds and Weinberg, 1994; Levine, 1990 and 1993; Sager, 1989; Weinberg, 1991). Inactivation of tumour suppressor genes causes dysregulation of growth and differentiation pathways and enhances the probability of neoplastic transformation.

Unlike proto-oncogenes which act in a dominant fashion, tumour suppressor genes are recessive genes so that only homozygotes for the gene give rise to the cancer phenotype (see Table 1.3). In experimental systems, the introduction of functional tumour suppressor genes from normal cells into cancer cells results in loss of tumorigenicity (Harris, 1971; Stanbridge, 1976).

Rb gene was the first tumour suppressor gene to be isolated and studied at the molecular level, but the identification and characterization of additional tumour suppressor genes has rapidly followed these initial studies. To date, more than twenty distinct tumour suppressor genes have been isolated by molecular cloning (Table 1.5; Hesketh, 1995).

The type of mutations in tumour suppressor genes are most frequently point mutation (nonsense and missense mutation), deletions, insertions that produce either an absent or truncated protein product, and loss of whole chromosome segments or in some cases epigenetic changes in gene expression (Table 1.5; Harris, 1996; Reik, 1989; Scrable et al, 1990; Stanbridge, 1990; Weinberg, 1991). The steps appear to be as follows: one chromosome of a pair is lost, and a deletion then occurs in the remaining chromosome, i.e. loss of heterozygosity (LOH).

Table 1.5 Examples of tumour suppressor genes involved in human cancer

Gene	Major types of mutation	Syndrome	Typical neoplasms
APC	Deletion and nonsense	Familial adenomatous polyposis	Carcinomas of colon, thyroid and stomach
ATM	Deletion	Ataxia telangiectasia	Leukaemia, lymphoma
NF1	Deletion	Neurofibromatosis type 1	Neural tumours
NF2	Deletion and nonsense	Neurofibromatosis type 2	Central schwannomas and meningiomas
p53	Missense	Li-Fraumeni	Carcinomas of breast and adrenal cortex; sarcomas; leukaemia; brain tumours
$p16^{INK4}$	Deletion and nonsense	Familial melanoma	Melanoma
RB1	Deletion and nonsense	Retinoblastoma	Retinoblastoma; osteosarcoma
WT1	Missense	Wilms' tumour	Wilms' tumour
VHL	Deletion	von Hippel-Lindau	Hemangioblastoma and renal cell carcinoma

1.3.4 Hereditary cancer

Although the development of cancer is primarily sporadic in nature, non-random aggregations of the disease have been recognized to occur since the middle of the last century (Broca, 1866). Virtually every type of cancer has anecdotally been reported to occur in a familial form, yet convincing statistical evidence for hereditary or familial predisposition exists in less than three percent of all cases (Knudson et al., 1973; Knudson, 1989 and 1996; Ponder, 1990).

The most striking form of genetic susceptibility involves Mendelian dominant inheritance with high penetrance and appearance of cancer at earlier than usual age, as shown for

colon cancer in persons with familial adenomatous polyposis. In this example, the heterozygous state of the germ-line mutation imparts a high risk for just one form of cancer, while in other examples, such as the Li-Fraumeni syndrome, it predisposes to several kinds of cancer (but not to every type). The most frequent cancer in Li-Fraumeni syndrome is carcinoma of the breast, although it does not afflict all female carriers. But no known mutations predispose to all forms of cancer. It has been estimated that there may be 50 or so different genes in which mutations can impart high susceptibility to heterozygous carriers.

The fact that penetrance is incomplete for cancer mutations in the germline indicates that heterozygosity is not a sufficient condition for the development of cancer: something else must occur (This fact also indicates that tumorigenesis is a multistage process). For hereditary retinoblastoma Knudson (1971) supposed that this may be a second mutation, occurring post-zygotically.

Under the Knudson 'two-hit' hypothesis, the only difference between hereditary and non-hereditary cases was the timing of the first event as prezygotic or postzygotic, respectively.

1.3.5 Tumorigenesis in transgenic and gene knockout mice

The study of molecular mechanisms of tumorigenesis has been greatly enhanced in recent years by the advent of transgenic mouse technology, and use of germline manipulation for the creation of targeted gene mutation. By these technologies, the particular genes thought to be important in the tumorigenic process can be mutated or deleted in the germ line (Fowlis and Balmain, 1993; Gonzalez, 1996; Viney, 1995). Mice inheriting disrupted genes will, if the disrupted genes have been correctly chosen, require fewer mutational events in

any somatic cell for malignant transformation to occur.

When an oncogene such as myc is transferred to the mouse germ line under the control of a breast-cell specific promoter, the transgenic animals develop breast tumours (Adams and Cory, 1991). However, of thousands of epithelial mammary stem cells in the mouse, only one or two become neoplastic. This suggests that the presence of a single oncogene is not sufficient for tumorigenesis, even when the gene is expressed at constitutively high levels for long periods. However, doubly transgenic mice, made by breeding myc transgenics with ras transgenic mice, develop tumours much earlier and more frequently (Sinn et al, 1987).

When a tumour suppressor gene such as Rb is disrupted by gene targeting, the knock-out mice develop tumours much earlier and frequently (Lee et al, 1992). However, most homozygous tumour suppressor gene knock-outs are lethal in mice at the fetal stage (Knudson, 1993).

1.4 Radiation carcinogenesis

Ionizing radiation may be the exogenous agent inducing more cancers than any other, because everyone is exposed to radiation from naturally occuring radioactivity, cosmic rays, medical applications and other sources. It induces cancer by mutating dominant-acting proto-oncogenes such as ras and myc to active form (Cox, 1994; Weichselbaum et al, 1989), or tumour suppressor gene such as p53 or Rb to inactive form (Cowell, 1990; Cox, 1994; Weichselbaum et al, 1989). Also, it kill cells, and thereby induces compensatory cell

proliferation, which in turn leads to more mutagenesis during DNA replication.

1.4.1 DNA damage, repair and cell mutation

There is strong circumstantial evidence to indicate that DNA is the principal target for the biological effects of radiation. Therefore, a consideration of the biological effects of radiation must begin logically with a description of the breaks in DNA caused by charged particle tracks and by the chemical species produced.

A wide spectrum of DNA lesions is produced by ionizing radiaiton. These include a variety of base alterations, single-strand breaks, double-strand breaks and multiply damaged sites (Goodhead, 1994; Hutchinson, 1985; Urlaub et al, 1986; Wallace, 1983; Ward, 1988). Repair, non-repair or misrepair of these types of damage leads to the genotypic change responsible for the biological effect, such as cell killing and transformation (Hall, 1994; Hutchinson, 1993; Thacker and Cox, 1975; Ward, 1995).

The effects of DNA damage are greatly reduced by repair. The most common type of repair is excision of damage from one strand, with the complementary strand serving as template for resynthesis of the damaged strand (Price, 1993). This mechanism cannot repair double-strand breaks. Repair of double-strand breaks requires homologous DNA as template to rejoin the correct strands and replace any missing sequences (Price, 1993). This is probably the major pathway for repair of radiation-induced double-strand breaks in yeast (Game, 1993) and *E. coli* (Krasin and Hutchinson, 1977; Kobayashi and Takahashi, 1988), but its contribution in mammalian cells is not known, although there is evidence in these cells for repair of double-strand breaks.

The nonrepair or misrepair of damage in DNA can lead to mutations and/or chromosomal abnormalities (Hutchinson, 1993; Thacker, 1992; Ward, 1995). Mutations can take a variety of forms including base alterations, frame-shift mutations and deletions (Hutchinson, 1993; Renan, 1992). Radiation can increase the frequency of specific mutations as well as chromosomal abnormalities (Sankaranarayanan, 1993; Ward, 1995).

1.4.2 Radiation tumorigenesis in experimental animals

Radiation carcinogenesis in animals is well documented (Fry and Storer, 1987; Fry, 1991; Silini, 1986; UNSCEAR, 1986 and 1988). Literally hundreds of studies have been done on animal models for radiation carcinogenesis, and it would be unproductive to review these reports in detail. Upton (1986) has, however, provided a very concise summary, as follows:

1. Neoplasms of almost any type can be induced by irradiation of an animal of suitable susceptibility, given appropriate conditions of exposure.
2. Not every type of neoplasm is increased in frequency by irradiation of animals of any species or strain.
3. The carcinogenic effects of irradiation are interconnected through a variety of mechanisms (see Section 1.4.3), depending on the type of tumour and conditions of exposure.
4. Some mechanisms of carcinogenesis involve direct effects on the tumour forming cells themselves, but others may involve indirect effects on distant cells or organs.
5. Though the dose-incidence curve has not been defined precisely for any neoplasm over a wide range of doses, dose rates, and radiation qualities, the incidence generally rises more steeply as a function of dose and is less dependent on dose rates with radiations of high LET than with radiations of low LET.

6. The development of neoplasia appears to be a multicausal and multistage process, in which the effects of radiation may be modified by other physical or chemical agents.

7. At low to intermediate dose levels, the carcinogenic effects of radiation often remain unexpressed unless promoted by other agents.

8. At high dose levels the expression of carcinogenic effect often tends to be suppressed by sterilization of the potentially tranformed cells or by other forms of radiation injury, resulting in saturation or a turning point of the dose-incidence curve.

9. The distribution in time of radiation induced tumours characteristically varies with the type of tumour, the genetic background and age of the exposed animal, the conditions of irradiation and other variables.

10. Because of the diversity of ways in which irradiation can influence the probability of neoplasia, the dose-incidence relationship may vary accordingly.

It is very important to note that tumour incidence does not necessarily continue to increase indefinitely with increasing total-body dose.

1.4.3 Mechanisms of radiation carcinogenesis

Given the multistage nature of carcinogenesis (see Section 1.2), the molecular mechanisms involved in radiation oncogenesis are expected to be highly complex with radiation acting as one of a series of interacting factors. It is however possible to subdivide the whole process in the following simplistic fashion (Cox, 1994):

(1) initial molecular damage to cellular DNA;

(2) post-radiation modification of that damage;

(3) the generation of specific gene or chromosomal mutations in appropriate target somatic cells that initiate the oncogenic process;

(4) the early clonal expansion of initiated cells generating preneoplastic lesions;

(5) the accumulation of additional genetic and epigenetic events;

(6) further clonal evolution-selection that drives the progression and metastasis of the final malignancy.

However it is quite possible that a need for further changes varies among tissues. Presumably these differences are due to differences in the importance of host factors and of the probability of the required change taking place spontaneously (Fry and Storer, 1987). It is also clear, and of considerable importance for risk estimation, that radiation of some tissues may convert normal cells into potential cancer cells that may never express their malignant phenotype or may do so only after a long time.

A frequent consequence of exposure to radiation is the loss of genetic material. Thus, activation of oncogenes and inactivation of tumour supressor genes by radiation may be part of a general mechanism of radiation-induced carcinogenesis (Cox, 1994). Guerreo et al (1984) found that mouse lymphomas induced by gamma radiation contained an active c-K-ras oncogene. After extensive molecular analysis, these investigators concluded that their results were compatible with gamma radiation as a source of point mutations which activated the c-K-ras gene in mouse lymphomas. The activation of the c-K-ras also appears important in the development of ^{90}Sr induced osteosarcoma in mice (Merregaert et al, 1986). Recently, Vahakangus et al (1992) found p53 mutations in lung cancer due to radon exposure.

1.5 The role of p53 tumour suppressor gene in tumorigenesis

p53, the best known of the tumour suppressor genes so far, is thought to play an important role in the regulation of cell proliferation (Harris, 1996; Michalovitz et al, 1990; Selivanova and Wiman, 1995), and it has been suggested that the loss of normal p53 function is associated with cell immortalisation or transformation in vitro (Dittmer et al, 1993; Eliyahu et al, 1985; Hsiao et al, 1994; Jenkins et al, 1984; Pohl et al, 1988; Rovinski et al, 1988) and development of neoplasms in vivo (Ahuja et al, 1989; Baker et al, 1989; Takahashi et al, 1989; Vogelstein et al, 1988; Yokota et al, 1987). The alterations within coding sequences of the p53 tumour suppressor gene are among the most frequent genetic changes detected in human cancers (Beroud et al, 1996; Caron de Fromental and Soussi, 1992; Harris, 1996; Hollstein et al, 1991 and 1996; Vogelstein, 1990). Reintroduction of p53 into transformed cells can induce either growth arrest (El-Deiry et al, 1993; Harper et al, 1993; Xiong et al, 1993) or apoptosis (Yonish-Rouach et al, 1991). The p53 gene or gene product is a common cellular target in tumorigenesis provoked by physical factors (Brash et al, 1991; Kemp et al, 1994), chemical carcinogens (Harvey et al, 1993; Kemp et al, 1993) or tumour viruses (Mowat et al, 1985; Wolf et al, 1987) (Figure 1.3).

1.5.1 The p53 tumour suppressor gene and its protein

The p53 gene encompasses about 16-20 kb of DNA located on the short arm of human chromosome 17 at position 17p13.1 (Isobe et al, 1986; Miller et al, 1986). In the mouse, the p53 gene is found on chromosome 11 (Czosnek et al, 1984; Rotter et al, 1984). Both human and mouse genes are composed of 11 exons (Table 1.6). The first exon is noncoding, contains 213 base pairs and is found 8-10 kb away from exons 2-11. Thus, the

first intron comprises about one-half of the gene. The p53 gene has been conserved during evolution. In cross-species comparison, the p53 proteins show five highly (>90%) conserved regions among the amino acid residues 13-19, 117-142, 171-181, 234-258, and 270-286 (Soussi et al, 1990).

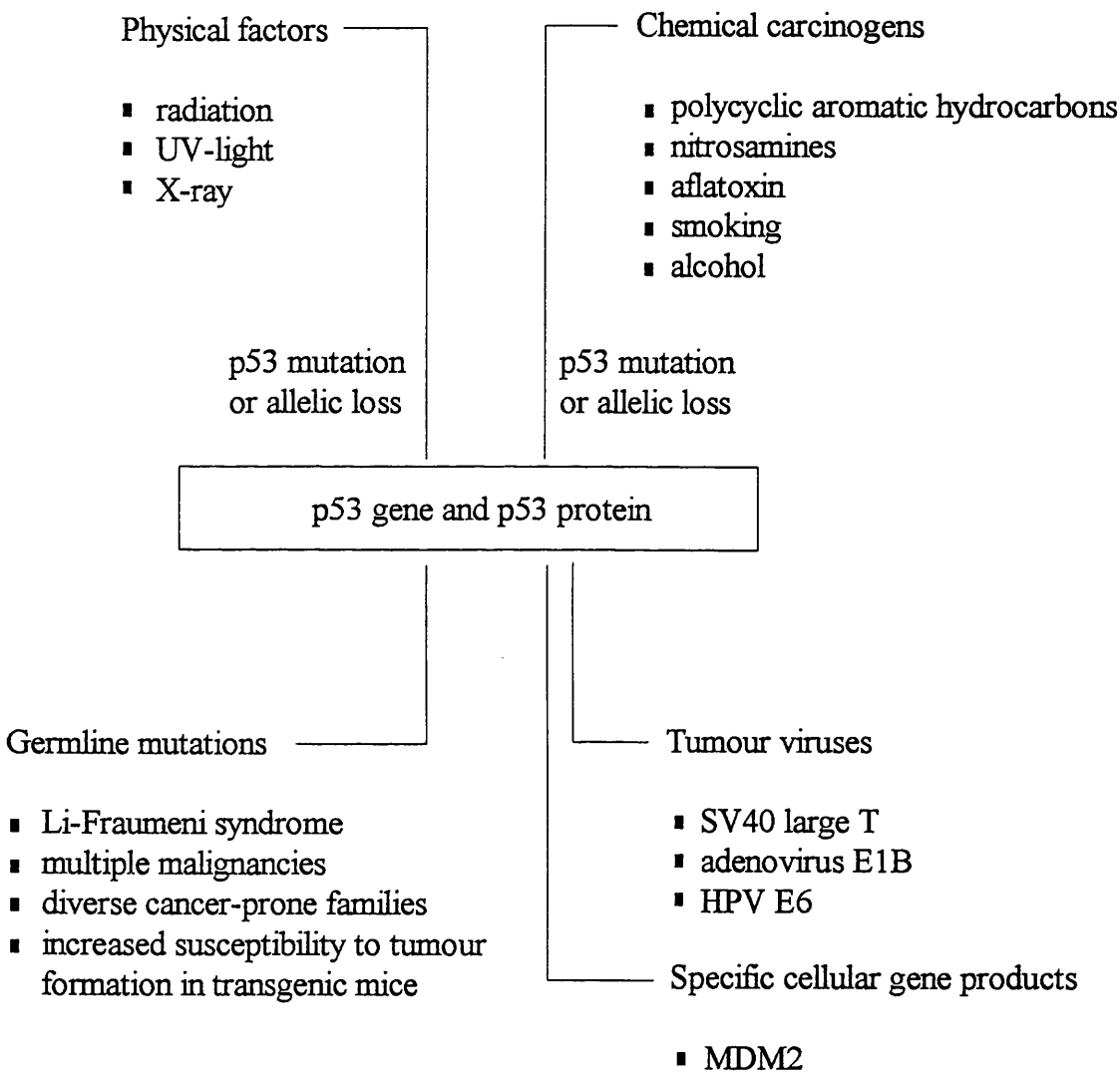


Figure 1.3 Schematic representation of p53 as a common cellular target in tumorigenesis (Chang et al, 1993).

The product of the p53 gene from which it gets its name is a 393 amino-acid nuclear phosphoprotein (approximately 53 kD in molecular weight) and was identified when co-

precipiteated in an antigen-antibody complex with extracts from the simian virus 40 (SV40) tumour antigen in 1979 (Lane and Crawford, 1979; Linzer and Levine, 1979). The p53 protein was found in very low quantities in normal cells, but larger quantities of p53 (5-100 fold) could be detected in transformed cells in culture and in human tumours (Gusterson et al, 1991; Purdie et al, 1991).

Table 1.6 Properties of p53 tumour suppressor gene

	p53
Nucleotides (kb)	16-20
Chromosome	
Human	17p13.1
Mouse	11
Exons	
Human	11
Mouse	11
mRNA(kb)	
Human	2.5
Mouse	2.0
mRNA half-life (h)	>12
Amino acids	
Human	393
Mouse	390
Mass (kDa)	
Human	pp53
Mouse	pp53
(expressed)	
protein half-life	5-20 minutes (normal cells)
	4-20 hours (transformed cells)

1.5.2 The function of the p53 gene

Much of the early work on p53 was difficult to interpret as it was performed with mutant alleles which have carcinogenic properties (Eliyahu et al, 1984; Jenkins et al, 1984; Parada et al, 1984; Rovinski and Benchimol, 1988), and it is now clear that this gene in its non-mutated form (wild-type) is a tumour suppressor gene which prevents carcinogenic change (Lane and Benchimol, 1990; Levine, 1992; Levine et al, 1991; Oren, 1992). Indeed the wild-type p53 is capable of reverting the transformed phenotype of many human tumour cell lines in vitro, i.e., causing human tumour cells in culture to revert to normal appearances and behaviour (Baker et al, 1990; Diller et al, 1990; Eliyahu et al, 1989; Finlay et al, 1989; Mercer et al, 1990; Harris, 1996).

The molecular mechanisms by which p53 functions normally, and by which it affects tumorigenesis remain unclear. As shown in figure 1.4, documented effects of wild-type p53 on cell proliferation include regulation of the transition from G1 to S-phase of the cell cycle (Diller et al, 1990; Kastan et al, 1995; Livinstone et al, 1992; Perry and Levine, 1993; Yin et al, 1992) and a role in determining cell death through apoptosis (Clarke et al, 1993; Donehower, 1994; Lowe et al, 1993; Midgley et al, 1995; Oren, 1994; White, 1996; Yonish-Rouach et al, 1991). Biological analysis indicated that p53 plays little part in normal cell cycle control, but plays an important growth-controlling role in stressed cells (Kastan et al, 1992; Lane, 1992). Emerging evidence also suggests that p53 appears to function normally as a G1-S checkpoint control for DNA damage (Hartwell, 1992; Kastan et al, 1992; Lane, 1992; Lee et al, 1994 Selivanova and Wiman, 1995). Accordingly, normal p53 may act as a 'molecular policeman' monitoring the integrity of the genome. If DNA is damaged, p53 accumulates and switches off replication to allow extra time for repair mechanisms to act. If this fails however, p53 may trigger cell suicide by apoptosis

(Lane, 1992).

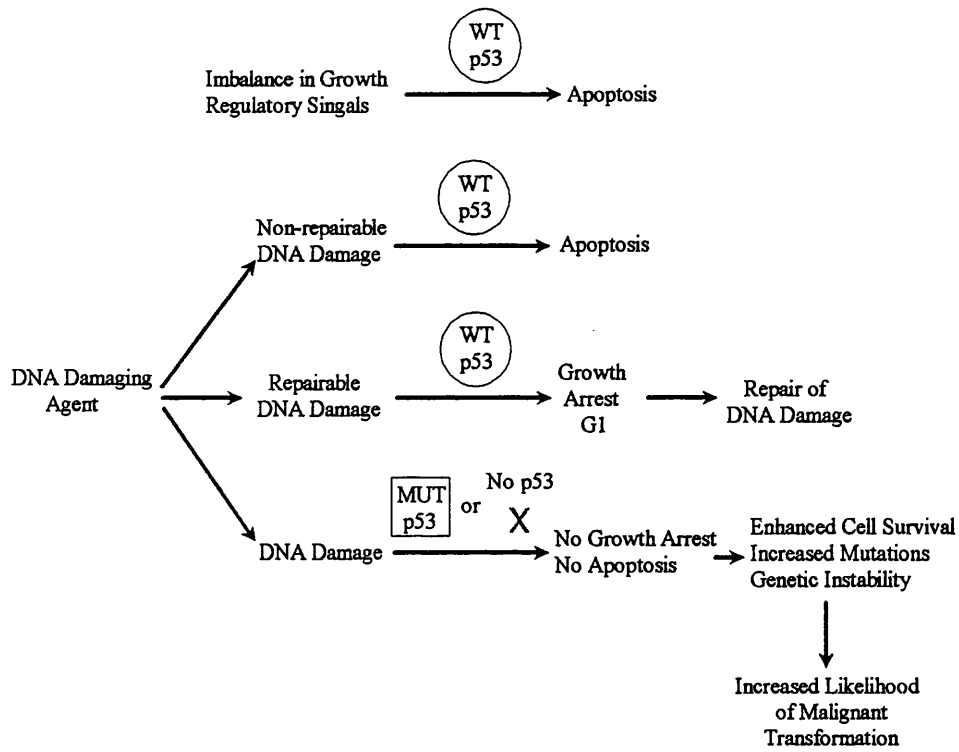


Figure 1.4 The hypothesized role of p53 in normal cells and the effects of its absence on development of a tumour cell.

These regulatory functions may be mediated by the interaction of p53 protein with specific DNA sequences (Hupp et al, 1992; Kern et al, 1991; Kern et al, 1992; Ko and Prize, 1996) which may allow regulation of other genes at transcriptional level (Donehower and Bradley, 1993; Farmer et al, 1992; Ko and Prizer, 1996), or perhaps by initiating DNA replication (Friedman et al, 1990; Ko and Prizer, 1996). It has been presumed that wild-type p53 could regulate the assembly or function of DNA replication-initiation complexes, or alternatively, p53 could act as a transactivator of gene transcription, either promoting or inhibiting mRNA synthesis(Levine et al, 1991; Ko and Prizer, 1996). In mutated p53 proteins, the DNA binding capacity, transcriptional activator function and initiation of

DNA replication are all altered (Friedman et al, 1990; Farmer et al, 1992; Hupp et al, 1992; Kern et al, 1992; Lane and Benchimol, 1990; Park et al, 1994; Pietenpol et al, 1994; Zhang et al, 1994).

1.5.3 p53 and genomic stability

p53 has been proposed to be involved in maintaining stability of the genome (Livingstone et al, 1992; Smith and Forance, 1995; Yin et al, 1992), and both cell cycle arrest and apoptosis can be considered mechanisms by which this may be accomplished. In the presence of DNA damage, cells will either arrest, presumably to allow DNA repair, or undergo cell death, in a p53-dependent manner. In either case, the propagation of potentially deleterious mutations can thus be averted.

Consistent with a role for p53 in protecting genomic integrity, fibroblasts from p53-deficient mice demonstrate chromosomal abnormalities that appear at early passage in homozygous null fibroblasts and at later passage in heterozygous fibroblasts (Harvey et al, 1993C). Aneuploidy and evidence of chromosomal instability was also found in tumours from p53 homozygous null mice and from mice with both a Wnt-1 transgene and homozygous for the null allele of p53. Finally, fibroblasts from p53 homozygous null mice become tetraploid and octaploid after exposure to spindle inhibitors, in contrast to those from wild-type mice, which undergo arrest (Cross et al, 1995).

p53-deficient cells exhibit a higher tolerance to genetic abnormalities arising from radiation as well as spontaneously. In response to γ-irradiation, cell from mice homzygous for the null allele show increased accumulation of double-stranded DNA damage as compared to heterozygotes or wild-type littermates (Bouffler et al, 1995; Lee et al, 1994).

1.5.4 Association of p53 with tumorigenesis

Is alteration of function of the p53 pathway a prerequisite for neoplastic transformation? The frequency of p53 mutations in human and animal tumours argues for a critical role (Beroud et al, 1996; Donehower et al, 1992; Hollstein et al, 1996; Purdie et al 1994). Of the about 6.5 million cancer cases worldwide each year, 2.4 million tumours are estimated to contain a p53 mutation (Harris, 1996). But for most tumour types mutations have been found only in 20-50% of cases (Table 1.7). Current techniques are unlikely to miss more than 15-20% of coding sequence abnormalities. How frequent and important to functional abrogation are mutations in the p53 promoter or unevaluated introns, epigenetic inactivation of p53, and/or alteration of gene or proteins downstream in the p53 pathway?

Is p53 only one of several pathways whose disruption is sufficient for neoplastic transformation? Support for this concept comes from the observation that both p53 and ras mutations are common events but often occur independently (Enomoto et al, 1993; Kalthoff et al, 1993; Lehman et al, 1991; Mitsudomi et al, 1992; Nikolaidou et al, 1993; Shaw et al, 1991), and either may be associated with aggressive tumour behaviour (Dowell and Hall, 1994; Harris and Hollstein, 1993; Rodenhuis and Slebos, 1992).

Does neoplastic transformation require a "critical mass" of genetic injury, in which p53 abberration is a frequent but not causally essential event? In this scenario, loss of p53 genomic stabilization properties would predispose a cell to an acceleration in the rate of genetic damage and greatly increase the likelihood of neoplastic transformation and/or malignant progression. Research which attempts to unify molecular and cellular theories of carcinogenesis should address these and other questions. Evaluation of mutation patterns of p53 and other genes in clinical tumours can be an important part of this process.

Table 1.7 Incidence of p53 mutations in human cancer

Cancer	% of samples in which p53 mutations occur	References
Acute myeloblastic leukaemia	6	Hu et al, 1992
Brain tumours	~10	Mashiyama et al., 1991
Breast cancer	53-86	Horak et al., 1991; Varley et al., 1991
Colorectal cancer	50	Rodrigues et al., 1990
Epithelial skin (basal cell) carcinoma	48	Moles et al., 1993
Oesophageal cancers	50	Bennett et al., 1991
Gastric carcinoma	57	Martin et al., 1992
HBV-positive hepatoma	18	Hosono et al., 1993
Lung tumours		
Small-cell carcinoma	44-73	Iggo et al., 1990; Takahashi et al., 1991
Non-small-cell carcinoma	45	Takahashi et al., 1991
Adenocarcinoma	57	Iggo et al., 1990
Squamous cell carcinomas	34-82	Gusterson et al., 1991; Iggo et al., 1990
Carcinoid	0	Iggo et al., 1990
Malignant astrocytomas	~30	Mashiyama et al., 1991
Melanomas (primary)	97	Akslen and Morkve, 1992
Multiple myeloma	20	Portier et al., 1992
Osteosarcomas	41	Mulligan et al., 1990
Ovarian carcinomas	44	Milner et al., 1993
Pancreatic carcinomas	40	Ruggeri et al., 1992
Rhabdomyosarcomas	45	Mulligan et al., 1990
Squamous cell carcinoma of the larynx	60	Maestro et al., 1992
Thyroid carcinomas	50	Dongi et al., 1992

Germinal mutations in the p53 gene have been observed in some families with the Li-Fraumeni syndrome, an autosomal dominant disease characterized by a greatly increased predisposition to cancer of various types (Malkin, 1994; Malkin et al., 1990; Srivastava et al., 1990). Roughly 50% of family members with Li-Fraumeni syndrome develop neoplasms by the age of 30, in contrast to an incidence of 1% cancer development by age 30 in the general population (Malkin et al., 1990). Furthermore, all the tumour-affected individuals retain the mutant allele and lose the wild-type p53 allele in their tumour tissue.

1.5.5 Tumorigenesis in p53 deficient mice

The animal model which has provided greatest insights into the activities of p53 in tumorigenesis is the p53-deficient mouse developed by gene targeting techniques (Donehower et al, 1992). It was demonstrated that mice homozygous for a null p53 allele were developmentally normal but highly susceptible to early onset of tumour. By the age of 6 months, 74% of the homozygotes had developed tumours and by ten months all of these mice had died or developed tumours. The spectrum of tumours observed were fairly diverse, although lymphomas predominated. However, the heterozygote mice also displayed an elevated level of tumour susceptibility, although the tumours in this group developed at a later age in a lower fraction of the mice. By 15 months of age, 27% of the heterozygotes had developed tumours of various types. However, instead of lymphomas, osteosarcomas and soft tissue sarcomas were the predominant tumour type in the heterozygotes. Clearly, these p53 deficient mice provide definitive proof that p53 behaves as a tumour suppressor gene. In addition, the heterozygous mice may serve as a useful model for the Li-Fraumeni syndrome.

Recently, preliminary studies on radiation-induced tumorigenesis showed that 4 Gy single treatment of gamma rays resulted in a marked reduction in median tumour latency in p53 heterozygous mice (Kemp et al, 1994), a lesser reduction in p53 null mice and negligible effect in the wild-type mice. Moreover, tumours developing in irradiated p53 heterozygous mice, when subjected to Southern blot analysis, were consistently found to be p53 null. These results imply that p53 inactivation may be a near-obligatory step in radiation tumorigenesis in these mice and pose critical questions as to the sensitivity of the p53 allele to radiation inactivation and the frequency of occurrence of full malignant transformation of a cell in which both p53 alleles have been inactivated. The present findings are consistent with high radiosensitivity of the p53 gene and a high probability of occurrence of further transforming events once p53 inactivation has taken place (i.e. an operational genomic instability). This could have important implications for Li-Fraumeni patients if the murine findings are more generally applicable.

Chapter 2

Multistage Models for Tumorigenesis

2.1 Introduction

In contrast to the extreme diversity and complexity of the biological observations and theories on tumour development described in Chapter 1, the quantitative description of tumorigenesis is still rudimentary. The conceptualiztion of tumorigenesis as a probabilistic multistage process led, in the 1950s, to the definition of the multistage model (Nording, 1953; Armitage and Doll, 1954 and 1957). The only assumption made by the multistage model is that, to become fully malignant, cells have to undergo a series of rate-limiting transformation events whose time of occurrence is exponentially distributed (see Section 2.1). Within such a framework, carcinogens are supposed to act by increasing the probability of transformation from one stage to another. With some methodological refinements (Crump and Howe, 1984; Crump et al, 1977; Krewski et al., 1983; Little et al, 1992; Murdoch and Krewski, 1988; Whittemore and Keller, 1978) this model is still the most widely used.

The discovery and confirmation that the loss of two copies of the retinoblastoma (Rb) gene could lead to a fully malignant tumour (Benedict et al, 1983; Hethcote and Knudson, 1978; Knudson, 1971; Knudson et al, 1975), and that the clonal expansion of pre-malignant cells was important (Nowell, 1976), led to the development of a series of closedly related clonal two-stage models for which a considerable literature now exists (Greenfield et al., 1984; Knudson, 1971; Moolgavkar, 1990; Moolgavkar and Knudson, 1981; Moolgavkar and Venzon, 1979; Tan, 1991). Most clonal models developed so far assume that two mutational events are required to form a fully malignant cell. Initiators are supposed to act by increasing the probability of these events (Moolgavkar et al, 1990; Portier, 1987), while promoters are supposed to induce the proliferation of intermediate stage cells (Cohen and

Ellwein, 1990; Moolgavkar, 1983), therefore increasing the size of the susceptible cell populations. In section 2.3, the Moolgavkar-Venzon-Knudson (MVK) model will be discussed in detail.

As demonstrated in Chapter 1, tumorigenesis is a multistage random process with intermediate cells subjected to stochastic cell proliferation and differentiation. These observations led to the extension of the MVK model into a multistage model (Little, 1995; Moolgavkar and Luebeck, 1992; Tan, 1991) that will be discussed in detail in Section 2.4.

Multistage models may be generalised to the situation of competing alternative pathways and to a still more general class of model, described by Portier et al. (1993) as multistage/multihit models in which traditional multistage pathways (requiring sequential occurrence of mutational events) coexist with multihit processes (for which a critical burden of events may be acquired in any order). These more general models are still quite new, their application to tumorigenesis will probably become more common as molecular genetic information on alternative pathways becomes available.

2.2 The Armitage-Doll multistage model

Multistage models for single cell carciongenesis were first advanced in the 1950s by Armitage and Doll (Armitage and Doll, 1954) to explain the observation that, in many adult carcinomas, the logarithm of the age-specific incidence rates increases linearly with the logarithm of age. Fisher and Hollomon (1951) suggested that this observation could be explained if a critical number of six or seven tumour cells were required for subsequent

independent tumour growth. The Fisher-Hollomon hypothesis is not now believed to be correct and modern tumorigenesis models are descendants of the Armitage-Doll concept - the multistage transformation of a single cell.

Figure 2.1 illustrates the basic theory behind the Armitage-Doll model. The model views carcinogenesis as the end result of a sequence of many steps, i.e., any single cell may transform from the normal state through many intermediate states into a malignant state. Consider a series of k steps through which a normal clonogenic cell must pass sequentially in order to reach the end state of full-blown malignant transformation, and λ_j (j=1,2,3, ..., k) represents the "rate of transition" from stage j-1 to stage j, where stage 0 is the normal state and stage k+1 is the malignant state.

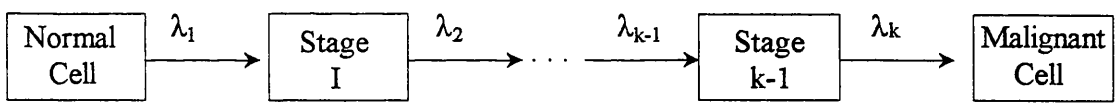


Figure 2.1 The Armitage-Doll multistage model of carcinogenesis

An intuitive derivation of the age-specific incidence predicted by this model then proceeds as follows: let $p_i(t)$ be the probability that the ith event has occured by time t. Then

$$p_i(t+\Delta t)=p_i(t)+\lambda_i \Delta t[1-p_i(t)] \qquad (2.2.1)$$

i.e. $$dp_i(t)/dt = \lambda_i[1-p_i(t)] \qquad (2.2.2)$$

so $$p_i(t)=1-\exp(-\lambda_i t) \qquad (2.2.3)$$

Thus the probability that all events have occured by time t is $p_1(t)p_2(t)\cdots p_k(t)$. It may be shown that P(t), the probability function of appearance of the tumour by time t or less, is

given by:

$$P(t) = N \prod_{j=1}^{k} [1 - e^{-\lambda_j (t-\tau)}] \tag{2.2.4}$$

where N the is the total number of susceptible cells in the tissue and τ is a fixed positive time for the growth of the tumor.

If the mutation rates λ_j (j=1, 2, ..., k) are very small, the probability of the appearance of the tumour, P(t) , is approximately given by:

$$P(t) \approx N A \lambda_1 \lambda_2 \ldots \lambda_k (t-\tau)^k \tag{2.2.5}$$

where A is a compound constant (a function of the λ terms).

The approximate age-specific incidence rate, I(t), predicted by the Armitage-Doll model is

$$\begin{aligned} I(t) &= \frac{dP(t)}{dt} \\ &= N A \lambda_1 \lambda_2 \ldots \lambda_k k (t-\tau)^{k-1} \\ &= B \lambda_1 \lambda_2 \ldots \lambda_k (t-\tau)^{k-1} \end{aligned} \tag{2.2.6}$$

where B is a compound constant.

It is noteworthy that the first exposition of the Armitage-Doll model multistage theory appeared only a few years after early, seminal work on initiation-promotion in mouse skin. It took many years for experimentalists to demonstrate that initiation and promotion were not idiosyncratic responses of mouse skin, but components of carcinogenesis of general relevance (Farber et al, 1987). It was evident much earlier, on statistical and epidemiological grounds, that a multistage process provided a parsimonious and coherent

explanation of many different phenomena.

In 1969, Cook, Doll and Fellingham undertook a comprehensive test of the relationship (2.2.4) for the age distribution of cancer. They tested this relationship in 31 types of cancer in 11 populations, and concluded that the constant k "might be a biological constant characteristic of the tissue in which cancer is produced." However, these authors also concluded that in a large number of data sets, the age-specific incidence rates showed significant departures from the simple relationship (2.2.4). Some possible reasons for these depatures were discussed by the authors. A fuller discussion is to be found in a thoughtful article by Peto (1977).

Epidemiologic data on exposure to specific carcinogens has been examined within the framework of the Armitage-Doll model by various authors (Brown and Chu, 1982; Day and Brown, 1980; Little, 1996; Little et al, 1992 and 1995; Thomas, 1982). By studying the pattern of evolution of risk after exposure to a carcinogen begins and after exposure stops, it is possible to deduce whether an early or a late stage (or both) is affected by the carcinogen.

That the Armitage-Doll model successfully describes the age-specific incidence curves of a large number of human tumors does not provide strong evidence in favor of its correctness. Many other models fit the age-specific incidence data equally well. The ultimate vindication of a model must derive from biological considerations. In this regard, the Armitage-Doll model is somewhat unsatisfactory. First, the Armitage-Doll model provides no satisfactory explanation for hereditary tumours such as retinoblastoma and Wilms' tumour. Second, initiation-promotion experiments and a host of other considerations suggest that both mutations and cell proliferation are important in carcinogenesis: the

Armitage-Doll model makes no explicit allowance for cell growth and differentiation. Thus, the initiation-promotion and initiation-promotion-initiation experiments cannot be explained by the Armitage-Doll mulitstage model.

2.3 Moolgavkar, Venzon and Knudson two-stage model

Moolgavkar, Venzon and Knudson (Moolgavkar and Venzon, 1979; Moolgavkar and Knudson, 1981) have proposed a model (MVK model) for carcinogenesis that assumes that a tumour develops from a single normal stem cell by clonal expansion and views carcinogenesis as the end result of two discrete, heritable and irreversible events in normal stem cells. Each event occurs during a single cell division. It differs from the Armitage-Doll model in that the normal stem cells and intermediate cells are subjected to cell proliferation and cell differentiation and death (see Figure 2.2). A distinct feature of this model is that the first event may occur either in germline cells or in somatic cells but the second event always occurs in somatic cells.

2.3.1 Assumptions

According to this model, there are three types of cells: the normal stem cells, the intermediate (or initiated) cells and tumour cells. Further, with probability one, a tumour cell will develop into a malignant tumour. We denote the number of normal stem cells, intermediate cells and tumour cells by X(t), Y(t) and T(t) at time t, respectively. To find the age specific incidence function of tumours and the probability distribution of the time required for a normal stem cell to develop into a tumour, we make the following

assumptions:

(1) Each susceptible stem cell in a tissue experiencess malignant transformation independently of the other susceptible stem cells.

(2) In the small time interval (t, t+Δt), a normal susceptible cell divides into two normal susceptible cells with probability $b_1 \Delta t + o(\Delta t)$; it dies or differentiates with probability $d_1 \Delta t + o(\Delta t)$; and it divides into one normal cell and one intermediate cell with probability $\mu_1 \Delta t + o(\Delta t)$; the probability of more than one event occuring is $o(\Delta t)$.

(3) Likewise at time t, an intermediate cell divides into two intermediate cells, dies (or differentiates), or divides into one intermediate and one malignant cell with parameters b_2, d_2, and μ_2, respectively.

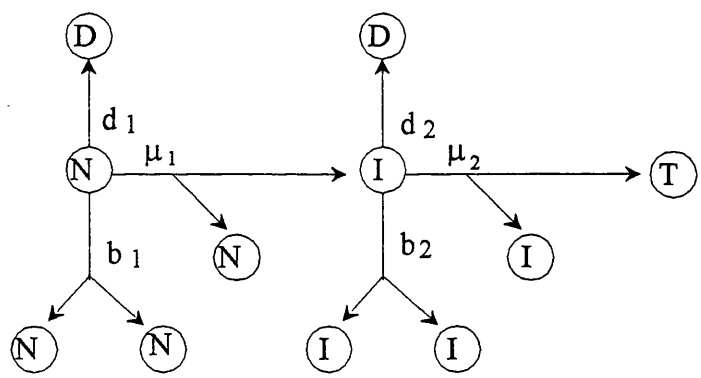


Figure 2.2 MVK two-stage model of carcinogenesis. N, I and T stand for normal stem cells, intermediate cells and tumour cells, respectively. D stands for death/differentiation.

2.3.2 Incidence function

There are two fundamental quantities: the first is the incidence function, I(t), at time t; the

second is the probability, P(t), that a malignant cell is generated by time t.

$$P(t) = 1 - \exp[-\int_0^t I(s)ds] \tag{2.3.1}$$

To obtain the incidence function and the probability of the occurrence of tumour, an important mathematical tool is the probability generating function (PGF) of the number of normal cells, intermediate cells and tumour cells, i.e., X(t), Y(t) and T(t). The PGF of X(t), Y(t) and T(t), $\Psi(x,y,z;t)$, is defined by:

$$\Psi(x,y,z;t) = \sum_{i,j,k} Pr\{X(t)=i, Y(t)=j, T(t)=k\} x^i y^j z^k \tag{2.3.2}$$

Given the PGF $\Psi(x,y,z;t)$, one may not only compute the probability of having k tumours by time t, but also can derive the incidence function I(t) of tumours using the following relationship:

$$I(t) = -\Psi'(1,1,0;t) / \Psi(1,1,0;t) \tag{2.3.3}$$

where $\Psi'(1,1,0;t) = d\Psi(1,1,0;t)/dt$.

To find out the PGF $\Psi(x,y,z;t)$, an important mathematical equation is the Kolmogorov forward differential equation.

Let p(i, j, k; t) = Pr{ X(t)=i, Y(t)=j, T(t)=k }; then, the differential equations of p(i, j, k; t) are obtained by considering two contiguous time intervals (0, t) and (t, t+Δt). The probabilities p(i, j, k; t+Δt) are:

$$
\begin{aligned}
p(i,j,k;t+\Delta t) &= \{1-[i(b_1+d_1+\mu_1)\Delta t + j(b_2+d_2+\mu_2)\Delta t]\}\, p(i,j,k;t) \\
&\quad + (i-1)b_1 \Delta t\; p(i-1,j,k;t) \\
&\quad + (i+1)d_1 \Delta t\; p(i+1,j,k;t) \\
&\quad + [(j-1)b_2 + i\mu_1]\Delta t\; p(i,j-1,k;t) \\
&\quad + (j+1)d_2 \Delta t\; p(i,j+1,k;t) \\
&\quad + j\mu_2 \Delta t\; p(i,j,k-1;t) + \circ(\Delta t)
\end{aligned}
\tag{2.3.4}
$$

Using (2.3.4), we obtain that,

$$
\begin{aligned}
\frac{d}{dt}\, p(i,j,k;t) &= -[i(b_1+d_1+\mu_1)+j(b_2+d_2+\mu_2)]p(i,j,k;t) \\
&\quad + (i-1)b_1 p(i-1,j,k;t) \\
&\quad + (i+1)d_1 p(i+1,j,k;t) \\
&\quad + [(j-1)b_2 + i\mu_1]p(i,j-1,k;t) \\
&\quad + (j+1)d_2 p(i,j+1,k;t) \\
&\quad + j\mu_2 p(i,j,k-1;t)
\end{aligned}
\tag{2.3.5}
$$

Equations (2.3.5) are called the Kolmogorov forward differential equations.

Hence, $\Psi(x,y,z;\, t)$ satisfies the following equations:

$$
\begin{aligned}
\Psi'(t) &= \partial\Psi \,/\, \partial t \\
&= \{\mu_1 xy + b_1 x^2 + d_1 - [b_1+d_1+\mu_1]x\}\; \partial\Psi/\partial x \\
&\quad + \{\mu_2 yz + b_2 y^2 + d_2 - [b_2+d_2+\mu_2]y\}\; \partial\Psi/\partial y
\end{aligned}
\tag{2.3.6}
$$

with the inital condition $\Psi(0)=x$ where the dependence of Ψ and Ψ' on x, y, z has been

suppressed for notational convenience. From (2.3.6) we have

$$\Psi'(1,1,0;t) = -\mu_2 \frac{\partial \psi}{\partial y}(1,1,0;t) \tag{2.3.7}$$

and thus

$$I(t) = \mu_2 E[Y(t) \mid T(t) = 0] \tag{2.3.8}$$

It should be noted that (a) the transition rates μ_1 and μ_2 are multiplicative factors and are important in determining the overall incidence rates of cancer in question (however, they do not influence the shape of the incidence curve), and (b) the shape of the incidence curve is determined by the growth curve of the normal tissue and the cellular kinetics of intermediate cells (Moolgavkar, 1986A; Moolgavkar and Knudson, 1981).

The MVK model has been widely used to analyze various experimental and epidemiological data (Moolgavkar and Knudson, 1981; Moolgavkar and Luebeck, 1990; Tan, 1991), and to interpret some important biological phenomena which include initation-promotion phenomenon (Moolgavkar and Luebeck, 1990; Tan and Gastardo, 1985), hormone effects in human female breast cancer (Moolgavkar, 1986B), familial human cancers (Moolgavkar, 1986A; Moolgavkar and Knudson, 1981; Tan and Singh, 1990), premalignant lesions (Dewanji et al, 1989 and 1991; Luebeck and Moolgavkar, 1991; Moolgavkar and Knudson, 1981), and *in vitro* analysis of carcinogenesis (Nettesheim et al, 1987). It appears that the initiation-promotion phenomenon is well explained by the two-stage models with the specification that initiators affect the rate of the first event while promoters facilitate the increase of the proliferation rate of intermediate cells. The familial human cancers are explained by the two-stage models by the feature that the first event of

the two-stage models may occur in germline cells.

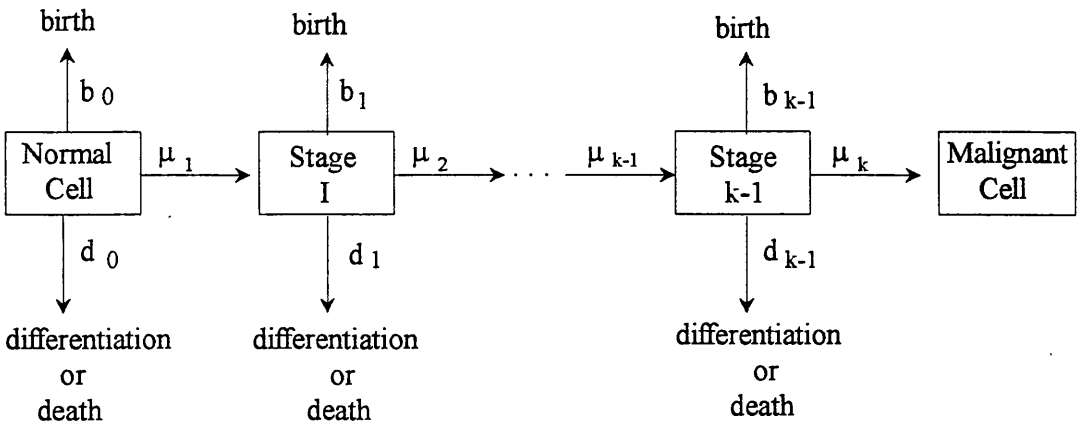
The MVK model may also be used to assess risks of environmental agents and to classify carcinogens (Luebeck et al, 1996; Moolgavkar, 1990; Moolgavkar et al, 1988, 1990A, 1990B and 1993; Van Ryzin, 1980; Tan, 1991).

2.4 Multievent model

As demonstrated in Chapter 1, cancers develop from individual normal cells which have undergone a series of changes affecting important genes. The number of mutations required depends on the tumour type and varies from 1 to 10 (Renan, 1993). This observation has led to the extension of the MVK two-stage model to a multistage model with cell proliferation and cell differentiation or death for normal stem cells and intermediate cells (Chu et al, 1987). This model is called as the multievent model to distinguish it from the classical Armitage-Doll multistage model.

A schematic representation of the multievent model is given in Figure 2.3. Like the Armitage-Doll multistage model, the transformation of a normal stem cell into a tumour cell requires a series of k sequential changes or stages. In addition, a cell in each stage may not only progress to the next more malignant-like stage, but may proliferate or differentiate/die.

The incidence rate of tumours at time t, I(t), can be derived by the same method for MVK model (for details see Tan (1991, chapter 6)).



Figuer 2.3 A multievent model involving k stages for carcinogenesis.

Recently, Moolgavar et al (1993) applied a multievent model to the development of human colon cancer and concluded that a three-event model provided a better description. Little (1995), studying radiation-induced cancers also found a three-stage model appropriate for several tumour types. Since the multievent models were only recently proposed, the implications of this category of model have not yet been fully worked out.

2.5 Multipath/multistage model

With recent progress in molecular biology, current research in cancer has moved beyond solely collecting tumour incidence data. Reseachers are able to obtain more sophisticated data that describe the mechanisms of tumorigenesis through a variety of experimental designs. The data collected from these experiments may include multiple stained enzyme-

altered premalignant and malignant focal lesions, labelling index, and other biomarkers. Schwarz et al. (1989) have observed heterogeneity between single phenotype lesions and multiple phenotype lesions (multiple stained lesions) in experiments where enzyme-altered foci were obtained from rat liver. A single-path process (multistage model) is not adequate to describe their observations, a multipath model is more appropriate. Observations in human colon tumorigenesis (Fearon and Vogelstein, 1990) also point to multiple pathways for the process of cancer. These observations have led Tan and Chen (1990), and Sherman and Portier (1994) to develop the multipath/multistage model.

A two-path/two-stage model is shown in Figure 2.4. There are two possible paths for a normal cell to be transformed into a malignant cell: (1) normal cell → initiated cell A → malignant cell and (2) normal cell → initiated cell B → malignant cell. A tumour develops independently by either of the two two-stage pathways.

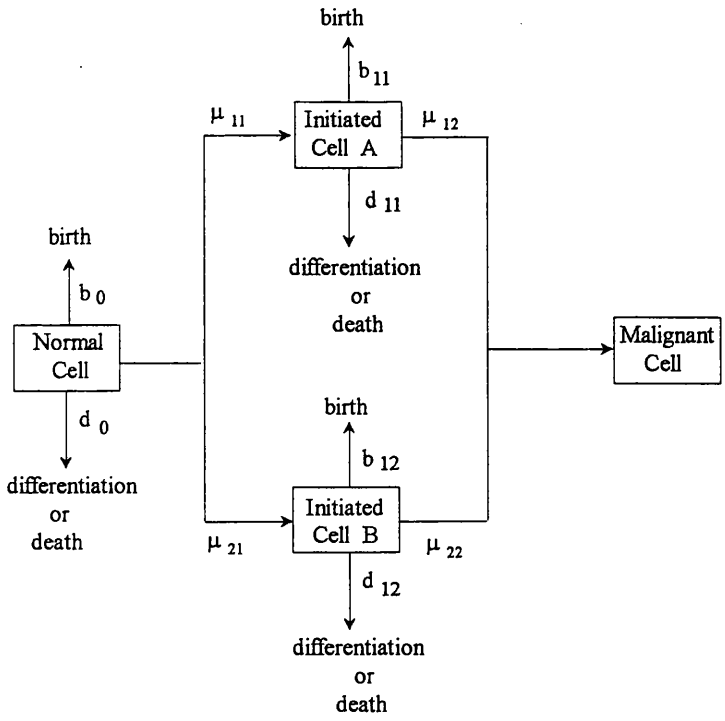


Figure 2.4 A two pathway model involving two-stage models of cacinogenesis.

For the model shown in Figure 2.4, it is also assumed that normal stem cell, intermediate cell A and intermediate cell B undergo growth via a linear birth-death process. As in the MVK model, it is also assumed that one malignant cell grows into a tumour with probability one. Let $P_1(t)$ and $P_2(t)$ be the probability of a malignant cell being generated via the upper and lower paths by time t, respectively. The probability, P(t), of a malignant cell being generated via by any of the two pathways is given by:

$$P(t)=[1-P_1(t)][1-P_2(t)]$$

Both two-path/two-stage and three-path/one-stage models have been used to describe the transformation of normal cells into single and multiple phenotype enzyme-altered cells in rat hepatocarcinogenesis by Sherman and Portier (1995). The three-path/one-stage model tends to fit the observed size distribution data for the multiple phenotype lesions better than the two-path/two-stage model. However, in both cases the fit of the models falls short of that for the single phenotype lesions.

2.6 Overview of present studies

Stochastic modelling of multistage tumorigenesis, reviewed as above, has been influential in consideration of general features such as number of stages but has not as yet provided insights at the molecular level.

Recent progress in molecular oncology, primarily the identification of candidate oncogenes and tumour suppressor genes in particular tumorigenic pathways has raised the possibility

of detailed mapping of some such pathways in molecular terms. In such investigations, it will be important to have analytic tools for experimental data processing which facilitate the testing of specific hypotheses regarding the tumorigenic pathway. In the investigations described in this thesis, mathematical tumorigenesis models will be developed, which are intended to help in the elucidation of tumorigenic pathways involving alterations to the structure or function of the p53 gene.

Prelimary modelling work was begun to explore p53 mediated spontaneous and radiation-induced tumorigenesis in mice (Chapter 3, 4, 5 and 6). The initial studies focused on the development of stochastic process (birth-death) models (extension of MVK model) in which 3 stages are required for malignant transformation of a somatic stem cell in a target tissue. These stages are intended to represent the inactivation of one and both p53 alleles with a third, genetically unspecified stage which may be composite. Then, tumorigenesis in wild-type ($p53^{+/+}$), p53 heterozygous ($p53^{+/-}$) and p53 homozygous null ($p53^{-/-}$) mice would require 3, 2 and 1 stage respectively. The model is intended to apply to all stages of mouse development post-conception and includes a stochastic reformulation of a deterministic 'Gomp-Ex' growth model (Wheldon, 1988) for stem cell number. Realistic parameter values were chosen where possible but the intention is to seek robust conclusions not heavily dependent on particular values. In the whole studies, we established a set of computer simulations for implementing all proposed models, explored the properties of these model in order to better understand the consequences of the model assumptions.

In Chapter 3, the work is confined to spontaneous tumorigenesis and involved exploration of the effect of changing number of stages, ultimate (i.e. steady-state) stem cell number and mutation rate on the timing of appearance of tumours. In Chapter 4, the model will

be extended to simulate the effect of single doses of irradiation. Irradiation was modelled as causing both transforming and lethal cellular mutations. The predicted dose-dependence of tumour latency for each genotype, for irradiation at different developmental stages, is examined, and the optimal time (age) of irradiation for maximum yield of tumours is predicted. In Chapter 5 and 6, attention is given to tumour multiplicity in non-irradiated and irradiated mice respectively in order to resolve a current paradox ($p53^{-/-}$ mice are seen to develop only a few tumors per mouse though all somatic stem cells are at risk). Both studies showed the model extended from the MVK models was not compatible with experimental data in p53 deficient mice.

Thereafter, two categories of explanation for this paradox were proposed. The first of these (Chapter 7), the multistage multipath model invokes a p53 independent pathway which exists in parallel with a p53 mediated route of tumorigenesis. The other, discussed in Chapter 8, which we called the multigate/multistage model postulated a single pathway (or gateway) with several gate-pass events (obigatory mutations) occuring at a rate which depends on p53 status.

Finally, the competitive tumour development in tissues that follow different growth patterns was introduced into multistage and multigate/multistage models and were used to explore the effect of growth pattern on tumour spectra (i.e tumours of differing pathological type) (Chapter 9). Some preliminary experimental studies, intended to estimate the *in vivo* mutation rate as a function p53 genotype, are described in Chapter 10.

Chapter 3

A Multistage Model for Tumorigenesis in Developing and Adult p53 Deficient Mice

3.1 Introduction

The study of molecular mechanism of tumorigenesis has been greatly enhanced in recent years by the advent of genetically engineered (transgenic) mice in which particular genes, thought to be important in the tumorigenic process, are mutated or deleted in the germ line (Fowlis and Balmain, 1993). Mice inheriting disrupted genes will, if the genes have been correctly chosen, require fewer mutational events in any somatic cell for malignant transformation to occur.

In particular, several laboratories have now developed transgenic mouse strains in which one or both normal copies of the p53 tumour suppressor gene have been inactivated in each somatic cell of the mouse (Donehower et al., 1992). Inactivation or mutation of p53 has been implicated in a wide range of tumour types in man (Caron de Fromental and Soussi, 1992; Hollstein et al., 1991) and in several animal species (Mowat et al., 1985; Levine et al., 1991). For tumours which develop via disruption of p53, and which usually require N mutations in total for malignant transformation, transgenic mice are available which have almost identical genotypes but require N, N-1 and N-2 mutational events for malignant transformation. The existence of mouse strains which differ only in the number of stages required for malignant transformation to any tumour type provides a powerful tool for the analysis of the tumorigenic process. In order to make use of this, it is important to have available theoretical models to study the influence of number of stages on tumorigenic processes. In this chapter, we will establish a model in which up to 3 stages are required to provide cellular malignant transformation. The stages are conceived as disruption of one or both p53 alleles, and an additional transformation event, which may

be composite. Analysis of experiments using p53 transgenic mice may help to reveal properties of the third stage which is initially introduced as a 'black box'. It is important also that the model should be able to represent the statistics of tumorigenesis in embryonic as well as post-natal mice, since transgenic mice are exposed to increased risk of tumorigenesis from time of conception onwards.

In this chapter, we will establish stochastic models for spontaneously occurring tumorigenesis in a one-, two- or three-stage mutational process in both growing (embryonic) and steady-state (adult) target tissue and explore the general properties of these models. In particular, it is intended to determine how the various parameters of the models within a reasonable range of values, affect the time (age) distribution of appearance of tumours, which is the most straightforward experimental observation. It is also intended to investigate the effect on the time distribution of number of mutational stages required for malignant change.

3.2 Model structure

The essential features of the three-stage model (Figure 3.1) are:

(1) Target cells for the first stage of malignant transformation are the stem cells of each tissue. Stem cells are presumed to replicate or differentiate/die (such as apoptosis) and to follow Gompertz-exponential or "Gomp-ex" growth kinetics during embryonic and post-natal development (see section 3.3 and 3.4). In the adult, the numbers of stem cells remain approximately steady-state (cellular loss rate balanced by cellular proliferation rate). Stem

cells are assigned to the initial compartment of the model and denoted $X_0(t)$.

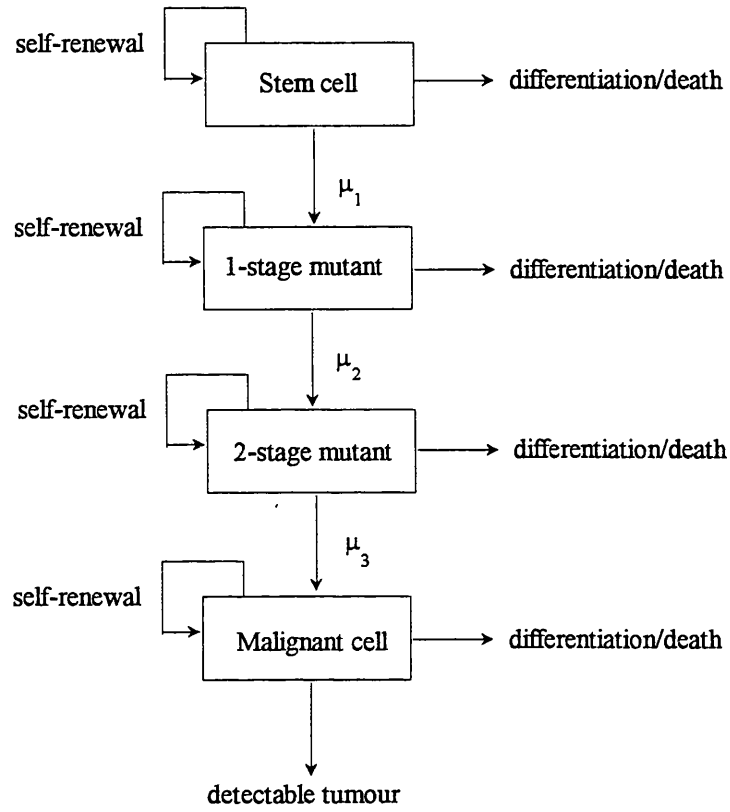


Figure 3.1 A three-stage model for tumorigenesis

(2) At each cell division, a daughter cell has a probability μ_1 of experiencing the first mutation leading to malignant change. These 1-stage mutants are assigned to the next compartment of the model and denoted $X_1(t)$. The 1-stage mutants are otherwise identical to stem cells. They are presumed to replicate or differentiate/die (such as apoptosis), and, in the embryo and post-natal development phase, follow Gomp-ex kinetics (see section 3.3 and 3.4), in the adult the cellular proliferation rate is equal to the cellular loss rate. The 1-stage mutants have no growth advantage.

(3) At each cell division, a 1-stage daughter cell has a probability μ_2 of experiencing the second mutation leading to malignant change. These 2-stage mutants are assigned to the next compartment of the model and denoted $X_2(t)$. The 2-stage mutants are also otherwise identical to stem cells. They are presumed to replicate or differentiate/die (such as apoptosis), and, in the embryo and post-natal development phase, follow Gomp-ex kinetics (see section 3.3 and 3.4), in the adult the cellular proliferation rate is equal to the cellular loss rate. The 2-stage mutants have no growth advantage.

(4) At each cell division, a 2-stage daughter cell has a probability μ_3 of experiencing the third - and final - mutation leading to malignant change. These 3-stage mutants are assigned to the next compartment of the model and denoted $X_3(t)$. A 3-stage mutant is a malignant cell. The malignant cells do have a growth advantage over all other cells. Their cellular proliferation rate is assigned a higher value than their cellular death rate (in the model this is done by setting the loss rate to zero).

(5) The cell cycle time of the stem cells, 1-stage mutants and 2-stage mutants remains the same and is not influenced by the mutational events.

(6) Detection of a tumour is recorded when the malignant cell population reaches 10^6 cells, provided a minimum time delay of τ_0 has elapsed. This allows for vascularization and morphogenesis of the tumour as well as growth of the cell population number.

The 1-stage and 2-stage carcinogenesis models have the same structure as the 3-stage model described above. They differ only in the number of mutational steps assumed necessary for malignant change.

3.3 Cell kinetic model for cell proliferation during mouse development

It is already known that growth of the whole mouse from conception onwards can be empirically described as initially following an exponential growth pattern which gives way to a Gompertzian pattern after some critical cell number N_c has reached, i.e., a Gompertz-exponential or Gomp-ex growth model (Wheldon, 1988), and it is reasonable to suppose that the various cell populations follow the same general pattern. Therefore if we consider a cell population N(t) we have, for the Gomp-ex growth model described by the piecewise continuous differential equation

$$\frac{1}{N(t)}\frac{dN(t)}{dt} = \begin{cases} a & t \le T_c \\ a - b * \ln[\frac{N(t)}{N_c}] & t \ge T_c \end{cases} \qquad (3.1)$$

for initial growth from a single cell with $T_c=\ln(N_c)/a$, where a represents the initial specific growth rate of the stem cell population (for N=1 when t=0) and b is the specific growth rate retardative parameter in the Gompertz equation.

However this formulation does not explicitly incorporate the cell division rate, which is important if mutational events occur preferentially in cells undergoing division. We need a cell kinetic model which explicitly includes the rate of cell division and also the rate of cell loss, but which gives the overall growth of the cell population to be same as for the Gompertz function. This problem was previously considered by Wheldon (1988) and the approach used here is similar to that analysis. A model which fits the stated requirements is

$$\frac{1}{N(t)}\frac{dN(t)}{dt} = \frac{0.693}{t_c} f[N(t)] - L \tag{3.2}$$

where t_c is the cycle time of proliferating cells, f[N(t)] is a function giving the proportion of cells proliferating at any time (the growth fraction) and L is a parameter giving the (constant) loss rate from population.

If we compare (3.1) and (3.2) we see that the new model will be identical to the Gomp-ex equation provided

$$f[N(t)] = \frac{t_c}{0.693} * \begin{cases} a + L & t \le T_c \\ [a - b * \ln(\frac{N(t)}{N_c})] + L & t \ge T_c \end{cases} \tag{3.3}$$

This provides the number of cells in the growth fraction as a function of the total cell number. We then have

Number of cells in cycle = f[N(t)]*N(t)

$$\text{Rate of cell devision} = \begin{cases} a + L & t \le T_c \\ a\, e^{-b(t-Tc)} + L & t \ge T_c. \end{cases}$$

For the three-stage model, $N(t)=X_0(t)+X_1(t)+X_2(t)$ and the numbers of stem cells, 1-stage mutants and 2-stage mutants in cycle are $f[N(t)]*X_0(t)$, $f[N(t)]*X_1(t)$ and $f[N(t)]*X_2(t)$, respectively. The same approach is used as for the two-stage model.

3.4 Outline of the simulation

The behaviour of a set of cells is followed using discrete events simulations (Maisel and Gnugnoli, 1972) in which the evolution of the population of cells is updated at each cell division. The simulation proceeds until a detectable tumour is generated, i.e. the population of malignant cells reaches the number 10^6 which corresponds to the smallest detectable tumour. Simulations were terminated by 600 days (see appendix 3).

3.4.1 Cellular behaviour at each division

(1) Stem cells: at each cell division, one stem cell may divide into two stem cells with probability $\alpha_0(t)$; may be lost with probability $\beta_0(t)$; may divide into one stem or lost cell and one 1-stage mutant with probability $\mu_1(t)$; or may divide into one stem cell and one lost cell with probability $\gamma_0(t)$ (Figure 3.2 (a)). The parameters $\alpha_0(t)$, $\beta_0(t)$, $\mu_1(t)$ and $\gamma_0(t)$ satisfy

$$\alpha_0(t)+\beta_0(t)+\mu_1(t)+\gamma_0(t)=1.$$

The numbers of cells undergoing these processes conform to a multinomial distribution (approximate Poisson or normal distribution when the number of stem cells is very large).

(2) 1-stage and 2-stage mutants: these cells have the same behaviour as stem cells (Figure 3.2 (b) & (c)). The rate parameters for 1-stage mutants are denoted $\alpha_1(t)$, $\beta_1(t)$, $\mu_2(t)$ and $\gamma_1(t)$; those for 2-stage mutants are denoted $\alpha_2(t)$, $\beta_2(t)$, $\mu_3(t)$ and $\gamma_2(t)$, respectively. The numerical values of these parameters are the same as those of stem cells.

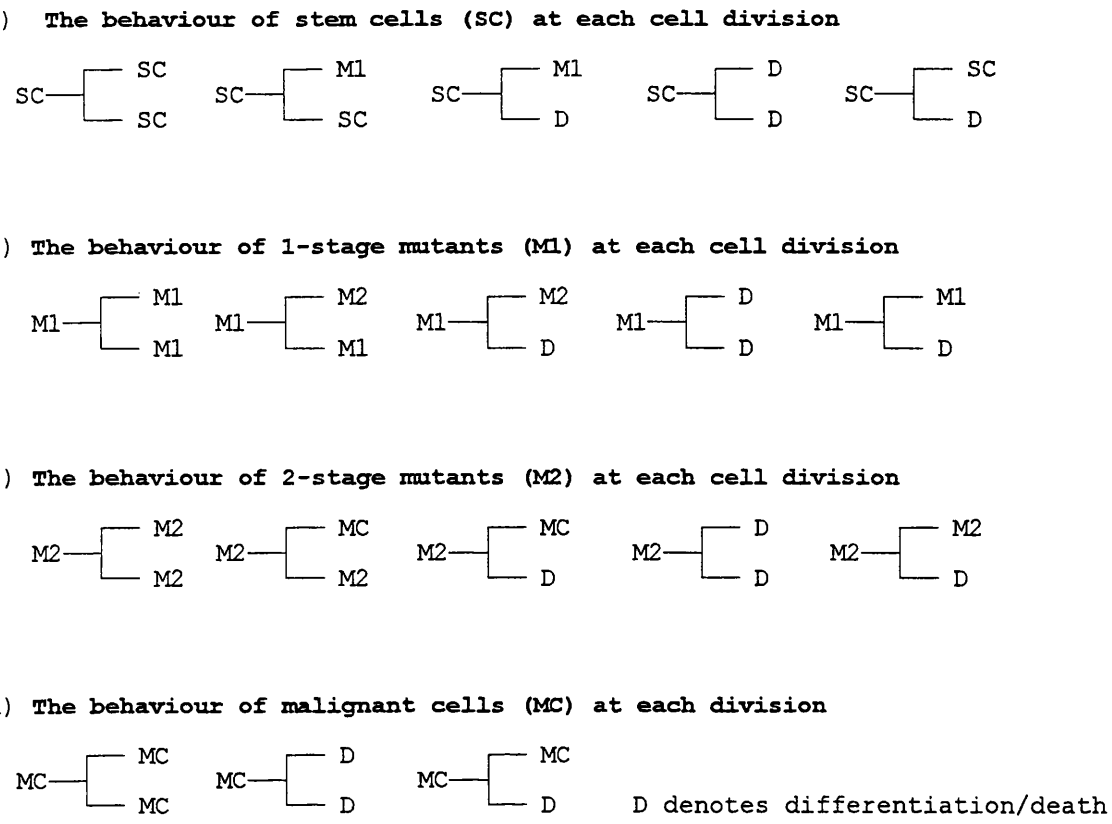


Figure 3.2 The behaviour of stem cells and stem cell mutants

(3) Malignant cells: at each cell division, one malignant cell may divide into two malignant cells with probability $\alpha_3(t)$; may be lost with probability $\beta_3(t)$; or may divide into one malignant cell and one lost cell with probability $\gamma_3(t)$ (Figure 3.2 (d)). The parameters $\alpha_3(t)$, $\beta_3(t)$ and $\gamma_3(t)$ satisfy

$$\alpha_3(t)+\beta_3(t)+\gamma_3(t)=1.$$

The numbers of cells undergoing these events again conform to a multinomial distribution (approximate Poisson or normal distribution when the number of malignant cells is very large).

In the simulation study, the parameter values are

$a = 1.063\ day^{-1}$

$b = 0.231\ day^{-1}$

$t_c = 0.4$ day

$L = 0.347\ day^{-1}$

$N_c = 10^6$ i.e. $T_c = 13$ days

chosen to be representative of cell kinetics in the developing mouse. The population achieves 35% of its final value (~ 10^8 cells) by 20 days (time of birth). The final steady state value corresponds to the number of target cells in a representative tissue in the adult mice (eg bone marrow or epidermis).

$\beta_0(t) = L^* t_c$,

$$\alpha_0(t) = t_c^* \begin{cases} a + L & t \leq 13 \text{ days} \\ a\, e^{-b(t-13)} + L & t \geq 13 \text{ days} \end{cases}$$

$\alpha_1(t) = \alpha_2(t) = \alpha_0(t)$ and $\beta_1(t) = \beta_2(t) = \beta_0(t)$

$\mu_1(t)$, $\mu_2(t)$ and $\mu_3(t)$ are constant and varied, and

$\alpha_3(t)$ is constant and equals to $\alpha_1(t_T)$, where t_T is time when malignant cell arose

$\beta_3(t)=0$.

Where the time-dependence of $\alpha_0(t)$ represents the slowing of cell division as embronic growth proceeds (see section 3.2).

3.4.2 Generation of random variables

The pseudo-random number generator was described by Wichmann and Hill (1982). The methods for generating the binomial, multinomial, Poisson and normal distribution variables are given by Devroye (1986).

3.4 Results

Each simulation begins with a single cell in the initial compartment and no cells in the others, i.e., the simulation follows growth and development from a fertilized ovum. The model structure is applicable to any mammalian species but kinetic parameters were initially chosen to represent mouse development. For each set of values of the parameters, the simulation repeats 1000 times. A useful figure of merit in analysing simulation data is the T_{50} value: the time until tumours are detected in 50% of the individuals. In situations where tumour incidence is low, the T_5 value is used instead.

3.4.1 General findings

After 70-day growth from conception, the total cell population in the target tissue remains approximately steady-state (Figure 3.3). Interestingly, a detectable tumour does not appear in any mice until a few days after birth (assuming 20 days gestation for mice) even for the one-stage model with a high mutation rate of 10^{-4} (Figure 3.4). When the mutation rate at each stage is low, for the 2-stage or 3-stage model, tumours are detected only in a small proportion of mice, however long the simulation time.

The most important parameters affecting the age-incidence of tumour appearance are the mutation rate at each stage, the steady-state number of target cells and the number of mutational events necessary for malignant change.

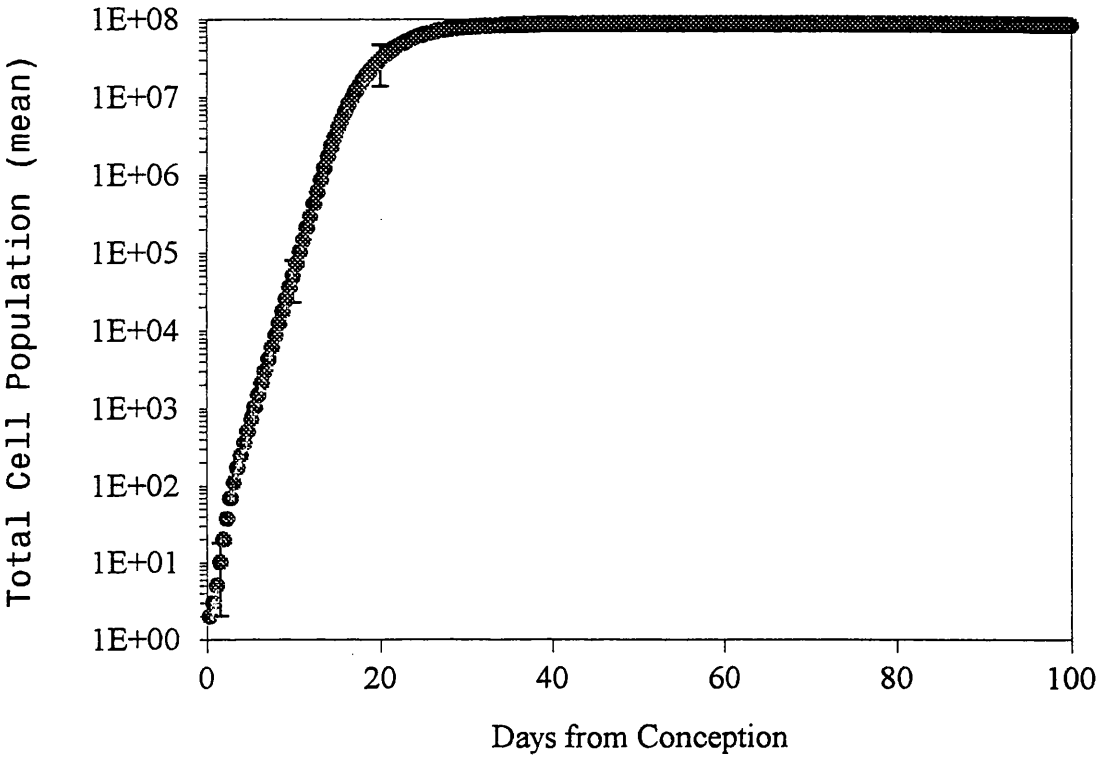
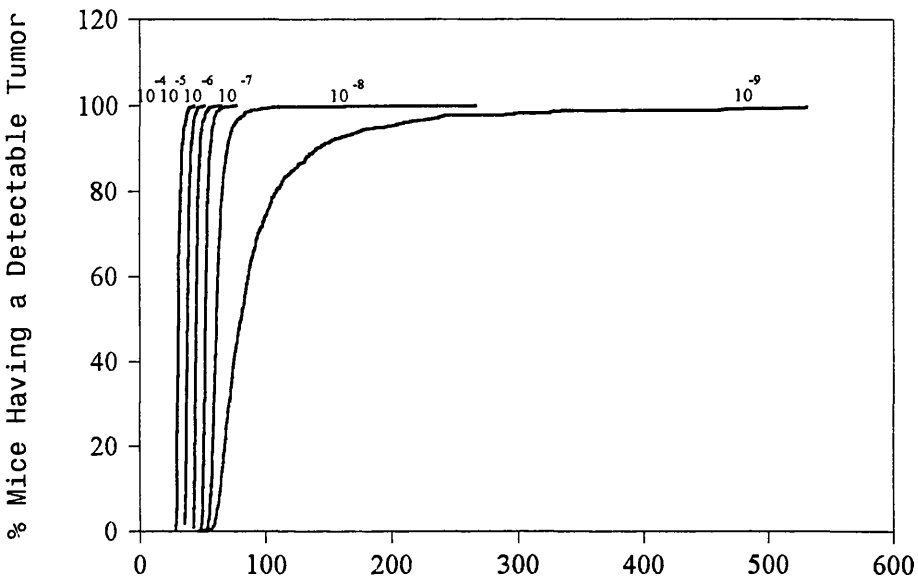


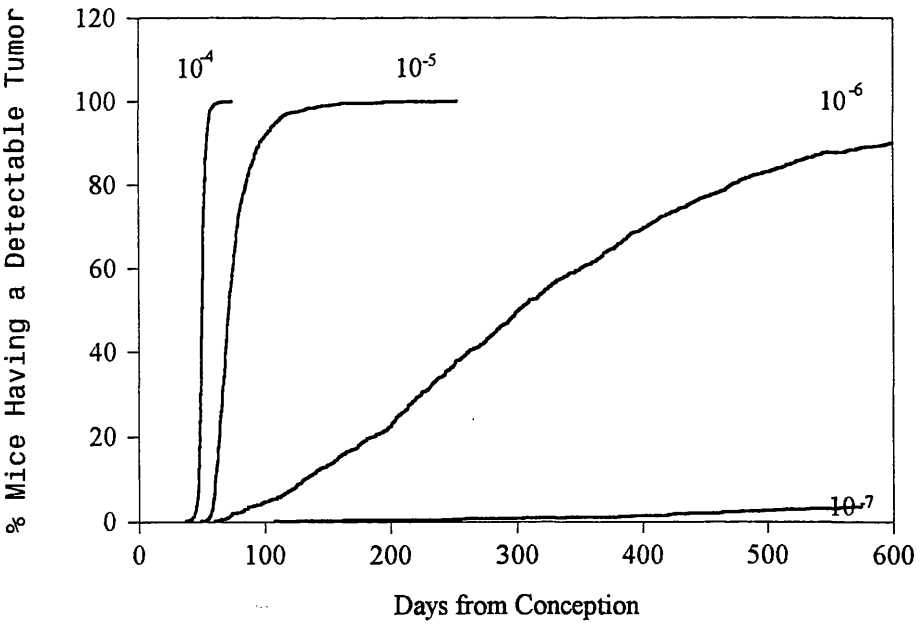
Figure 3.3 Kinetics of growth of a representative mouse tissue from conception to maturity with standard deviations shown at low, intermediate and high cell populations.

3.4.2 Mutation rate

For all simulations, increasing mutation rate leads to a steeper and left-shifted (earlier) age-incidence curve (Figure 3.4). For lower mutation rates, the T_{50} value is steeply decreasing with mutation rate; at higher values the relationship tends to flatten out (Figure 3.5).

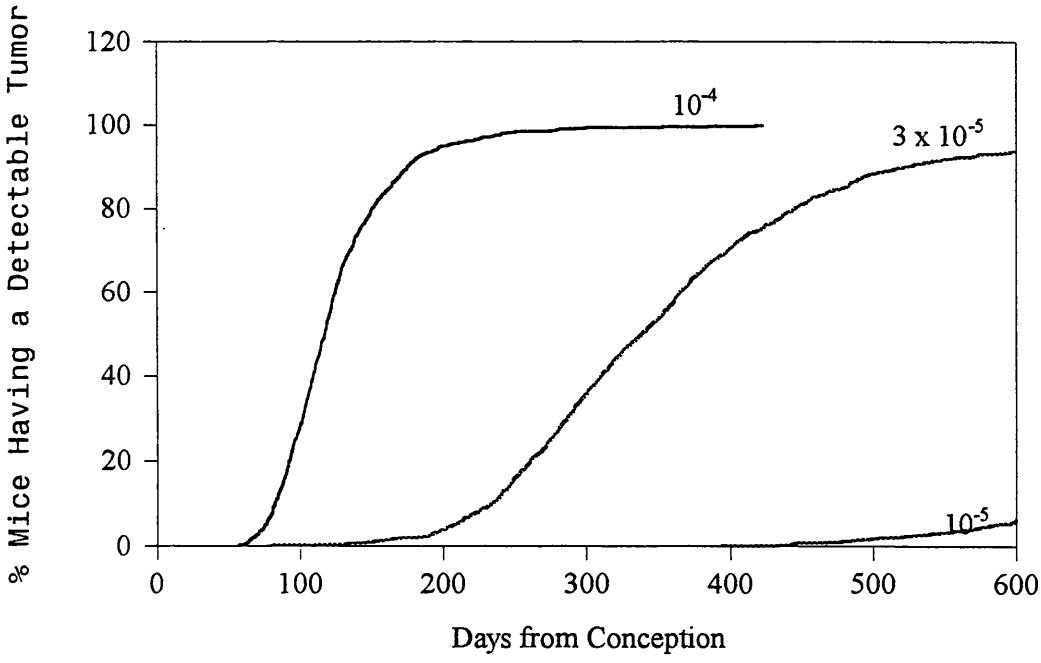


(A) One-stage model



(B) Two-stage model

(Figure 3.4 to be continued)



(C) Three-stage model

Figure 3.4 Kinetics of appearance of tumours in mice. (A) one-stage model; (B) two-stage model; (C) three-stage model. The graphs are labelled by the value of spontaneous mutation rate. The mutation rates in each stage are same and are labeled in graphics.

3.4.3 Target cell number

For all models the T_{50} value is less for higher steady-state target cell number. However the steepness of this relationship is strongly dependent on the mutation rate. With low values of mutation rate, the T_{50} value declines rapidly with increasing target cell number; at higher values this relationship also tends to flatten out (Figure 3.6).

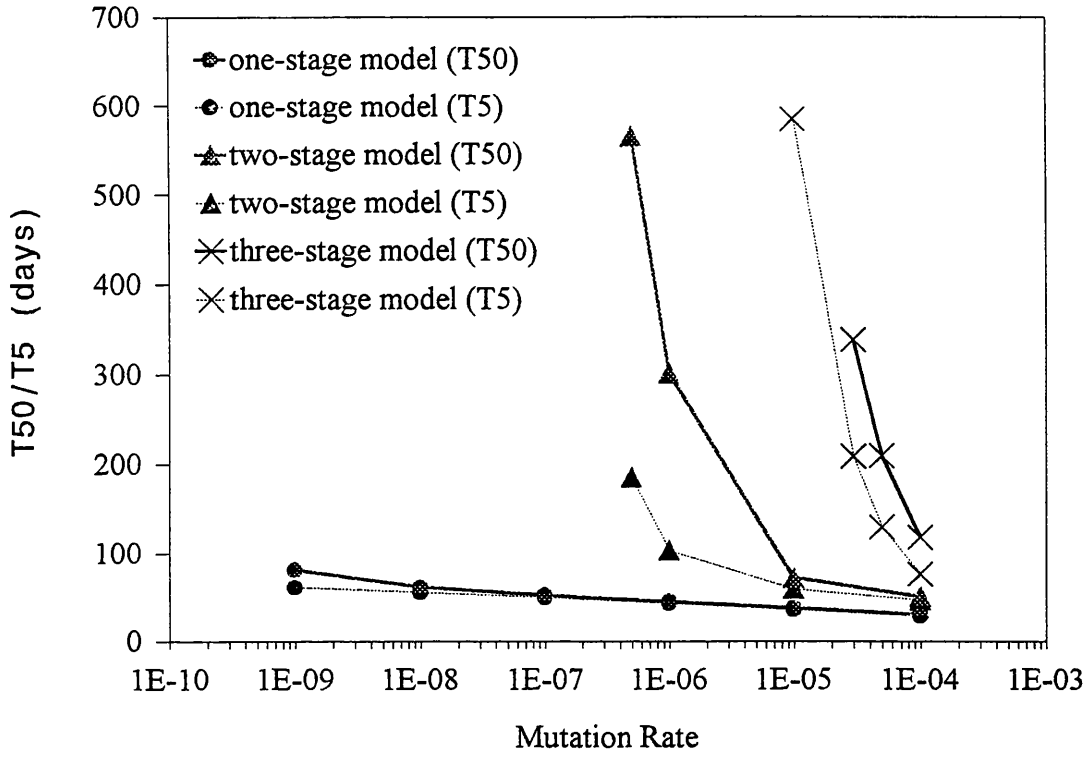
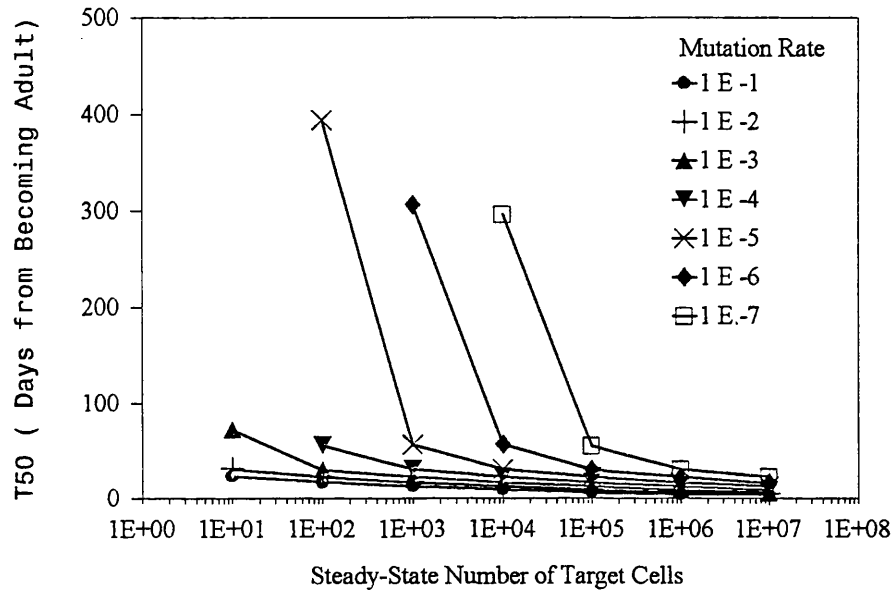


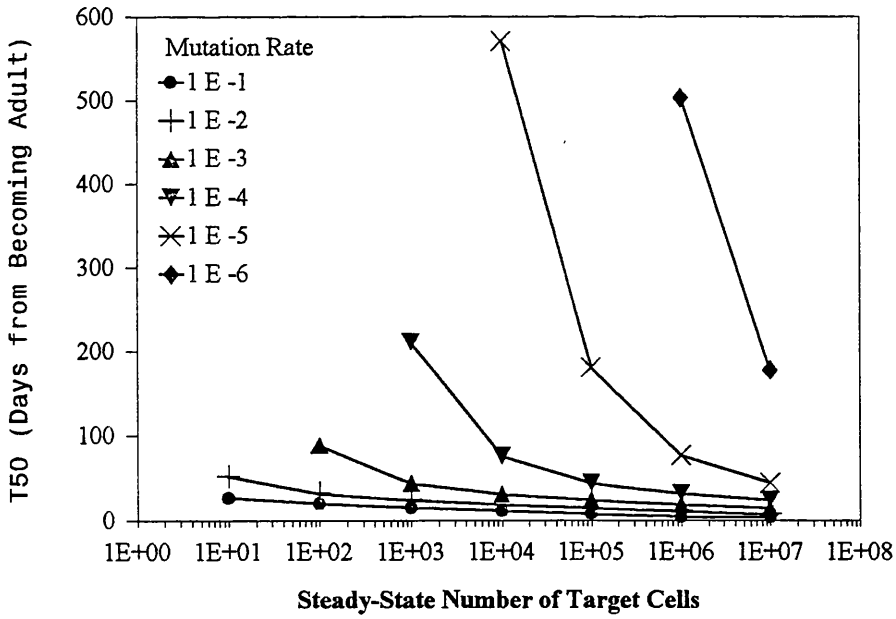
Figure 3.5 Time to appearance of first tumour in 50% mice (T50) and 5% mice (T5). The mutation rates in each stage are same.

3.4.4 Mutational events

With increasing numbers of mutational events required (1 to 3) the age-incidence curve moves to the right (later) (Figure 3.4) and the T_{50} value increases. However the difference is less marked for higher mutation rates (Figure 3.5).



(A) One-stage model



(B) Two-stage model

(Figure 3.6 to be continued)

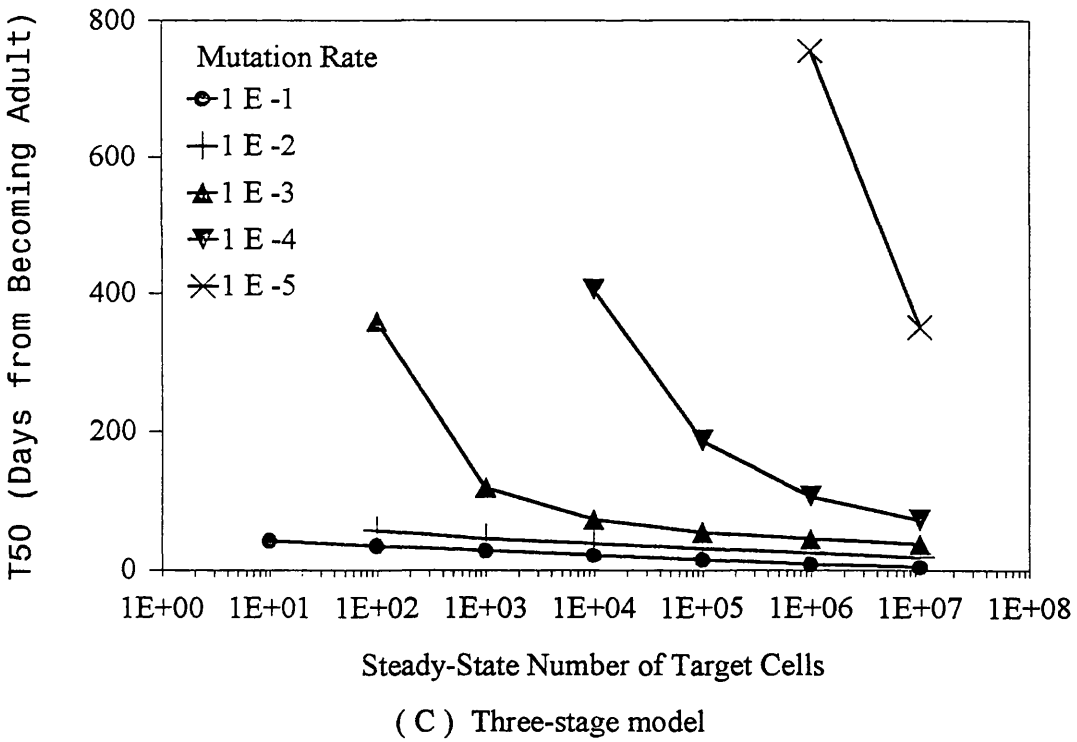


Figure 3.6 Time to appearance of the first tumour in 50% mice (T50). (A) one-stage model; (B) two-stage model; (C) three-stage model. The mutation rates in each stage are same.

3.5 Discussion

Besides the features that have been incorporated in early models considered by Moolgavkar and colleagues (1979, 1981), the models used in this study take into account the number of cells in cycle (growth fraction) during mouse development. This is important because most tumours probably originate from these renewing cell populations (Buick and Tannock, 1992). We assume that the growth fraction is a function of the total cell number. In the embryo, most cells are in cycle whereas in the adult, in which net growth has ceased, only a small proportion of the cells are actively proliferating to balance the cell loss.

There is no doubt that carcinogenesis is a multistage process due to sequential mutations (or equivalent epigenetic events) of specific genes. The number of mutations required for genesis of specific cancers is not definitely known, but is unlikely to be fewer than two and may be as many as ten (Renan, 1993). Here, we have developed one- two- and three-stage models in order to study some general features. As expected, larger numbers of mutational stages lead to later appearances of tumours, but the dependence of latency on stages is most clearly seen at lower than higher mutation rates. This observation may be important in future work.

Albanes and Winick (1988) proposed that, within any one species, cancer risk is proportional to both cell number and the rate of cell division. Carcinogenesis can be increased by either increasing the number of cells at risk or increasing the mitotic activity of a tissue. Our studies have also shown the more tumours occur with increasing the target cell number, however this effect is influenced by the spontaneous mutation rate and number of mutational events required for malignant transformation.

In general, the parameters examined, i.e., mutation rate, target cell number and number of mutational events, affected the age-incidence curves in the expected fashion. However, a striking observation was that the effects of each of the parameters were less dramatic at higher than lower mutation rates. The mutation rate itself was less influential once a higher range of mutation rates was reached. Physically, this may be because cells accumulate rapidly in the malignant cell compartment at higher mutation rates and the cellular growth rate becomes the most important parameter. This observation may be especially important for the analysis of tumorigenesis in p53 deficient transgenic mice since it is presently believed that p53 inactivation may result in genetic instability, i.e., in higher mutation rates

applicable to the remaining stages of tumorigenesis (Livingstone et al, 1992; Yin et al; 1992).

The present findings also suggest that the role of target cell number (as measured by its final steady-state value) is also most clearly seen at lower rather than at higher mutation rates. This could be applicable to experiments in which (for example) variable numbers of p53 deficient bone marrow stem cells are transplanted into wild-type mice, and when target cell number is effectively changed by killing by radiation and drugs.

Chapter 4

A Multistage Model for Radiation Tumorigenesis in p53 Deficient Mice: the Effect of Single Doses of Radiation

4.1 Introduction

Radiation produces a wide spectrum of lesions in the DNA of cells (Hutchinson, 1993). These lesions may be repaired or misrepaired (Elkind, 1984; Price, 1993). The lack of repair, or misrepair, can lead to chromosomal aberration, further inducing mutations and malignant transformation, and eventually giving rise to tumours.

In order to assess the quantitative cancer risk of exposure to radiation, many theoretical models have been developed to construct the dose-response relationship for tumorigenesis (UNSCEAR, 1986). These models, however, are often empirical rather than incorporating the biological mechanisms known to exist. In this chapter, it is intended to incorporate the effects of irradiation, i.e., cell killing and mutation, in a model established for spontaneous tumorigenesis in Chapter 3 and to investigate the carcinogenic effect of single doses of radiation. In particular, it is intended to explore how age at exposure, spontaneous mutation rate and number of mutational events required for neoplastic transformation influence the appearance of tumours and the dose-response relationship of tumour latency. The model will be used to predict the optimal time (age) of radiation for tumorigenesis.

4.2 Model and assumptions

Radiation tumorigenesis, like spontaneous tumorigenesis, is multi-stage (Cox, 1994; Fry et al., 1982). The stages of radiation tumorigenesis are believed to be same as those of spontaneous tumorigenesis. The role of radiation in this whole process is induction,

producing mutations, and acceleration, shortening the latency period. A major effect of radiation is the deletion of DNA sequences, which frequently result in cell death. In particular, clonogenic cells — cells which are usually capable of prolonged proliferation — may be sterilised, or deprived of their proliferative ability. In radiobiology, this is considered synonymous with 'cell killing'. However, some cells will survive and may have undergone mutations predisposing to malignancy, represented here as the migration of cells from their current stage to next one.

As discussed in Chapter 3, we will consider a 3-stage tumorigenesis model which requires inactivation of each of two p53 alleles and one other mutational step. The third step is envisaged as a 'black box' and may prove to be composite. The availability of p53 deficient transgenic mice (heterozygote and homozygote null) means that the number of stages required for tumorigensis may be reduced from 3 to 2 or 1, facilitating study of the tumorigenic process. The structure of three-stage radiation tumorigenesis model is schematically represented in Figure 4.1.

4.2.1 General assumptions

For the model shown in Figure 4.1, it is assumed that, before exposure to single dose radiation, the tumorigenic process is spontaneous, so the features of model is same as described in section 3.2 in Chapter 3. At time zero, corresponding to fertilization, only a single stem cell (fertilized ovum) is present. Post radiation, cells at different stages may be killed or survive. Amongst surviving cells, some may be migrated to the next mutational stage. Suppose that there are no surviving cells which experience two tumorigenic mutations by radiation at single exposure. The total cell population is reduced by the killing effect of radiation although repopulation by surviving cells will occur.

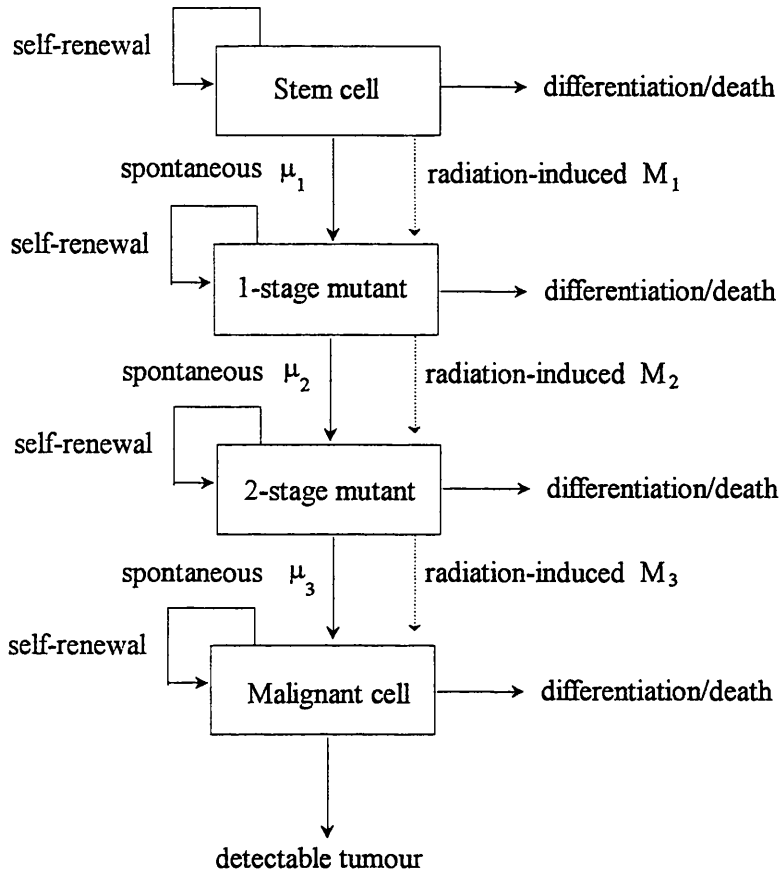


Figure 4.1 Three-stage model for radiation tumorigenesis

The one- and two-stage radiation tumorigenesis model have the same structure as the three-stage model. They differ only in the number of mutational events necessary for malignant change.

4.2.2 Cell surviving fraction

Numerous radiobiological models, which predict the probability of cell survival post

radiation, have been proposed, such as linear-quadratic model (Hall, 1994), repair-misrepair (RMR) model (Tobias 1985), lethal-potentially lethal (LPL) model (Curtis, 1986), etc.. In this study, we will make use of the linear-quadratic model, for which the probability of cell survival (S) is predicted as

$$S=\exp\{-(a_1 D+ a_2 D^2)\}$$

where D is a single dose, and a_1 and a_2 are cell survival parameters. In this study we will assume a_1 and a_2 values which are typical of cells growing exponentially in culture (a_1 =0.3 Gy^{-1} and a_2=0.03 Gy^{-2}) (Leenhouts and Chadwick, 1989).

Also, we assume that stem cells, 1-stage mutants, 2-stage mutants and malignant cells have the same probability of surviving post radiation, i.e., the malignancy-predisposing mutation does not alter the radiosensitivity of the cells.

4.2.3 Mutation frequency

The dose response relationship for tumorigenic mutation is not well established. However, it is reasonable that the probability that any cell carries a mutation of given (tumorigenic) type should be approximately linear at lower doses, but asymptotic to unity at higher dose. Suppose that mutation frequency (M) per surviving cell is given by

$$M=1-\exp\{-q D\}$$

where D is a single dose, and q=0.001 Gy^{-1} is a reasonable parameter value (from study by Leenhouts and Chadwick, 1989). Notice that for small values of q and lower dose,

$$1-\exp\{-q D\}\approx q D$$

i.e., mutation frequency is approximately linear with dose in the low dose range.

We assume that the mutation frequency of surviving stem cells, surviving 1-stage mutants and surviving 2-stage mutants is the same ($M_1=M_2=M_3=M$) , i.e., no change in genomic stability as a result of acquiring the first mutations (in future studies, we shall relax this condition).

4.2.3 Repopulation process

The cell population is reduced because of cell killing. The repopulation process is assumed to be same as that of normal development (Figure 4.2), i.e., an exponential growth process when the population is well below steady-state value, followed by Gompertzian growth as the original steady-stae value is approached (see Section 3.3 in Chapter 3).

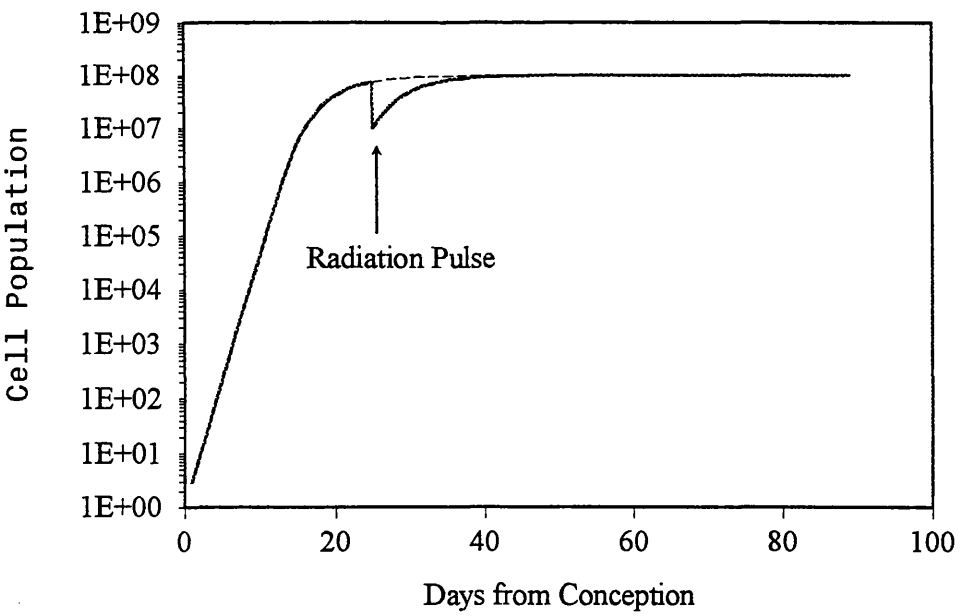


Figure 4.2 Kinetics of repopulation following radiation: this is initially exponential but becomes Gompertzian as population number increases and recovers with similar kinetics following single dose radiation.

4.3 Computer simulation study

In this analysis, radiation-induced tumorigenesis is represented by a pulse, or perturbation in numbers of cells at various stages, due to the brief (effectively instantaneous) radiation exposure (Figure 4.2). Post radiation, the numbers of cells that survive and surviving cells that undergo mutation are simulated as a Poisson distribution with expectation XS and YM, respectively, where X and Y represent the number of cells just before radiation and the number of surviving cells at each stage, S and M are given in Section 4.2.2 and 4.2.3, respectively. The whole process is simulated as described in Chapter 3. The value of the parameters is same as that given in Chapter 3. For each set of parameters, 1000 cases are simulated. T_{50}, i.e the time until tumours are detected in 50% of the individuals, is used to present the dose-response relationship.

In this study, we have used a computer simulation model rather than seeking analytic results as can be done for simpler models of this type. The advantage of the computer simulation approach is its generality. Although used only for single dose irradiation here, we will in future consider more complex patterns of irradiation for which analytic solutions would not be possible.

4.3.1 General findings

Usually, a single dose of radiation results in earlier appearance of tumours, but when the spontaneous mutation rate is higher and age at exposure is late, irradiation does not noticeably decrease the tumour latency (Figure 4.3, 4.4, 4.5 and 4.6) despite increasing the

number of mutants. When tumorigenesis is 2- or 3-stage with lower values of the spontaneous mutation rate at each stage, tumours are detected only in a small proportion of mice. For example, if tumorigenesis is 3-stage with a spontaneous mutation rate of 10^{-6} at each gene locus per cell division at each stage, only about 0.3% of the mice have tumours during a 600-day period after 6 Gy irradiation is given at neonatal age. This theoretical prediction is consistent with a very low rate of tumorigenesis for wild type mice (with intact p53 alleles); on the model this means three stages are required for transformation (Kemp et al., 1994).

4.3.2 Dose-tumour-latency relationships

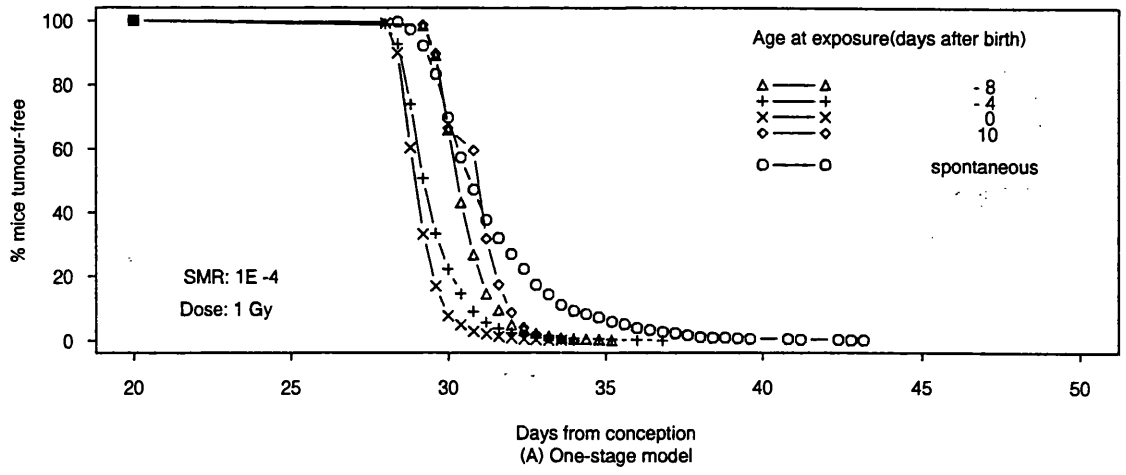
The dose-tumour-latency relationships for tumorigenesis post single whole-body exposure are influenced by age at exposure, the spontaneous mutation rate and the number of mutational events required for malignant transformation (Figure 4.4), but they may be grouped under three categories of relationship:

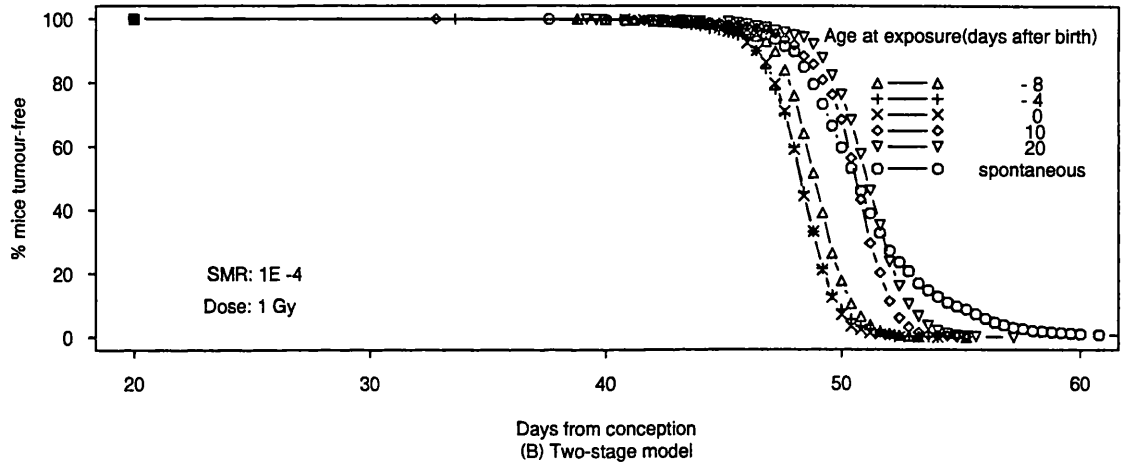
(1) T_{50} decreases with increasing dose down to a minimum, with an increase following that minimum (the increase is due to the increasing role of cell killing);

(2) T_{50} is flat with increasing dose;

(3) T_{50} increases with increasing dose.

Contrary to 'common sense' expectation, the tumour frequency does not increase as a simple function of dose in all cases. Only in a limited class of scenarios would be a clear dose-response relationship be observable in experimental studies.





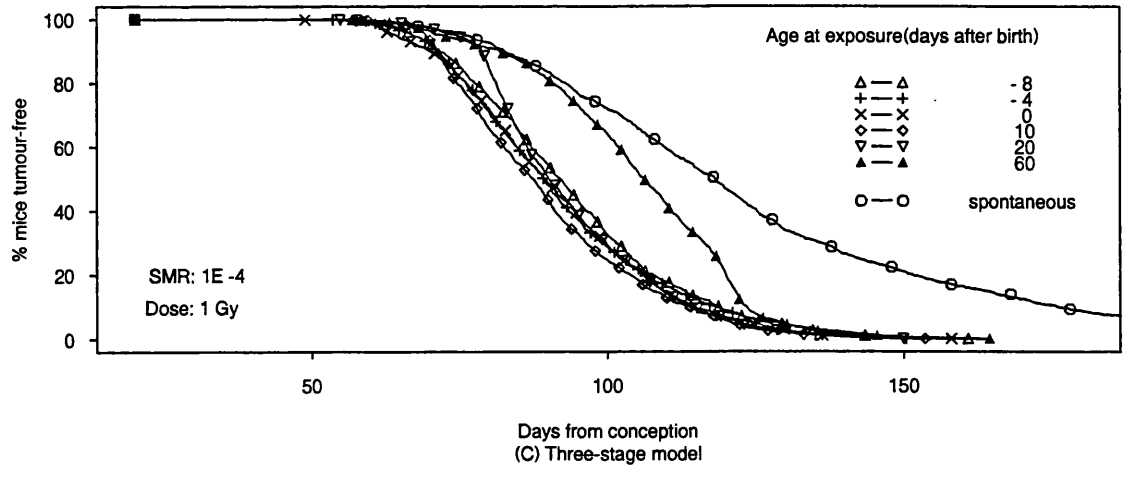


Figure 4.3 Kinetics of appearance of tumours in mice induced by single dose radiation. SMR: spontaneous mutation rate (per generation per cell)

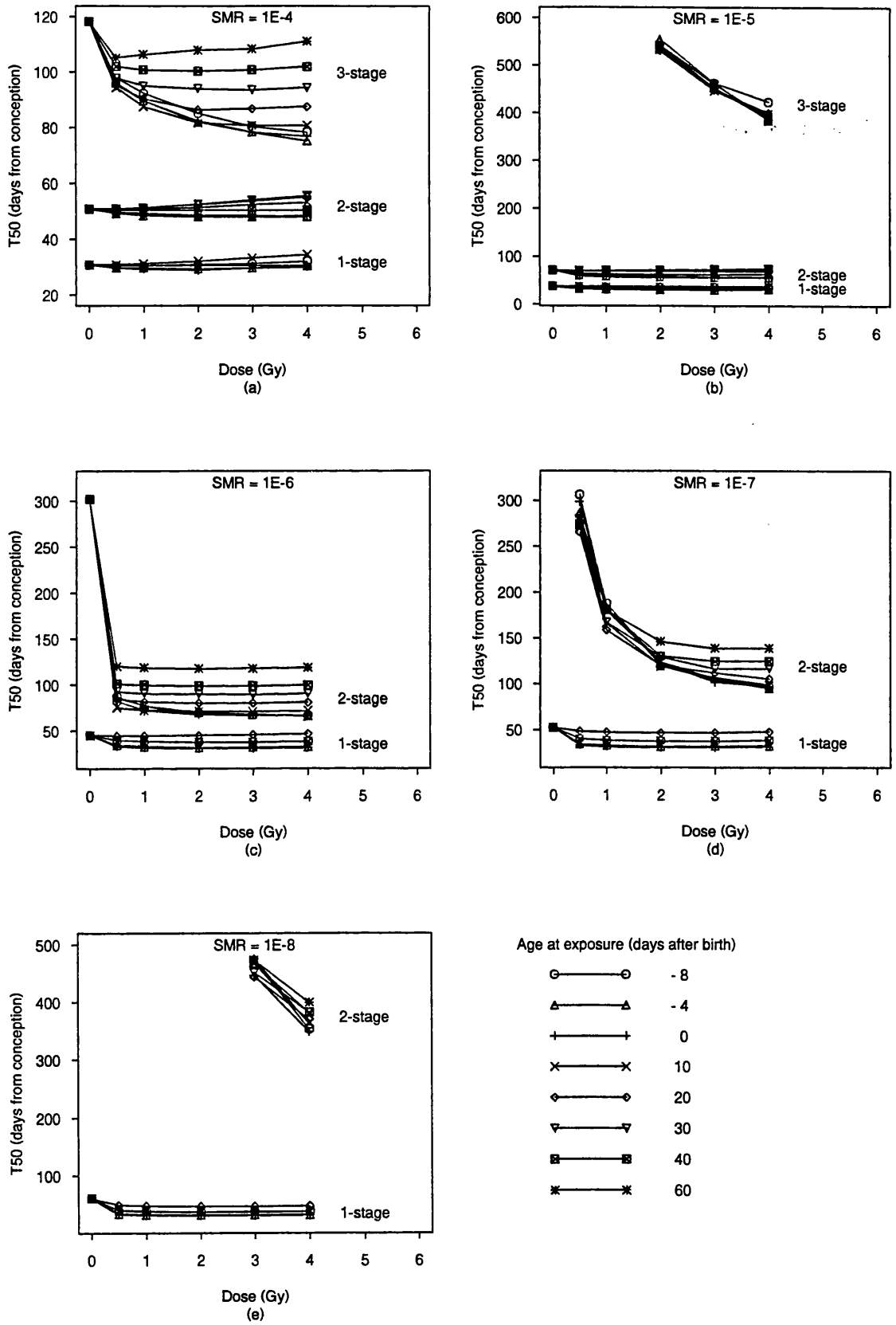


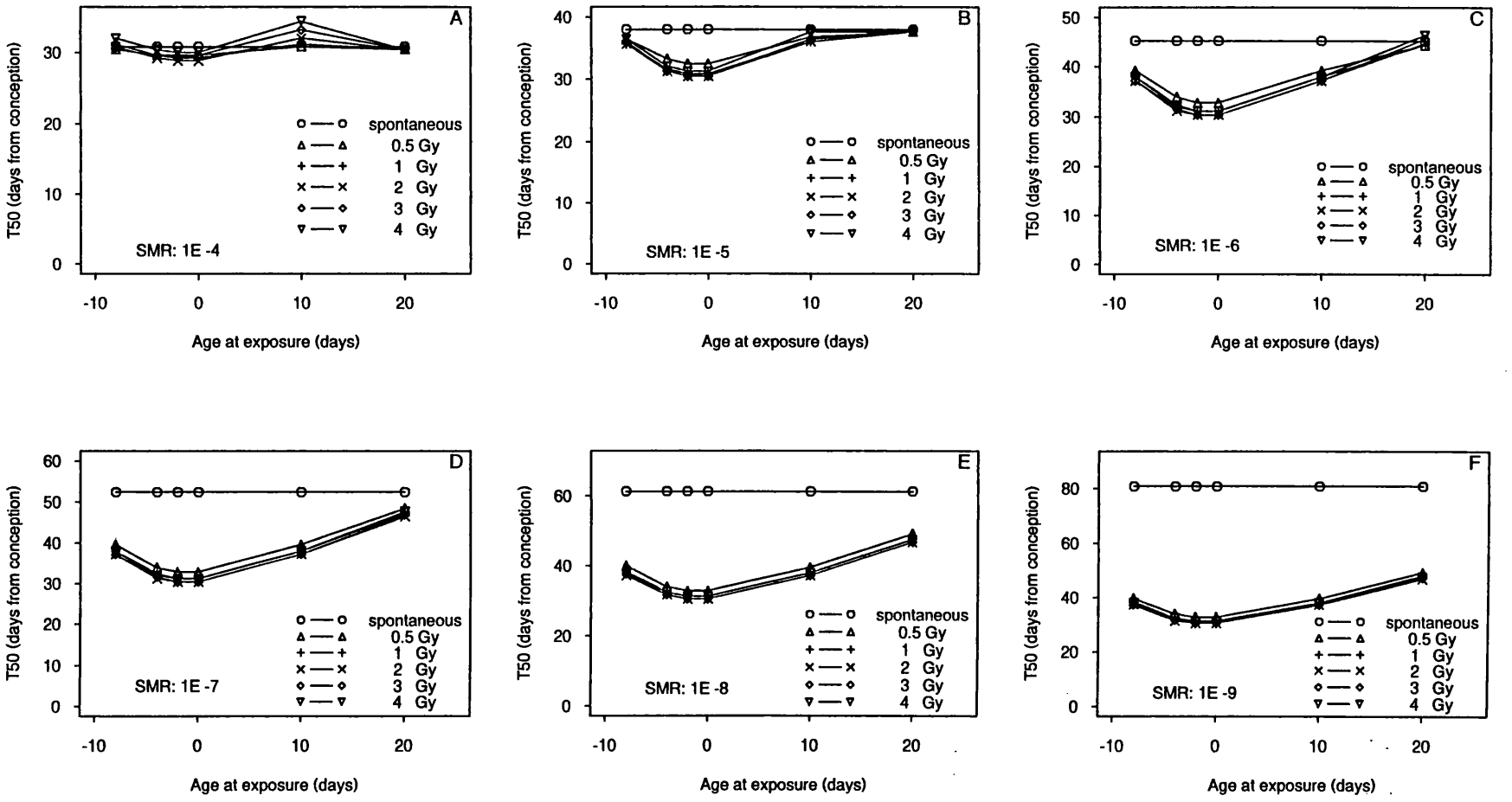
Figure 4.4 Dose-tumour-latency T_{50} (time of appearance of first tumour in 50% of mice) relationship post exposure to a single dose of radiation. SMR: spontaneous mutation rate (per generation per cell)

4.3.3 Age at exposure

In the simulation study, the T_{50} value decreases with increasing age at exposure down to a minimum, with a slight increase following that minimum for one-stage tumorigenesis (Figure 4.5, A to F). However, for two- and three-stage tumorigenesis, this appearance only occurs at higher values of spontaneous mutation rate. With lower values, T_{50} tends to flatten out (Figure 4.5, I to N).

In the following simulation, the spontaneous mutation rate was allowed to vary between stages. Interestingly, for 2- and 3-stage tumorigenesis, when the spontaneous mutation rate in the primary stage is lower, and in the later stages is higher, T_{50} increases with age at exposure (Figure 4.6, 1A, 1B, ..., & 1J). But, conversely, with higher spontaneous mutation rate in the primary stage and lower spontaneous mutation rate in later stages, the results are critically different for three-stage tumorigenesis, T_{50} always decreases with increasing age at exposure (Figure 4.6, 2J), whereas for two-stage tumorigensis, T_{50} rapidly decreases down to a minimum, then increases (Figure 4.6, 2A, 2B, ..., & 2I). These results suggest that the spontaneous mutation rate in later stages may be much more important.

The optimal time (age) of irradiation, i.e., age at exposure when the minimum value of T_{50} is reached, is in the neonatal stage and slightly varies with dose, spontaneous mutation rate and number of mutations required for tumorigenesis. When mutation rate is very low in each stage of two-stage and three-stage model, it is difficult to tell the optimal age of radiation tumorigenesis.



(Figure 4.5 to be continued)

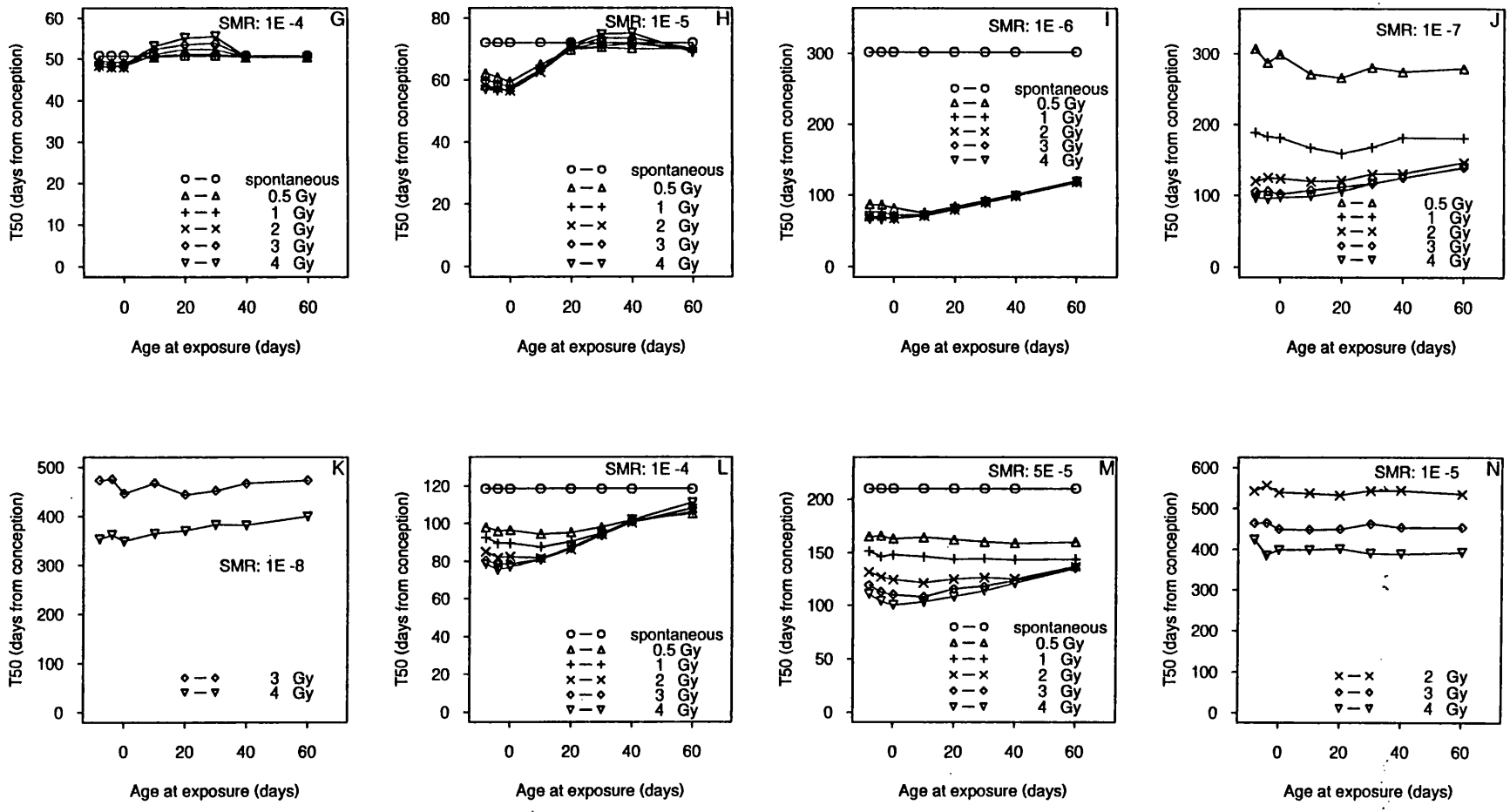
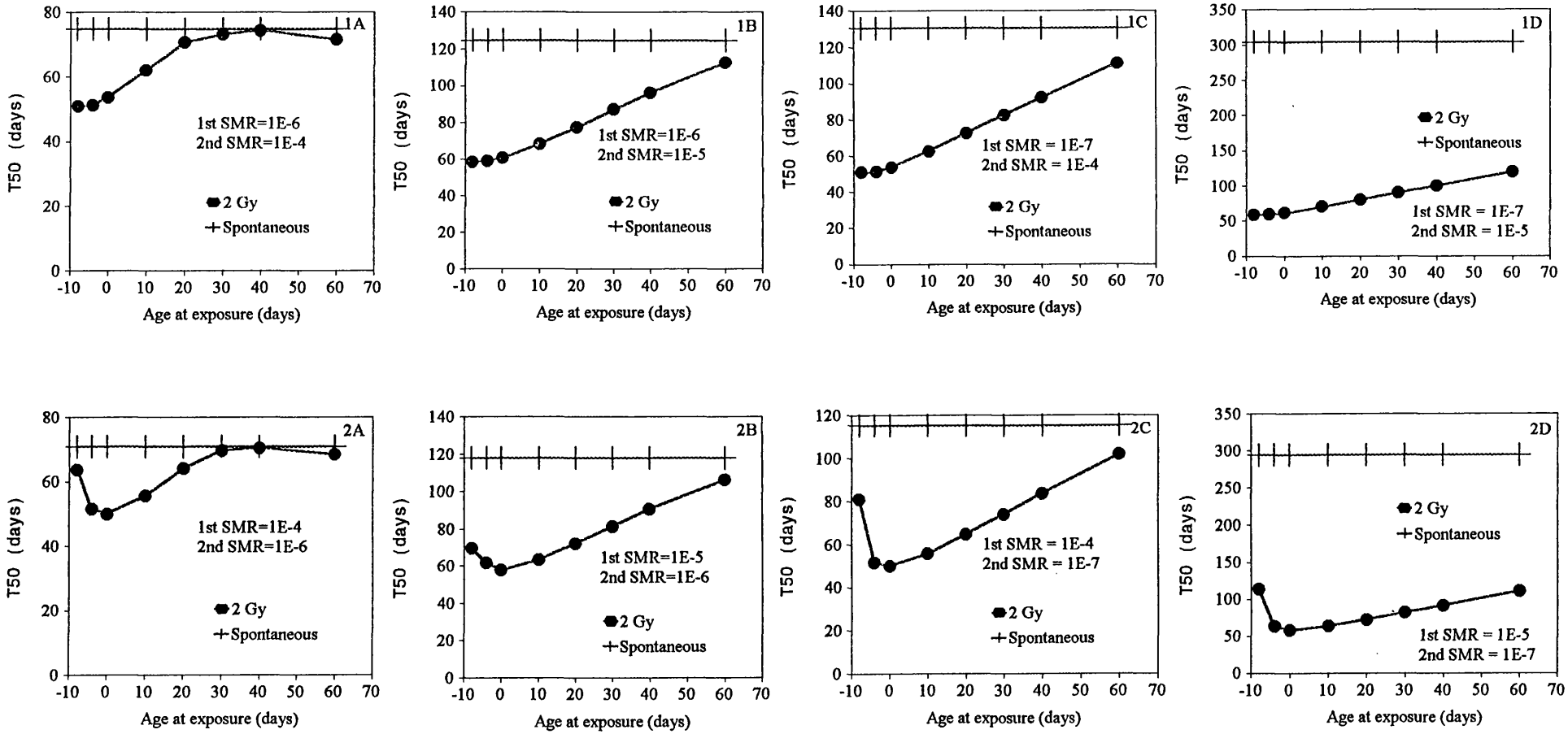
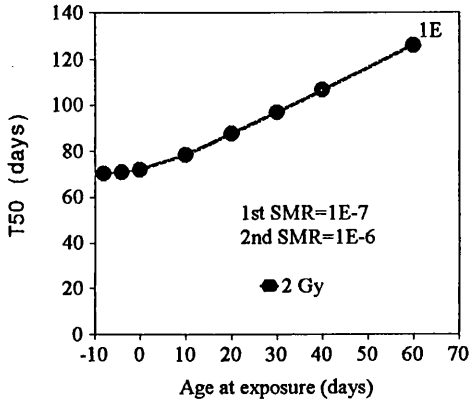
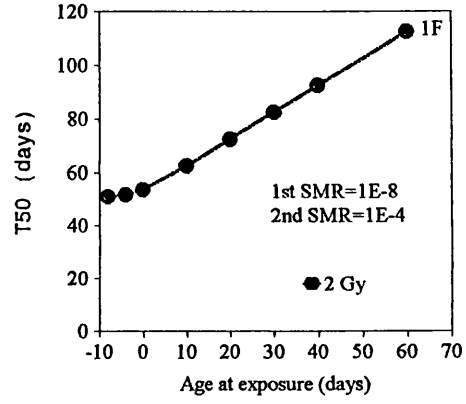


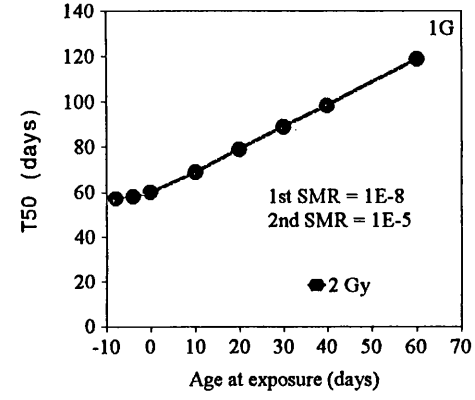
Figure 4.5 The relationship between tumour latency and age at expousre predicted by the multistage models with the same mutation rate at each stage. (A)-(F) one-stage model (G)-(K) two-stage model (L)-(N) three-stage model

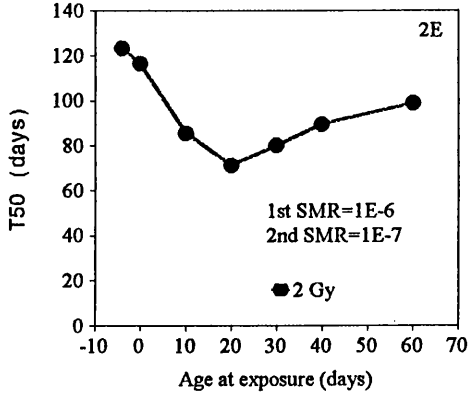


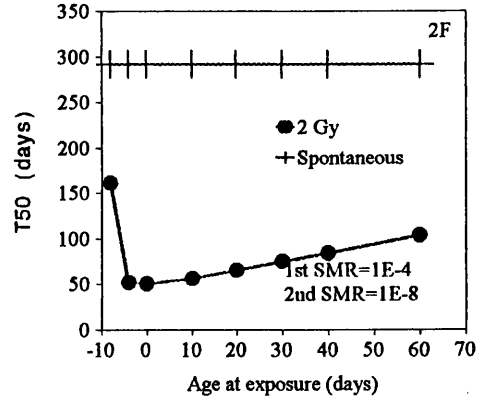
(Figure 4.6 to be continued)

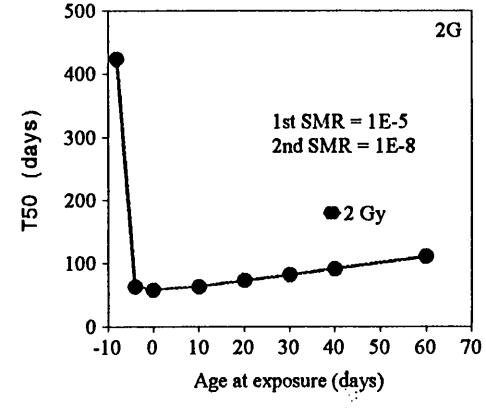












(Figure 4.6 to be continued)

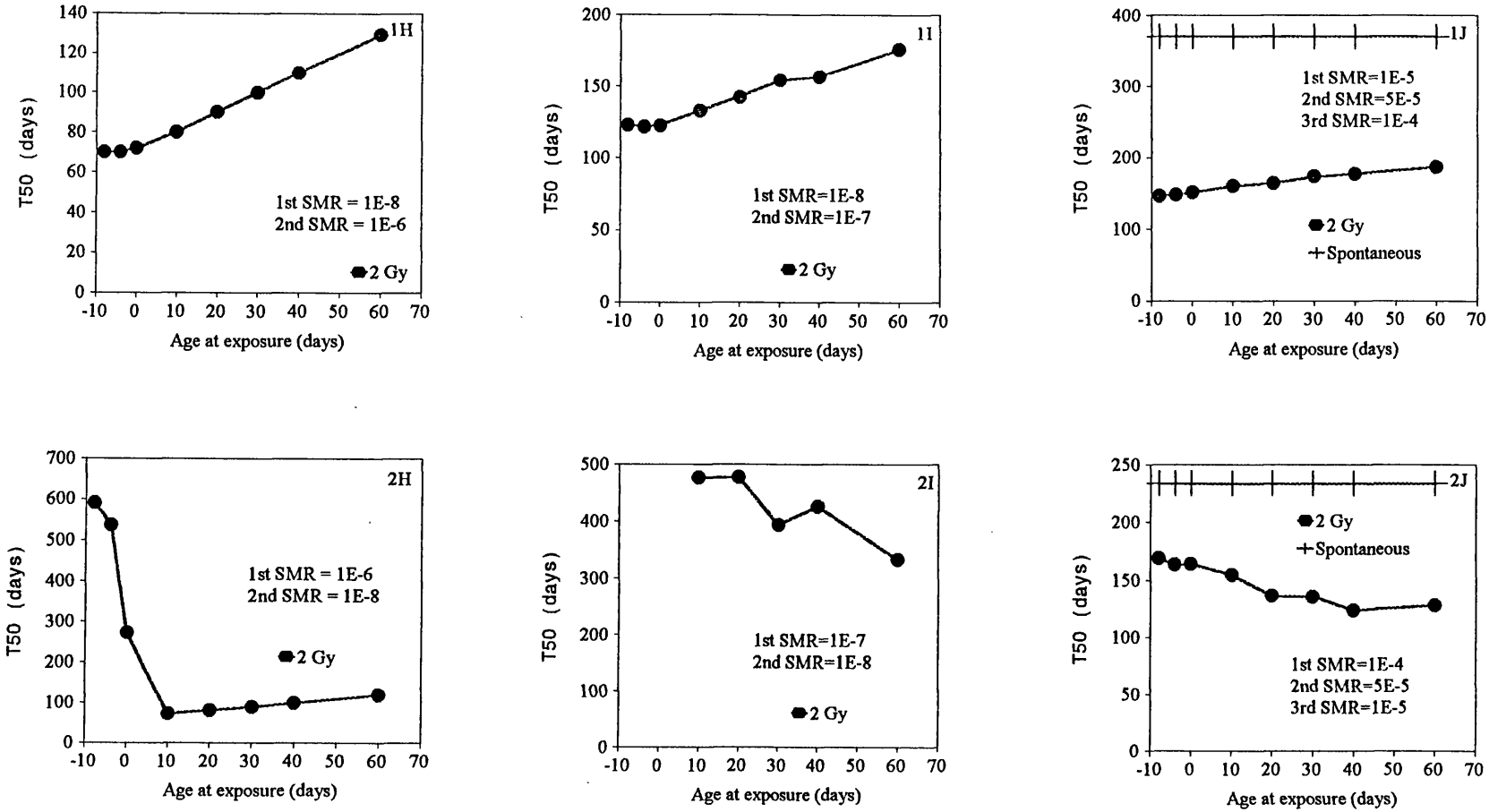


Figure 4.6 The effect of spontaneous mutation rate (SMR) in each stage on the relationship between latency and age at exposure. (1A)- (1I) and (2A)-(2I) two-stage model; (1J) and (2J) three-stage model

4.3.4 Spontaneous mutation rate: influence on dose-tumour-latency

For one-stage tumorigenesis, the most important thing is the growth of tumour to become overt, the spontaneous mutation rate does not seem to be important because many cells will transform by radiation even at low mutation rates, whereas, for two- or three-stage tumorigenesis, the steepness of the dose-tumour-latency relationship is strongly dependent on the spontaneous mutation rate. With low values of spontaneous mutation rate, the T_{50} value declines rapidly with increasing dose in the lower dose regions; at higher values, the dose-response relationship tends to flatten out (Figure 4.4).

4.3.5 Number of mutational events

With a smaller number of mutational events, T_{50} is always lower (the latency period is shorter) and the steep portion of the dose-tumour-latency curve always comes earlier (Figure 4.4). However the difference is less marked for higher spontaneous mutation rates. Therefore, the number of stages is most important at low spontaneous mutation rate.

4.4 Discussion

We have established 1-, 2- and 3-stage stochastic models for radiation tumorigenesis and have shown that, in the majority of cases, a single dose of radiation usually results in earlier appearance of tumours, but with higher value of spontaneous mutation rate, there is no decrease in the tumour latency compared to spontaneous when age at exposure is

later (Table 4.1). The reason may be that, while providing greater opportunity for tumour development, radiation kills tumour cells that are spontaneously converted. There exists a competition between induction and cell killing. Therefore, if it is found experimentally that the latency increases at low doses (0-4 Gy), this may indicate high spontaneous mutation rate and possibly low number of stages. This observation may allow us to understand why 7-week-old p53 null mice exposed to a single of 4 Gy whole body γ radiation did not experience decreased tumour latency although latency was decreased in irradiated p53 deficient heterozgous mice (Kemp et al., 1994). It is presently believed that p53 inactivation may result in genetic instability, i.e. in higher mutation rates applicable to the remaining stages of tumorigenesis (Livingstone et al., 1992; Yin et al., 1992). Thus, there may have been already tumours developing spontaneously, and irradiation would make little difference to time of appearance of the first one.

In the multistage theory of tumorigenesis, the events necessary to produce a tumour cell might occur spontaneously which means that they might accumulate with age. In fact, it has been found that the T_{50} value changes with age at exposure, but this relationship is influenced by the spontaneous mutation rate and number of mutational events required. With lower value of spontaneous mutation rate, for 2- and 3-stage tumorigenesis, there is no clear change in the value of T_{50} with age at exposure increasing.

When the spontaneous mutation rate in the primary stage is lower, and in the later stages is higher, radiation may mainly have effect on primary stages, it will be acting on the population of stem cells, whereas, with higher spontaneous mutation rate in the primary stage and lower spontaneous mutation rate in later stages, it may mainly have effect on the later stages, it will be acting on the population of the cells that have already reached an premalignant stage. So, in the former case, we observed that T_{50} increases with age at

exposure, but in the latter case, T_{50} decreases with age at exposure, however, after a certain age, T_{50} increases with age at exposure, possibly because some malignant cells may have already arisen from stem cells, and these are then killed by radiation.

It has been found the forms of the dose-response relationships vary. The number of stages, spontaneous mutation rate and age at exposure are of importance in determining the shape of the dose-response curve. The dose-response relationship observed in a particular situation may be helpful in providing some mechanistic information about radiation tumorigenesis.

Table 4.1 Effect of radiation (0-4 Gy) on tumour latency

Age at radiation exposure	Number of stages	Latency
Low spontaneous mutation rate		
early	1	↓
	2	↓
	3	↓
late	1	↓
	2	↓
	3	↓
High spontaneous mutation rate		
early	1	↓
	2	↓
	3	↓
late	1	↑
	2	↑
	3	↓

In conclusion, a single dose of radiation usually leads to earlier appearance of tumours, but such effects depend on age at exposure, the spontaneous mutation rate and the number of stages required for malignant change. The dose-response relationship has a steepness which is strongly dependent on age at exposure, the spontaneous mutation rate and the number of stages. The number of mutational events is most influential at low spontaneous mutation rate. The optimal time of irradiation for maximum yield of tumours in mice is predicted to be around time of birth and slightly changes with dose, spontaneous mutation rate and the number of mutational events. This information will be necessary for analysis and interpretation of experimental data on radiation-induced tumorigenesis in transgenic mice with p53 genetic abnormalities.

Chapter 5

Tumour Multiplicity Analysis in p53 Deficient Mice

5.1 Introduction

As reviewed in Chapter 1, inherited p53 mutations in transgenic mice and in Li-Fraumeni human patients lead to significant increases in tumour incidence. However, although each somatic cell inherits the mutation with presumably millions of target cells at increased risk, the number of tumours that develop in each individual is usually quite small (Vogelstein, 1990), i.e. there are no more than a few independent malignant clones, rather than the many which might have been expected. This observation led us to use the carcinogenesis model, which is extended from Chapter 3, to simulate tumour clone multiplicity in wild-type and p53 deficient transgenic mice.

The time taken for second and other tumours to occur after the first tumour has formed, i.e. the distribution of the lag between the first tumour and others, is also very important. If the lag between the first tumour and others were very long, the first tumour could result in mouse death (or humane sacrifice) before the second and other tumours could be detectable. This would lead to a gross underestimate of tumour clonality, if single-tumour bearing mice were to harbour additional undetected tumours at time of death.

In this chapter, it is firstly intended to generally investigate how the spontaneous mutation rate, number of target cells and the number of mutational events required for malignant transformation affect tumour multiplicity (i.e. number of independently-arising tumours) during the same period and the lag distribution between the first-appearing tumour and others.

Later, we will study tumour multiplicity in wild type and p53 deficient mice, and show that multistage models in which inactivation of each p53 allele represents a distinct stage in a single pathway of tumorigenesis invariably predict excessively large numbers of tumours in both p53 deficient heterozygotes and homozygotes, allowing this category of model to be decisively rejected.

5.2 Mathematical modelling and computer simulation

The model of tumorigenesis, illustrated in Figure 5.1, is a k-stage model, which is generalised from the model in Chapter 3. So the features of this model are same as described in Section 3.2 of Chapter 3. Each stem cell at division may reproduce, die or differentiate, or experience a mutation advancing the cell to the next stage of the model. Cells experience these competing processes independently. Pre-malignant mutants are taken to follow the same self-limiting growth pattern as unmutated stem cells, i.e. the pre-malignant mutants have no growth advantage. However, each malignant cell, once it exists, follows an unrestrained growth pattern (linear birth-death process) until the tumour is large enough to be detectable (10^6 cells).

The precise value of the expected number of tumours is not analytically expressible and has to be calculated numerically (see appendix 1). The exact distribution of number of tumours is also not describable analytically, although an approximation to the distribution can be obtained (Sherman et al, 1994).

We predicted tumour multiplicity and explored the distribution of lag time between the

first-appearing tumour and the detection of any others by using computer simulation, with the simulation continued to 600 days, i.e. close to mouse lifespan. The process of computer simulation will be divided into two stages, i.e. the conversion of a normal stem cell into a malignant cell and the growth of each malignant clone to form a tumour (See Appendix 3). Ideally, each malignant cell should be followed separately, but this becomes prohibitive when the number of malignant cells generated is large. In that case, the detection (or not) of a tumour is simulated as a binomial distribution with the probability $F_D(s,600)$ that a tumour cell at time s is detectable as a tumour at or before 600 days and is obtained by Feller-Arley birth-death process (Tan, 1990), where s is the time at which the malignant cells are generated.

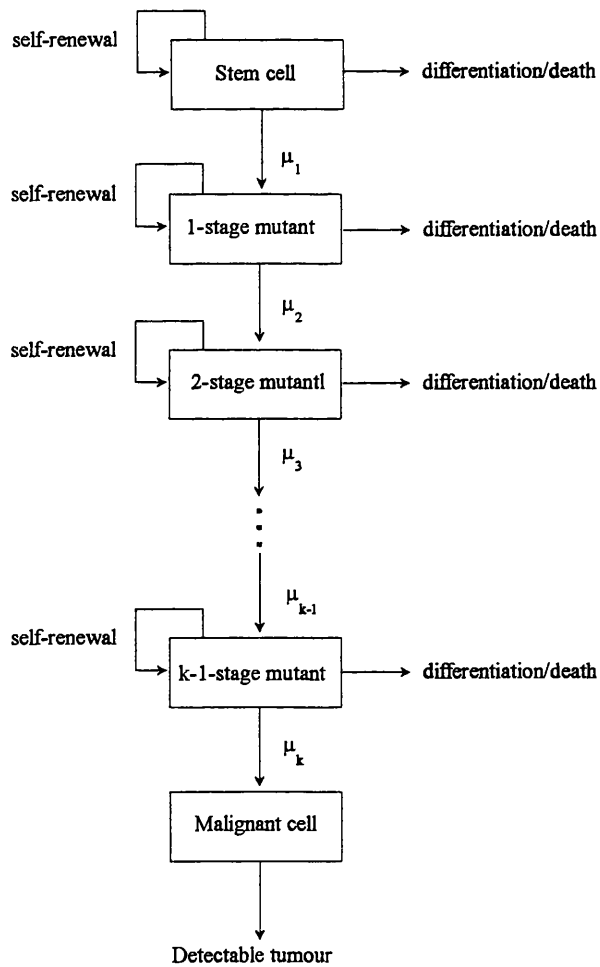


Figure 5.1 General structure of the multistage single path model.

In our simulation, the growth kinetics of each cell and the value of parameters are as described in Chapter 3. 10^4 simulations were carried out for each choice of parameters in the models. The total number of malignant cells generated from stem cells and the total number of tumours detected during 600 days, the time of appearance of the first 10 tumours are recorded for studying the distribution of the lag between the first-appearing and subsequent tumours.

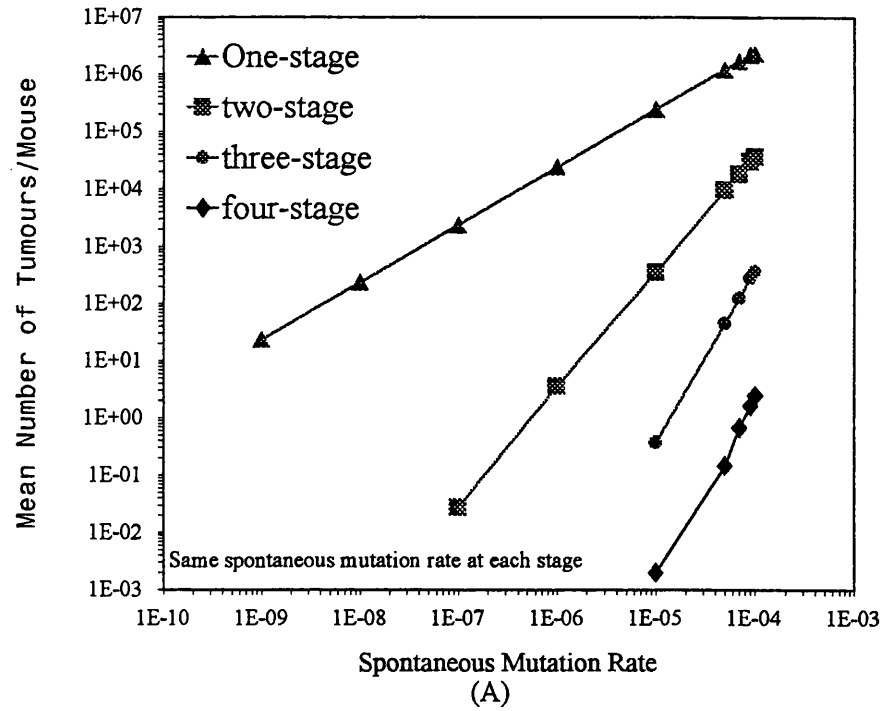
5.3 General findings

5.3.1 Mutation rate

The mean number of tumours per mouse increases with mutation rate at each stage (Figure 5.2). Furthermore, it is also shown that an increase in the spontaneous mutation rate results in a corresponding increase in the probability of more than one detectable tumour in a mouse (Figure 5.3).

5.3.2 Number of mutational events

With a smaller number of mutational stages required for malignant transformation, the mean number of tumours per mouse is always larger during the same period (Figure 5.4). Reduction of the number of mutational stages also results in a corresponding increase in the probability of more than one tumour. However, a large number of independent tumours appear together in a mouse only in the situation with few stages and high mutation rate.



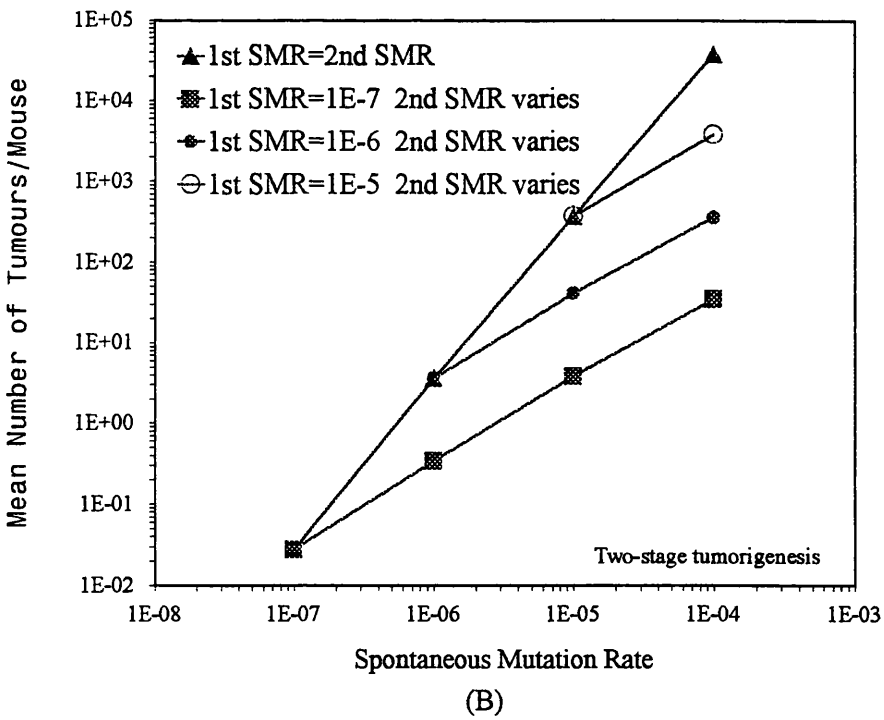
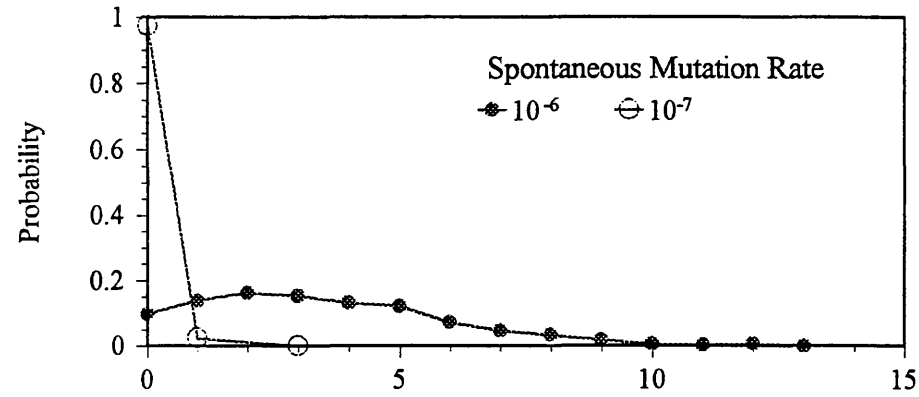
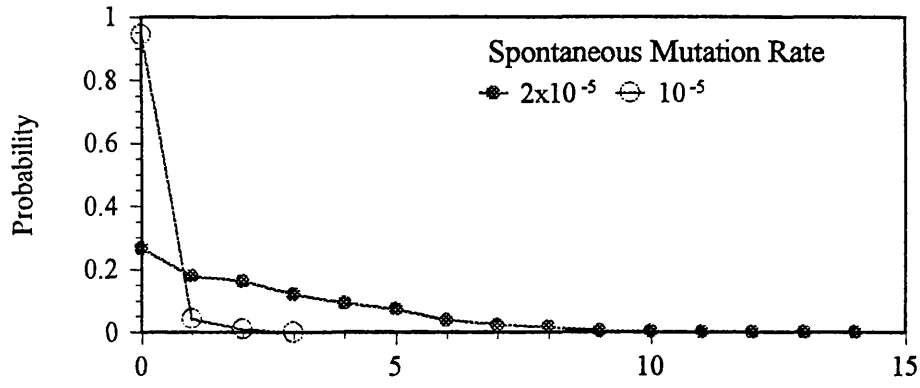


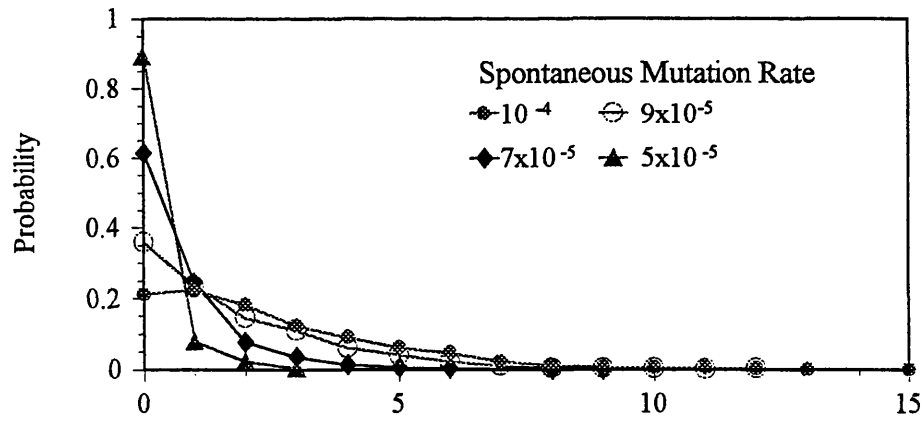
Figure 5.2 The relationship between the mean number of tumours and spontaneous mutation rate (SMR). (A) SMR are same in each stage; (B) SMR varied in each stage of two-stage tumorigenesis.



(A) Two-stage tumorigenesis



(B) Three-stage tumorigenesis



(C) Four-stage tumorigenesis

Figure 5.3 The probability density of the number of tumours. (A) two-stage tumorigenesis; (B) three-stage tumorigenesis; (C) four-stage tumorigenesis.

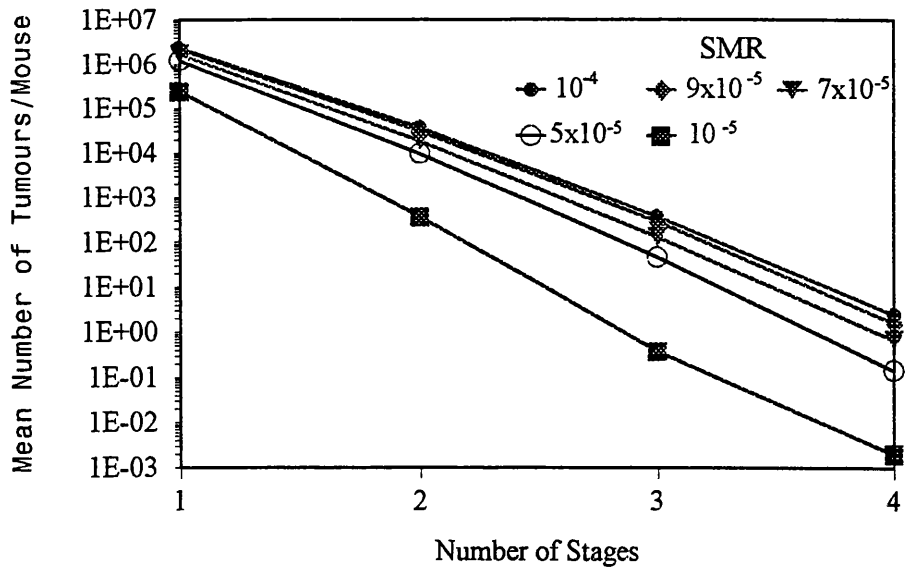


Figure 5.4 The relationship between the mean number of tumours per mouse and the number of stages required for malignant transformation.

5.3.3 Number of target cells

For the all models, the mean number of tumours increases with the number of target cells (Figure 5.5). This relationship is shifted by spontaneous mutation rate and is influenced by the number of mutational events required for malignant transformation (Figure 5.5). These observations provide a possible experimental test of the model predictions. (For example, if variable numbers of p53 deficient bone marrow stem cells are transplanted into wild-type mice.)

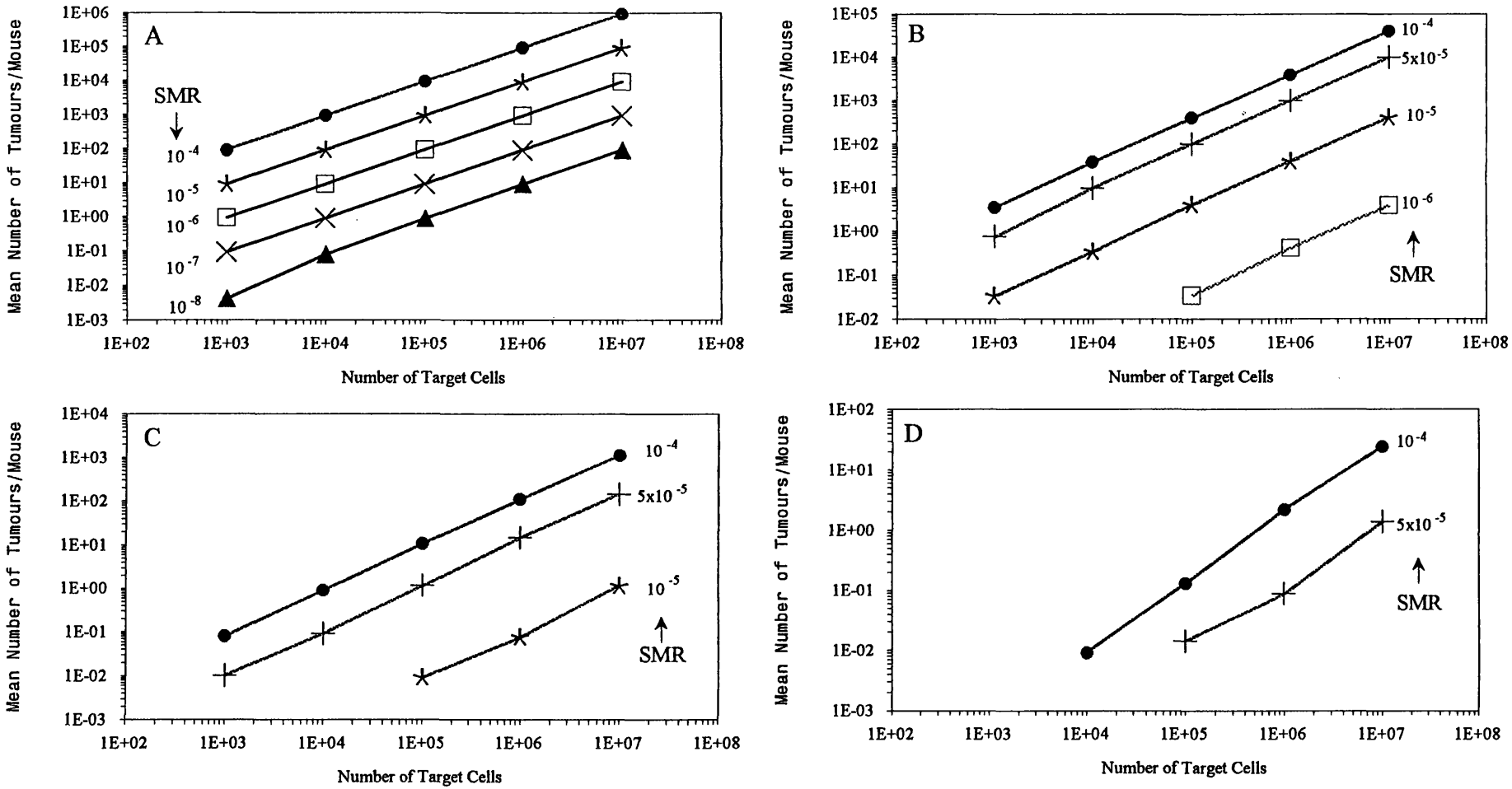


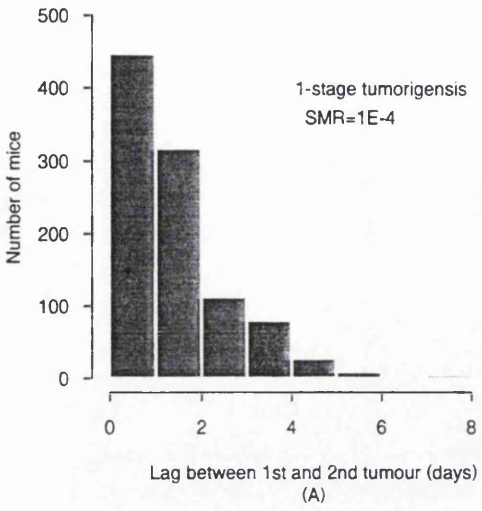
Figure 5.5 The relationship between mean number of tumours per mouse and number of target cells. (A) 1-stage model; (B) 2-stage model; (C) 3-stage model; and (D) 4-stage model.

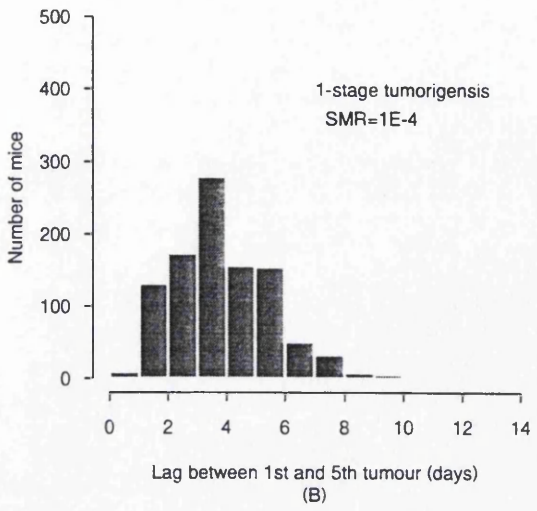
5.3.4 Distribution of the lag between the first and subsequent tumours

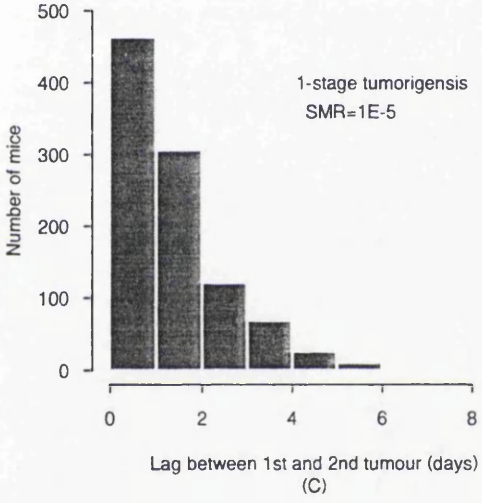
The distribution of lag time between the first and subsequent tumours is dependent on the spontaneous mutation rate and the number of mutational events required for malignant transformation (Figure 5.6). With a higher mutation rate and fewer mutational events, the distribution of lag always shifts left (Figure 5.6), that is the time difference between first tumour and others become shorter. This result indicates that, in this situation, many independently-arising tumours may appear together in a mouse.

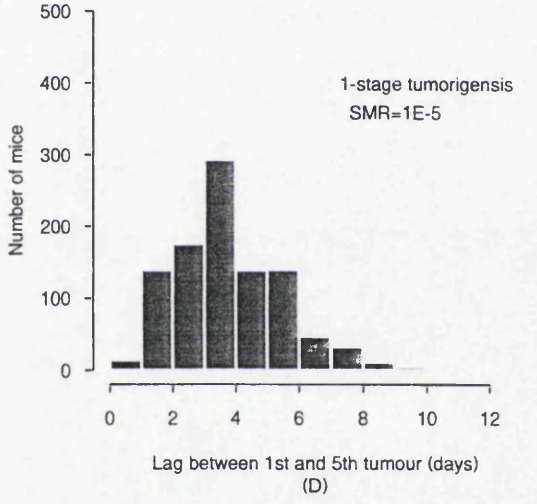
5.4 Tumour multiplicity in p53 deficient mice

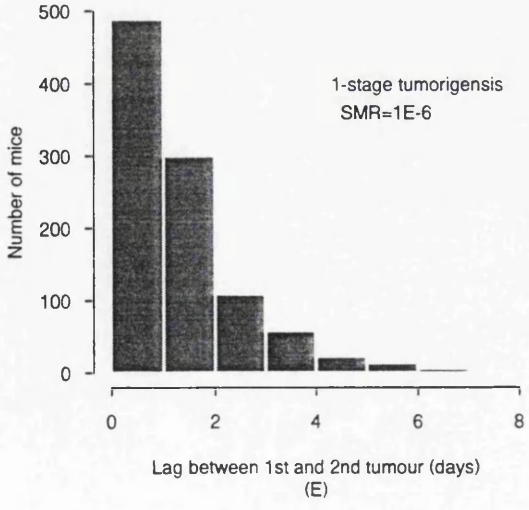
As shown in Chapter 3, tumorigenesis in p53 deficient mice is interpreted using the multistage model in which inactivation of each p53 allele represents a distinct stage. For wild-type genotypes, we have considered 3-stage, 4-stage and 5-stage models and have in each case chosen the mutation rate to match the tumour incidence (about 10%) observed experimentally (Donehower et al, 1995) in wild type mice by 600 days. The corresponding tumour incidences for p53 null heterozygous and p53 null homozygous mice may then be computed by subtracting 1 or 2 stages respectively without changing the mutation rate. For presentation, we have computed the tumour incidence by 80 weeks, and the mean number of tumours per mouse predicted to have appeared by 16 and 80 weeks, close to the observed median latency in $p53^{-/-}$ and $p53^{+/-}$ mice, in each of these situations.

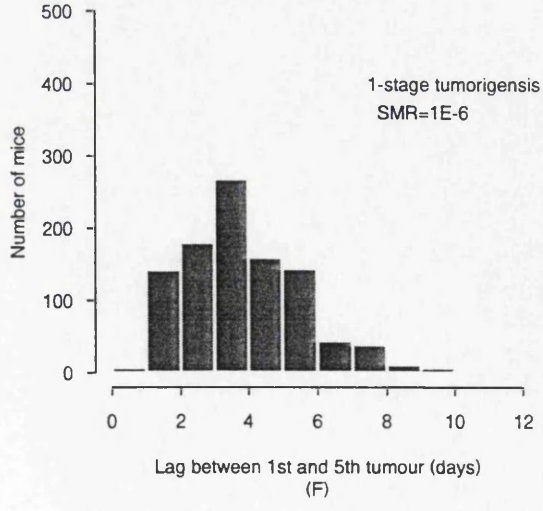




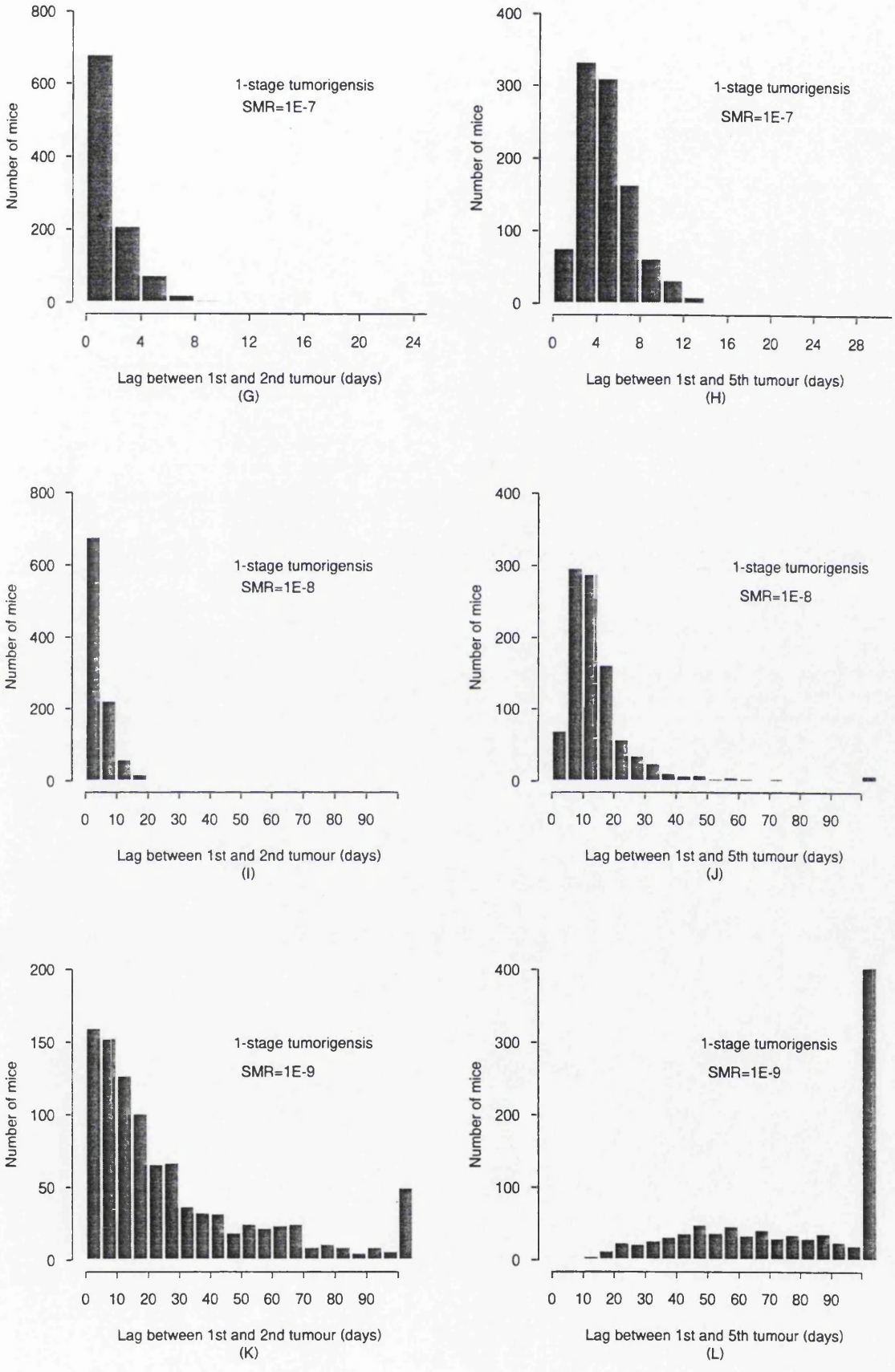




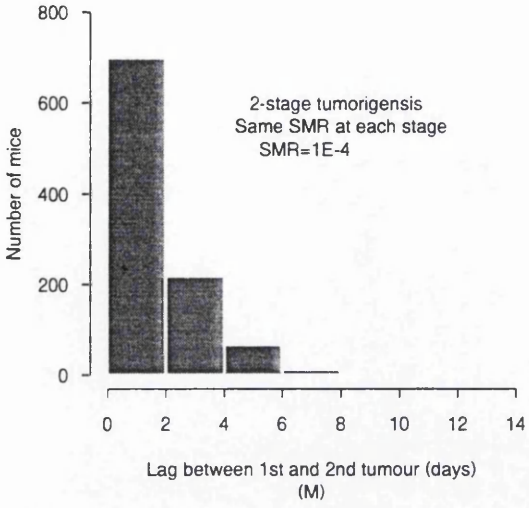


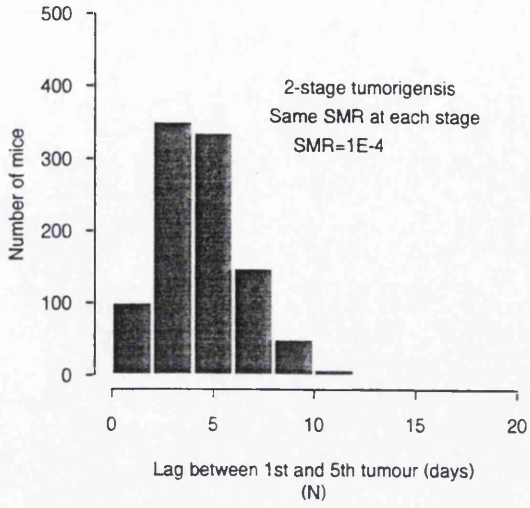


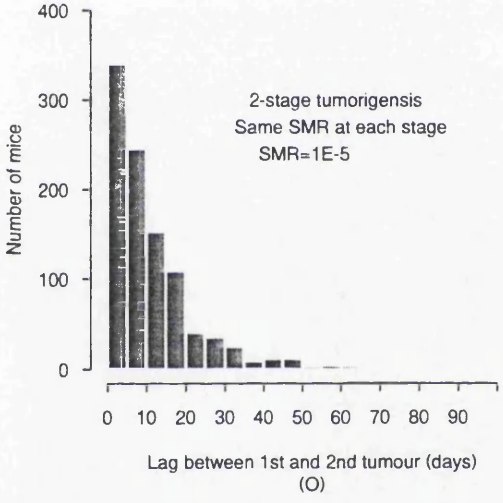
(Figure 5.6 to be continued)

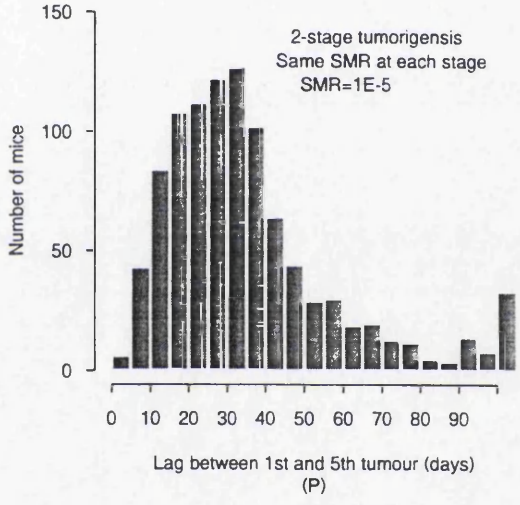


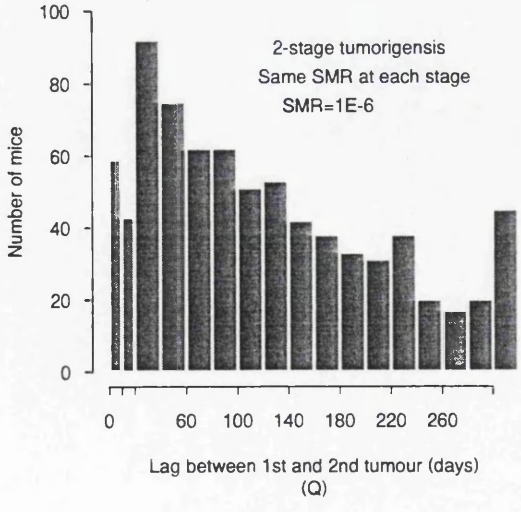
(Figure 5.6 to be continued)

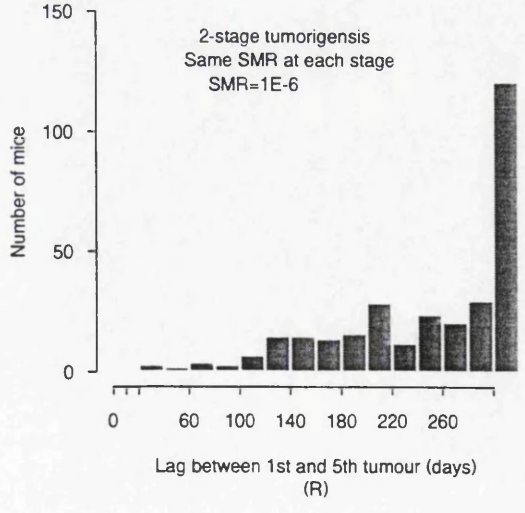












(Figure 5.6 to be continued)

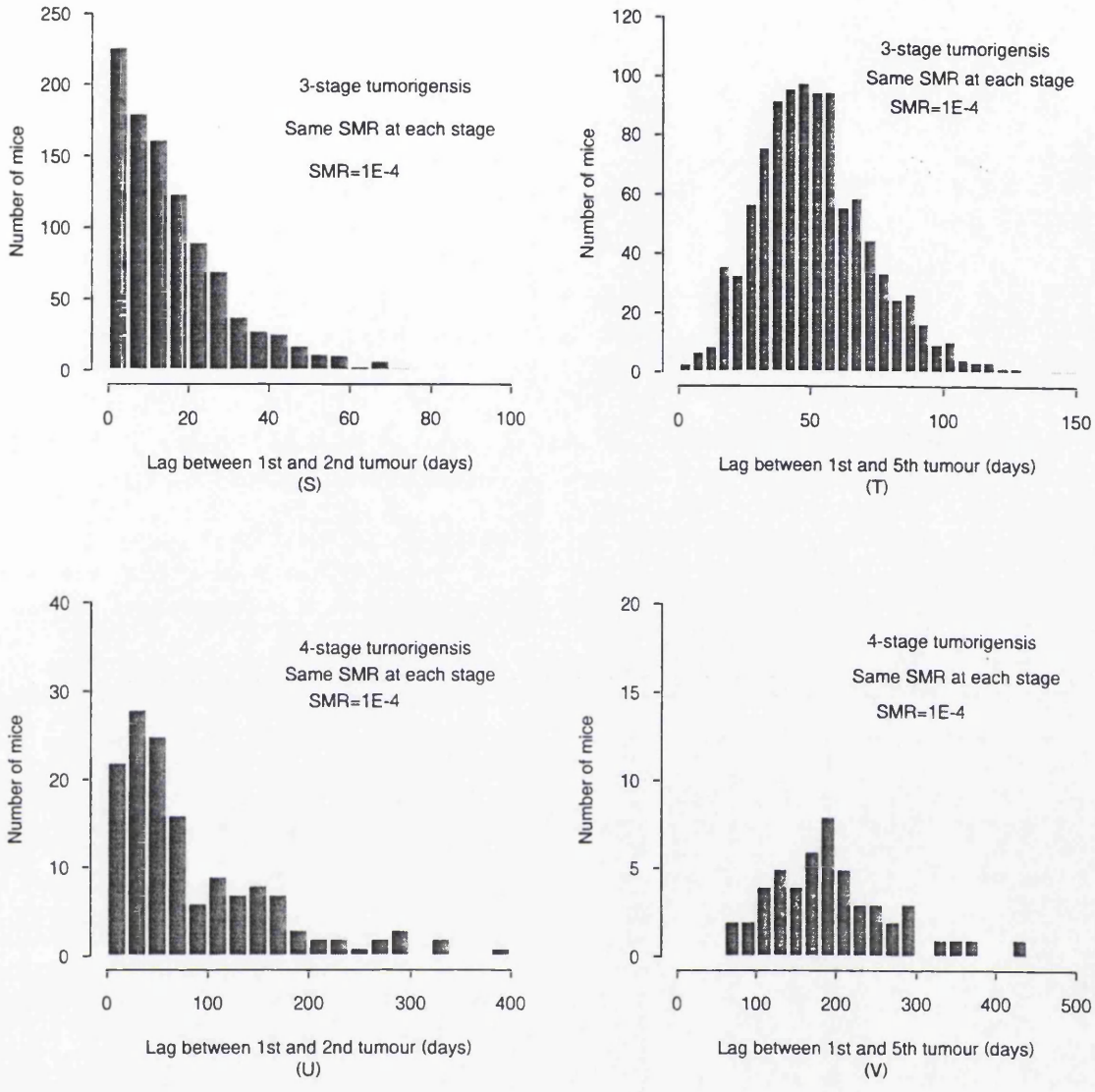


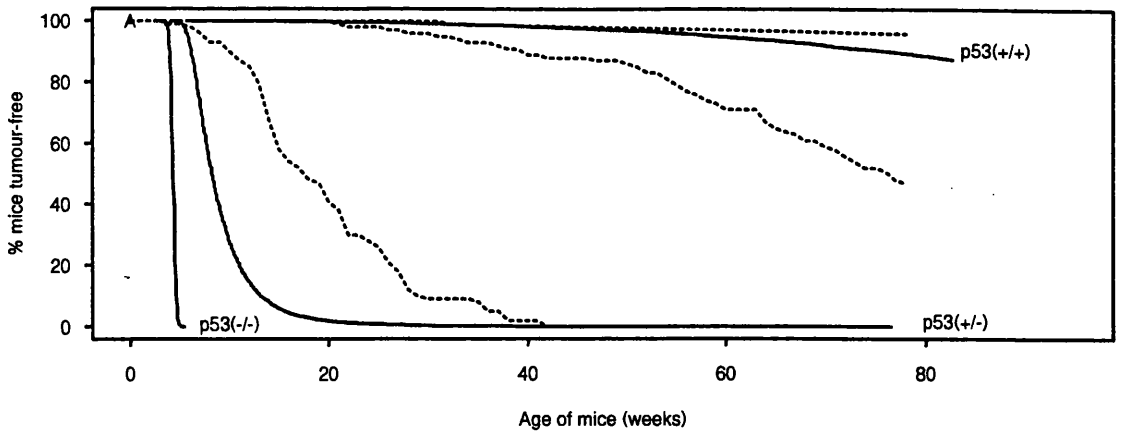
Figure 5.6 Distribution of lag time between the 1st and 2nd, 1st and 5th tumour. (A) to (L) 1-stage model, (M) to (R) 2-stage model, (S) and (T) 3-stage model, (U) and (V) 4-stage model. SMR = spontaneous mutation rate.

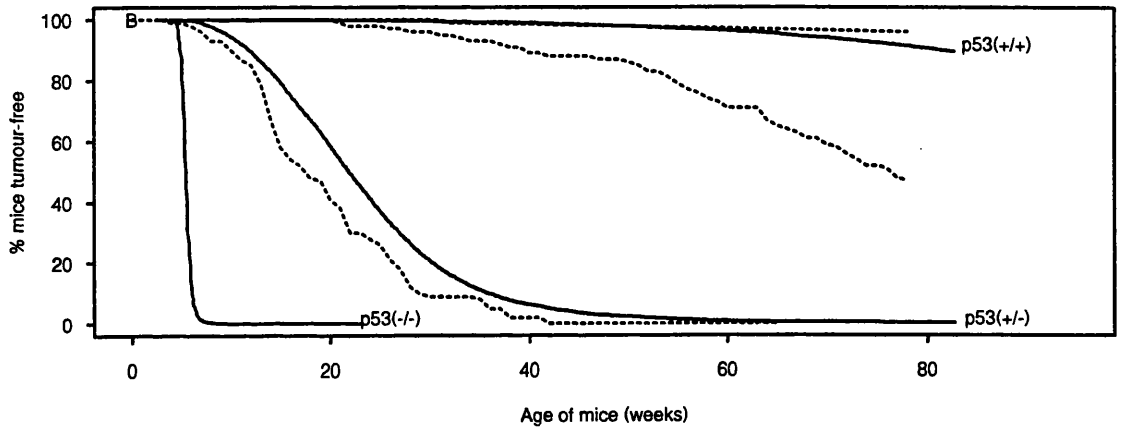
Table 5.1 shows that 3, 4 and 5-stage models all predict the early development of large numbers of tumours in both $p53^{+/-}$ and $p53^{-/-}$ mice. Although experimentally observed numbers of tumours will certainly be underestimates of the number destined to develop, it hardly seems possible that the predicted thousands of tumours could be reconciled with the typical observation of one or two. The corresponding age-incidence curves are shown in Figure 5.7 and demonstrate much faster tumour development predicted by the model for both p53 deficient genotypes than occurs in practice (The latent periods seen experimentally for p53 deficient mice are also shown in figure 5.7 for comparison). This discrepancy has been found to occur for all combinations of model parameters giving 10% lifetime incidence of tumours in wild-type mice; it appears to be a robust feature of this class of model. We have concluded that it is not possible to accommodate data on tumour incidence in wild type and p53 deficient mice by the classical multistage single-path model.

Table 5.1 Tumour incidences in mice of different genotypes as predicted by 3, 4 and 5 stage single path models. The mutation rates were chosen to match tumour incidence (ie fraction of mice developing tumours) observed experimentally in wild type mice. In this scenario, all tumours develop by a route involving p53 inactivation.

Genotype	Experimental data		3-Stage Model (Mutation rate=7.5 x 10^{-6}) Tumours /mouse (SE)*		4-Stage Model (Mutation rate = 5 x 10^{-5}) Tumours /mouse (SE)*		5-Stage Model Mutation rate=1.65 x 10^{-4}) Tumours /mouse (SE)*	
	16 weeks	80 weeks	16 weeks	80 weeks	16 weeks	80 weeks	16 weeks	80 weeks
$p53^{+/+}$	0	0.08	0.002 (0.0007)	0.12 (0.006)	0.003 (0.0001)	0.09 (0.005)	0 (0)	0.2 (0.007)
$p53^{+/-}$	0	0.53	6.8 (0.26)	1.9×10^{2} (1.8)	0.26 (0.02)	45 (0.55)	0.03 (0.004)	19 (0.17)
$p53^{-/-}$	0	1.00	3.3×10^{4} (1.6×10^{2})	1.7×10^{5} (8.4×10^{2})	5.5×10^{2} (9.7)	9.6×10^{3} (61.7)	9.4 (0.43)	9.1×10^{2} (11.9)

* SE = standard error of mean number of tumours per mouse





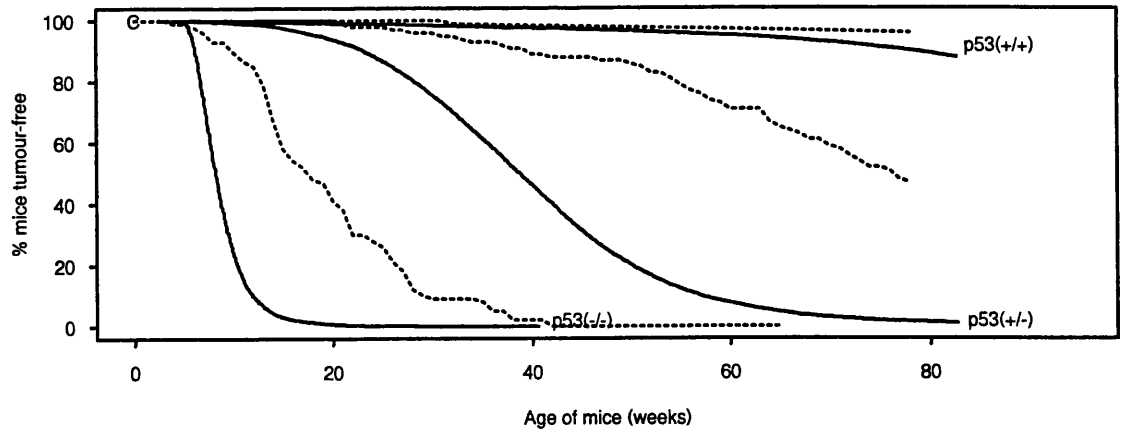


Figure 5.7 Predicted age-incidence pattern of tumour development in mice of three genotypes for (A) 3-, (B) 4-, and (C) 5-stage single path models. In each case the mutation rates have been chosen to match tumour incidence observed experimentally in wild type mice by 80 weeks; the models then predict excessively rapid development of tumours in both p53 deficient genotypes. The broken lines in the diagrams show the incidence of experimental tumours (data from Donehower et al (1995), dashed line).

5.5 Discussion

Computer simulation studies of tumour multiplicity by the multistage model has been carried out. In these studies, the issue of the tumours within tumours (i.e. intratumoural multiclonality) was not taken into account, however, this is not likely to be an important issue in practice.

If the rate of death (including apoptosis, differentiation, etc.), i.e. cell loss, is greater than zero, then there is non-zero probability that a tumour cell will become extinct without giving rise to a detectable tumour. Also, some of tumour cells may not have enough time to grow into a detectable tumour before the host is dead. This could lead to a gross underestimate of tumour multiplicity (Table 5.2).

The simulation studies generally show that the mean number of tumours per mouse strongly depends on the spontaneous mutation rate, the number of mutational events required for malignant transformation, and number of target cells. However, there is a considerable stochastic variation around this mean (Table 5.2), and this variation is also dependent on the spontaneous mutation rate, and the number of mutational events (Table 5.2).

The analysis has demonstrated a fundamental problem in the application of the classic multistage model to spontaneous tumorigenesis in p53 deficient mice. On a multistage model, with a single pathway of tumorigenesis, the reduction in stage number by one, resulting from germ line inheritance of one of the tumorigenic mutations, without change of mutation rate, results in a marked increase in predicted tumour frequency. Transgenic

mice have provided a unique opportunity to test the prediction that the inheritance of two tumorigenic mutations (inactivated p53 alleles), corresponding to a reduction in stage number by two, would produce an astronomical number of tumours per mouse. Our analysis shows that this prediction applies for up to five stages being required for tumorigenesis in wild type mice.

Table 5.2 The mean number of tumour cells arising from stem cells and the mean number of tumours

Number of Stages	Spontaneous Mutation Rate	Mean Number of Tumour Cells (SD)*	Mean Number of Tumours (SD)*
1	10^{-4}	$2.53\times10^{6}(1.15\text{x}10^{6})$	$2.35\times10^{6}(1.07\text{x}10^{6})$
	10^{-5}	$2.63\times10^{5}(1.24\text{x}10^{5})$	$2.44\times10^{5}(1.16\text{x}10^{5})$
	10^{-6}	$2.61\times10^{4}(1.21\text{x}10^{4})$	$2.42\times10^{4}(1.12\text{x}10^{4})$
	10^{-7}	$2.56\times10^{3}(1.21\text{x}10^{3})$	$2.38\times10^{3}(1.12\text{x}10^{3})$
	10^{-8}	$2.60\times10^{2}(1.23\text{x}10^{2})$	$2.41\times10^{2}(1.14\text{x}10^{2})$
	10^{-9}	$2.55\times10^{1}(1.30\text{x}10^{1})$	$2.37\times10^{1}(1.22\text{x}10^{1})$
2	10^{-4}	$4.29\times10^{4}(2.17\text{x}10^{4})$	$3.74\times10^{4}(1.91\text{x}10^{4})$
	10^{-5}	$4.25\times10^{2}(2.77\text{x}10^{2})$	$3.67\times10^{2}(1.92\text{x}10^{2})$
	10^{-6}	$4.29\times10^{0}(3.29\text{x}10^{0})$	$3.63\times10^{0}(3.51\text{x}10^{0})$
	10^{-7}	$4.20\times10^{-2}(2.30\text{x}10^{-1})$	$2.80\times10^{-2}(1.82\text{x}10^{-1})$
3	10^{-4}	$4.64\times10^{2}(2.69\text{x}10^{2})$	$3.75\times10^{2}(2.23\text{x}10^{2})$
	10^{-5}	$4.54\times10^{-1}(7.67\text{x}10^{-1})$	$3.72\times10^{-1}(6.69\text{x}10^{-1})$
4	10^{-4}	$3.38\times10^{0}(3.01\text{x}10^{0})$	$2.49\times10^{0}(2.45\text{x}10^{0})$
	10^{-5}	$2.00\times10^{-3}(4.50\text{x}10^{-2})$	$2.00\times10^{-3}(4.50\text{x}10^{-2})$

* The figure in brackets is the standard deviation.

We may argue that by the first-appearing tumour causing the death (or humane sacrifice) of mice, the other tumours do not have the chance to appear, but it seems not to be true. From the distribution of lag times between the first-appearing tumour and the others, we predict that many tumours will occur in $p53^{-/-}$ mice within few days even when the tumorigenesis in the wild-type mice is assumed to require 5 mutational stages (Figure 5.8).

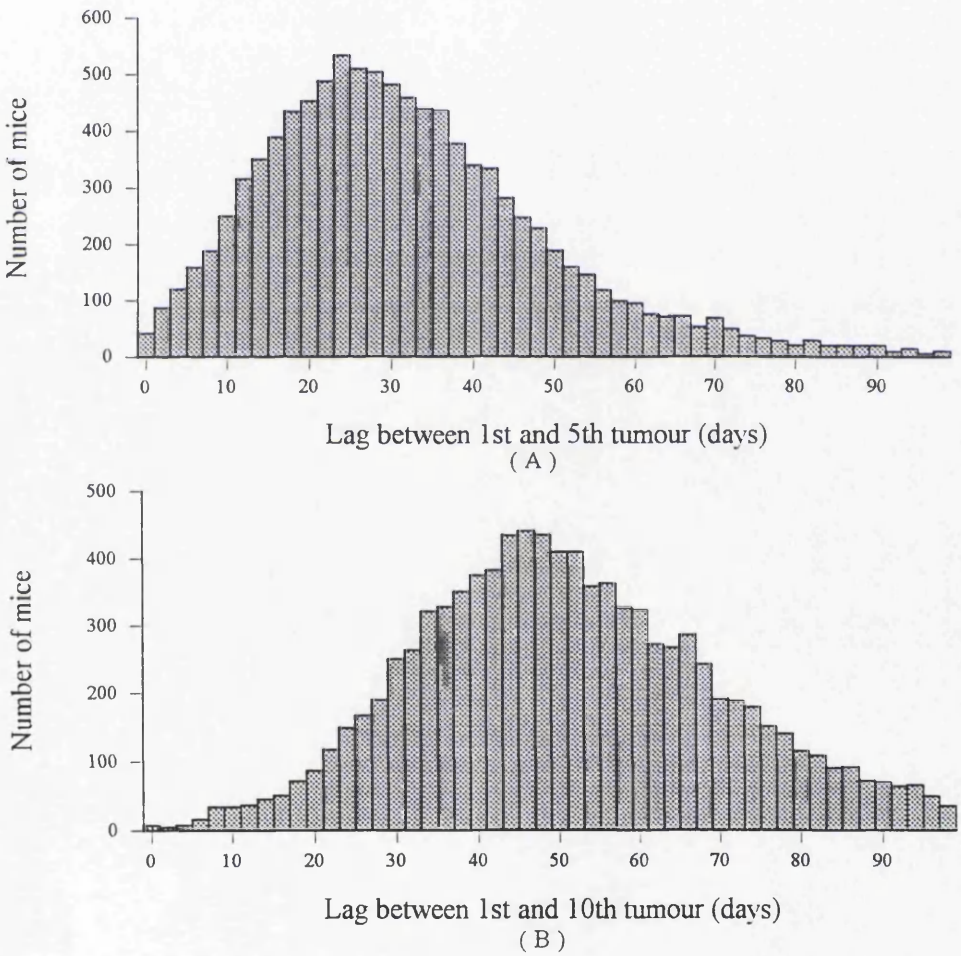


Figure 5.8 The distribution of the lag between 1st tumour and others in $p53^{-/-}$ mice as predicted by the 5-stage tumorigenesis model. (A) 1st and 5th, (B) 1st and 10th

This difficulty has been recognised previously. In 1990, Vogelstein commenting on tumour incidence in human Li-Fraumeni patients (who inherit a single defective p53 allele) posed the question 'Why don't these patients develop more tumours?' and commented 'Given the diverse tumours occurring in Li-Fraumeni patients it would seem that many human cell types are susceptible to the effect of inherited p53 mutations; yet the median age of tumour development is over 30 years and the median number of lifetime tumours is less than two' (Vogelstein 1990). Vogelstein's paradox also occurs for the double-defect $p53^{-/-}$ mice, which still show no more than a few tumours per mouse, although thousands would be predicted. Two categories of explanation for this paradox will be given in Chapter 7 and 8 respectively.

Chapter 6

Effect of Radiation on Tumour Multiplicity in p53 Deficient Mice

6.1 Introduction

In the previous chapter, we have shown that the number of events required for malignant transformation, the spontaneous mutation rate, and number of target cells influence the tumour multiplicity. In this chapter, we incorporate the effect of radiation, i.e., cell killing and mutation induction, into the model described in Chapter 5. In general, it is intended to explore:

(1) The dose-tumour-multiplicity relationship;

(2) Time of appearance of 1st, 2nd, and subsequent tumours;

(3) How age at exposure, the spontaneous mutation rate and the number of mutational events required for malignant transformation influence the dose-tumour-multiplicity relationship and the distribution of times of appearance of tumours.

Finally, we will consider the situation where there is a certain background incidence of tumorigenesis in p53 wild type mice (as we discussed in chapter 5) and a single dose exposure is introduced, and we will study tumour multiplicity in wild-type and p53 deficient mice.

6.2 Modelling considerations

Figure 6.1 shows the structure of the k-stage model for radiation tumorigenesis, which is generalized from the model presented in Chapter 4. Before exposure to a single dose of radiation, the tumorigenic process is spontaneous. Post radiation, the cells (normal stem

cells, 1-stage mutants, ..., k-1-stage mutants, and malignant cells) may survive or may be killed. Amongst the surviving cells (normal stem cells, 1-stage mutants, ..., k-1-stage mutants), some may undergo tumorigenic mutation produced by radiation.

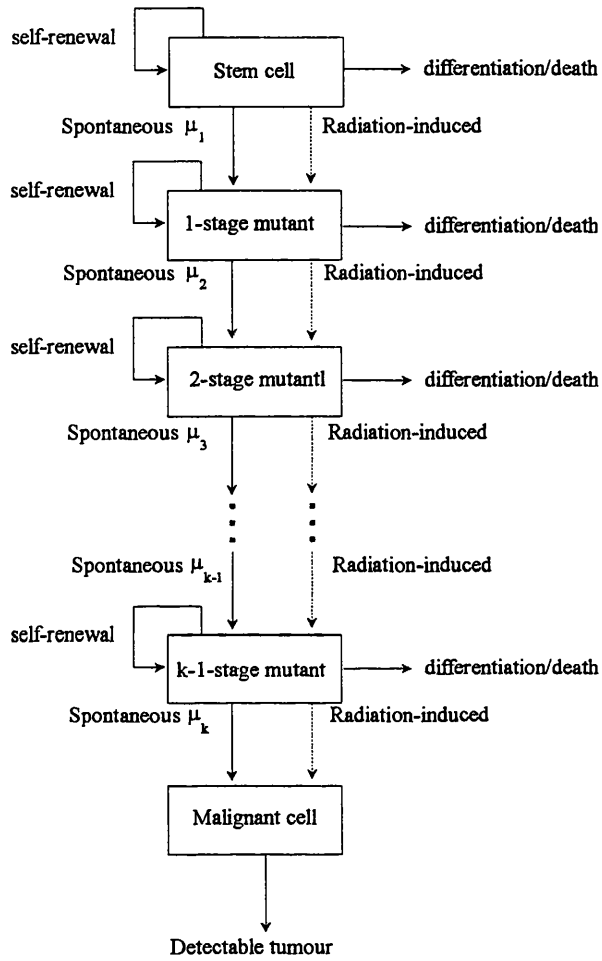


Figure 6.1 Structure of k-stage model for radiation tumorigenesis.

The tumour multiplicity is predicted by computer simulation, with the simulation continued to 600 days. The process of computer simulation is divided into two stages, i.e. conversion of a normal stem cell into a malignant cell and growth of each malignant cell clone to form a tumour. Each stage is affected by radiation. When exposure to a single dose of

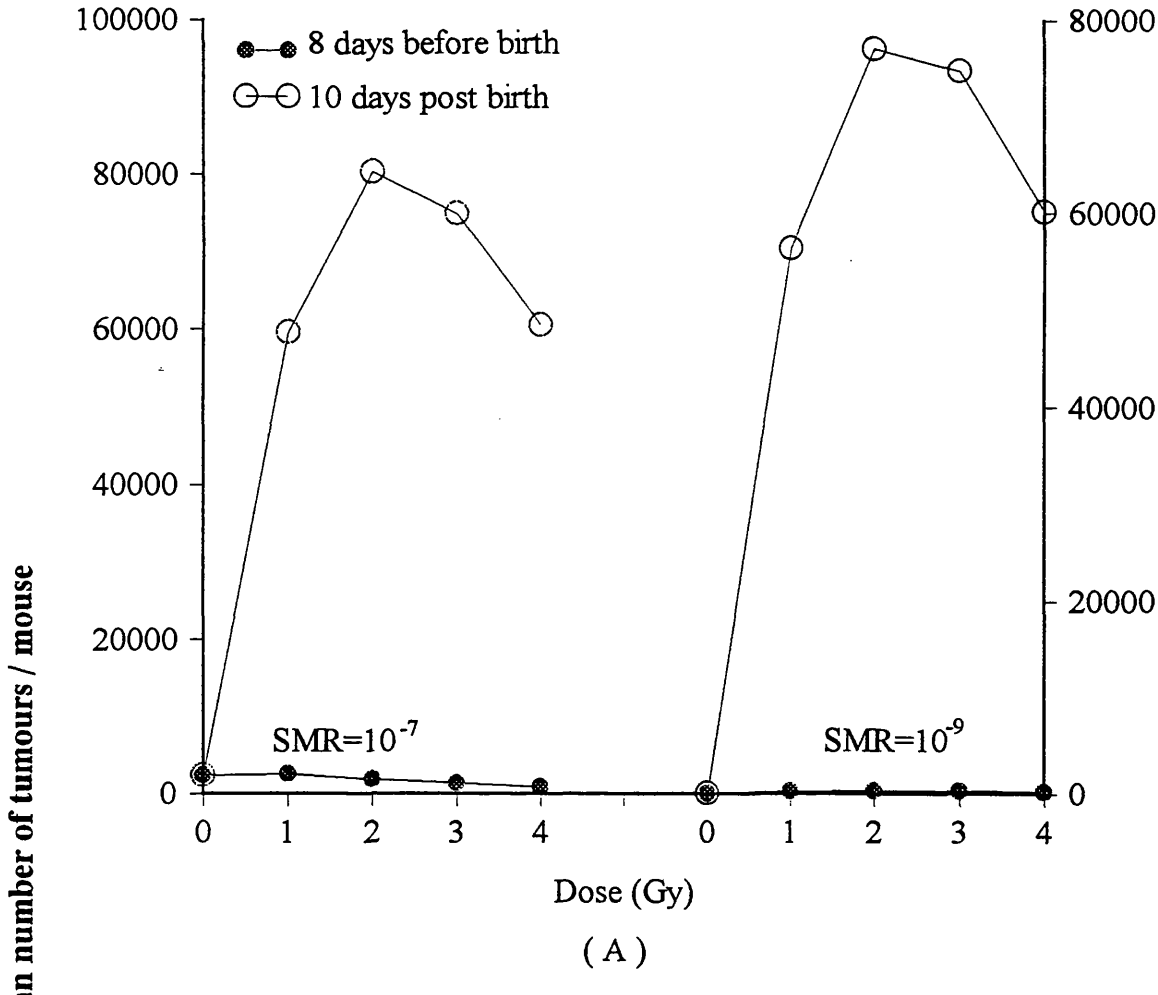
radiation occurs, it is required to find the number of cells killed and the number of cells which have undergone tumorigenic mutation (both radiation-induced and spontaneous). These numbers are simulated as a Poisson distribution, where the probability of cell survival is predicted by the linear-quadratic model, and the probability that any surviving cell carries a tumorigenic mutation is approximately linear at lower doses (details referred to Chapter 4).

6.3 General findings

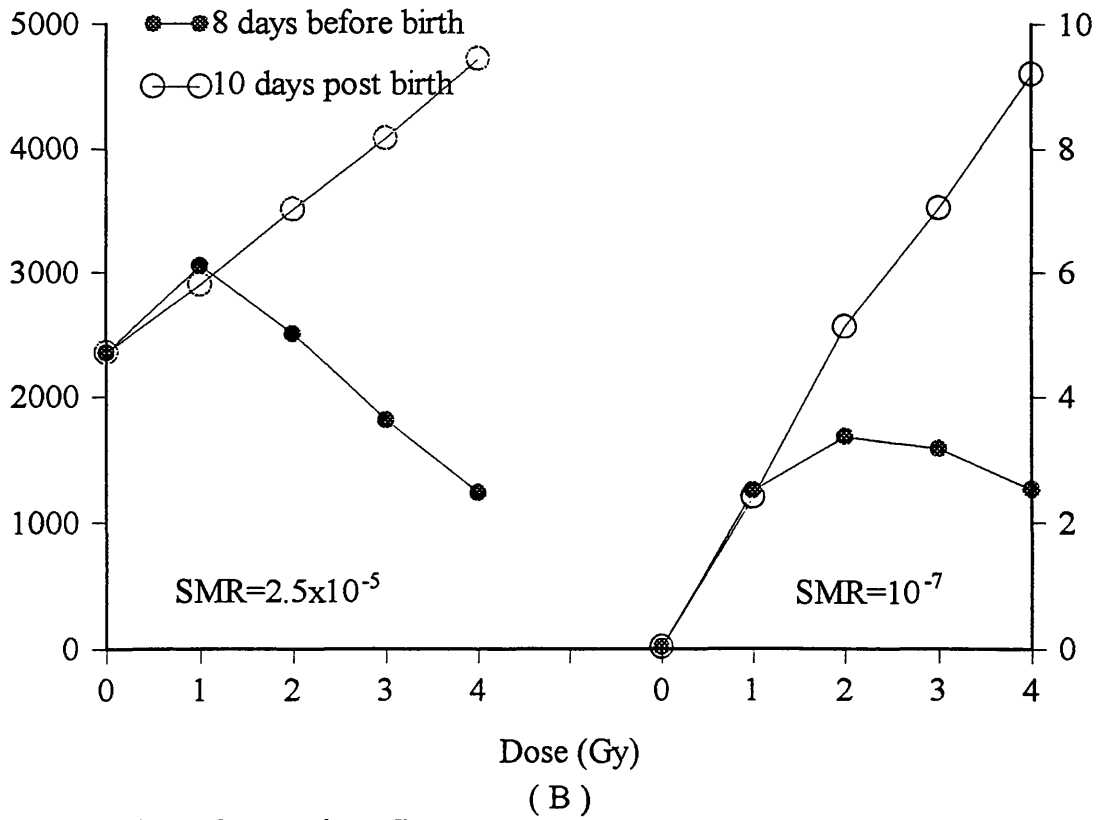
The values of most parameters in the model are the same as stated in Chapter 4. For each set of parameters, 10^4 simulations were carried out. The mean number of tumours per mouse and T50 (the time until the 1st tumours, 2nd tumours, etc. are detected in 50% mice) are used to present the simulation results.

6.3.1 Dose-tumour-multiplicity relationship

In general, the dose-tumour-multiplicity relationship is strongly dependent on the number of mutational events required for malignant transformation, the spontaneous mutation rate, and age at exposure. The mean number of tumours per mouse initially increases with irradiation dose, reaches a maximum, and then decreases (Figure 6.2). However, when the exposure is given post-natally, the multiplicity has not reached the maximum by 4 Gy (at which dose the simulations were stopped) except in the case of the one-stage model (Figure 6.2).



(A)



(B)

(Figure 6.2 to be continued)

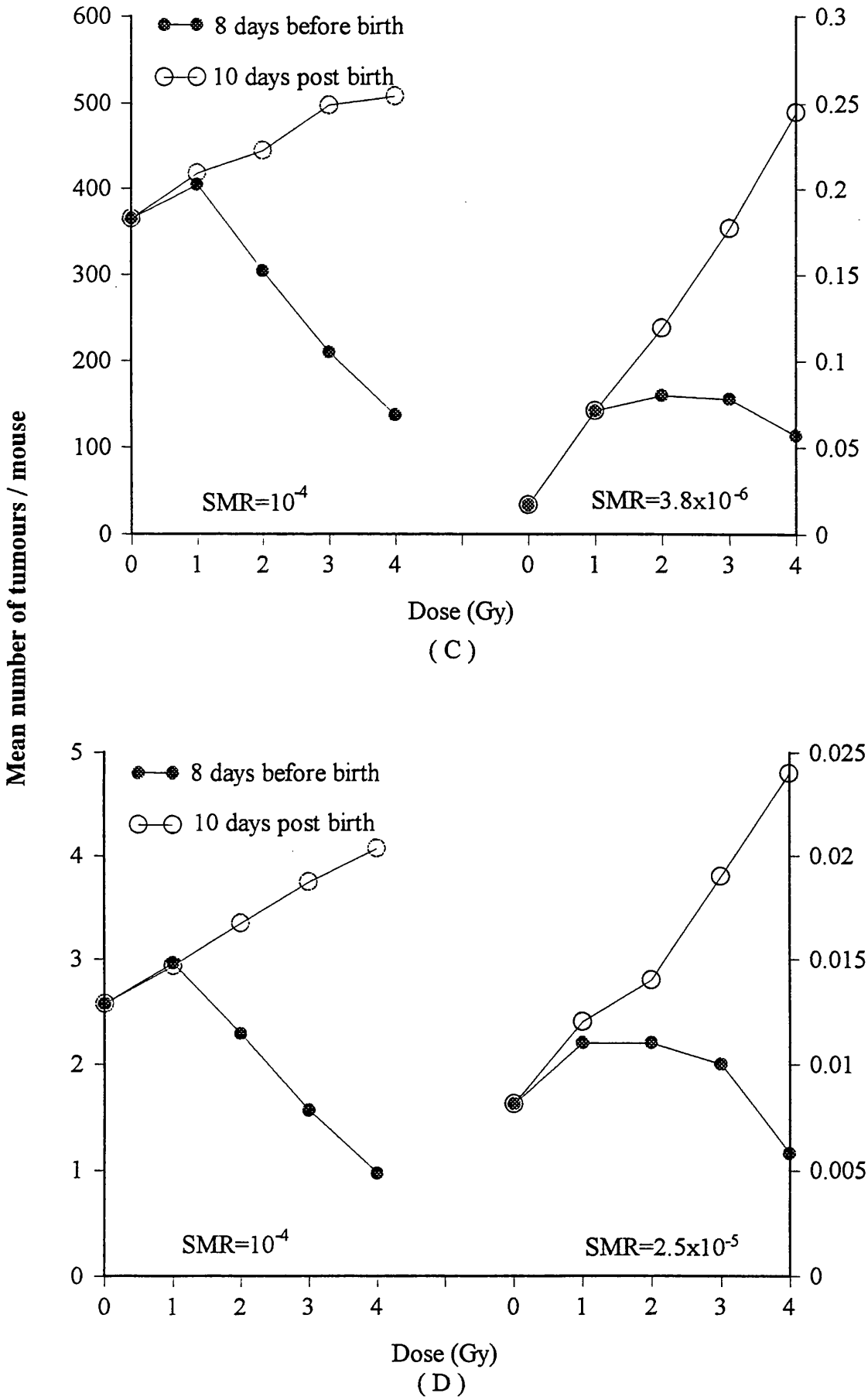


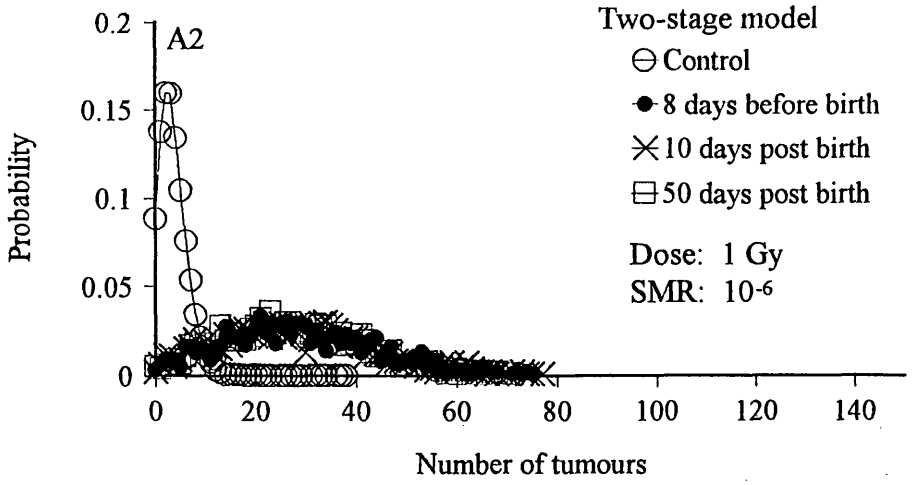
Figure 6.2 Dose-tumour-clonality response curve. (A) one-stage model; (B) two-stage model; (C) three-stage model; and (D) four-stage model

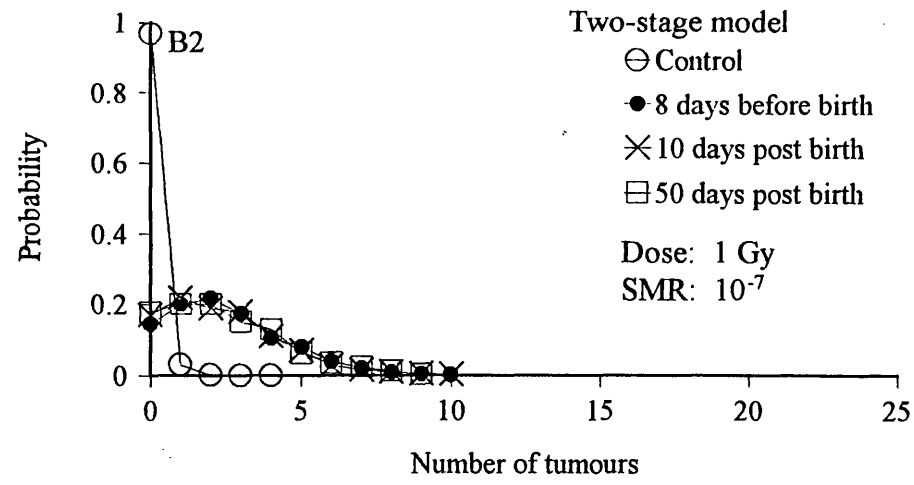
It has also been found that exposure to a single dose of radiation results in a corresponding increase in the probability of more than one tumour in a mouse (Figure 6.3). Very interestingly, lower doses (such as 1 Gy) always increase this probability (Figure 6.3 A1-A4 and B1-B4) whatever the time of irradiation, whereas, for pre-natal exposure, with higher value of spontaneous mutation rate, the higher doses reduce this probability (Figure 6.3 C1-C4 and D1-D4), which is considerably influenced by age at irradiation. These different observations provide a possible experimental test of the model predictions.

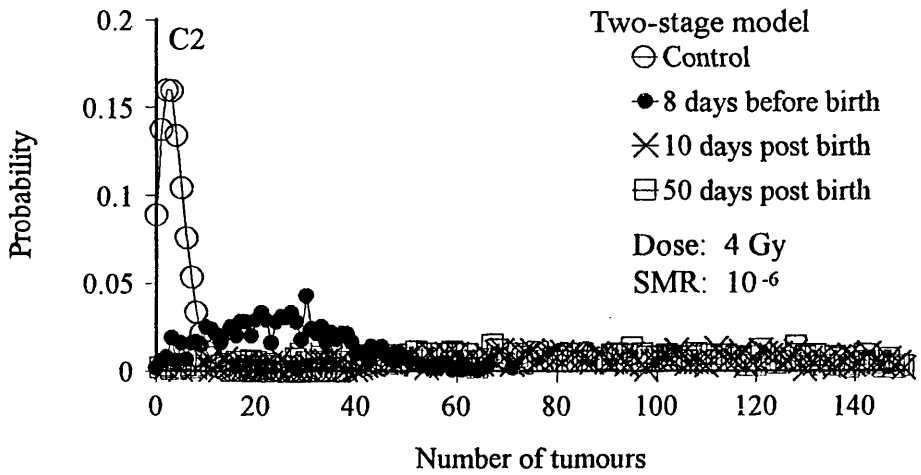
Figure 6.3 also shows that the number of tumours per mouse has a large variation, which is dependent on the number of mutational events required for malignant transformation, the spontaneous mutation rate, the dose of irradiation, and age at irradiation. Such variation reduces with the increase in the number of mutational events, and with decrease in the spontaneous mutation rate and dose (Figure 6.3).

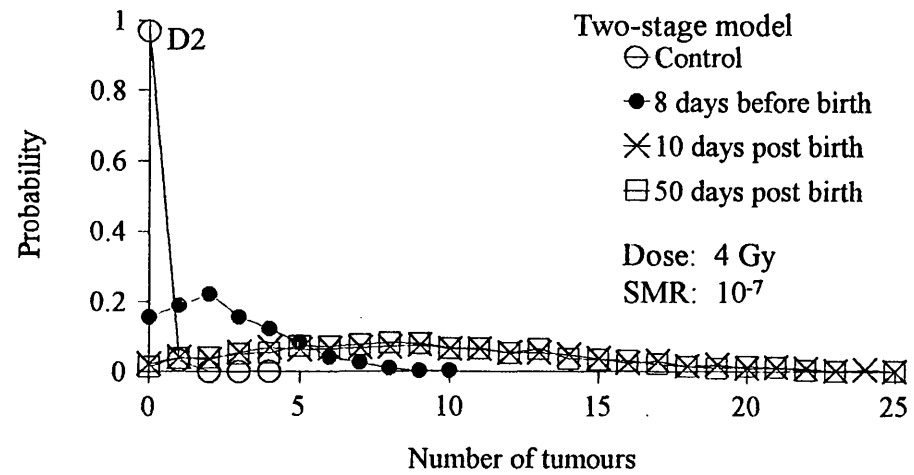
6.3.2 Times of tumour appearance

The time of the first, second and subsequent tumour appearance is strongly dependent on the number of mutational events required for malignant transformation and the spontaneous mutation rate. It is also influenced by the dose of radiation and age at exposure (Figure 6.4). With higher values of spontaneous mutation rate, for pre-natal exposure, higher doses of radiation do not accelerate tumour appearance, but lower doses do shorten the latency of tumour appearance (Figure 6.4). Interestingly, with the smaller number of mutational events and higher value of the spontaneous mutation rate, when the radiation is given at older age, the time of the first couple of tumours appearing does not shorten, but the time to appearance of subsequent tumours shortens (Figure 6.4 B3). This may be a consequence of radiation cell killing competing with tumorigenesis.

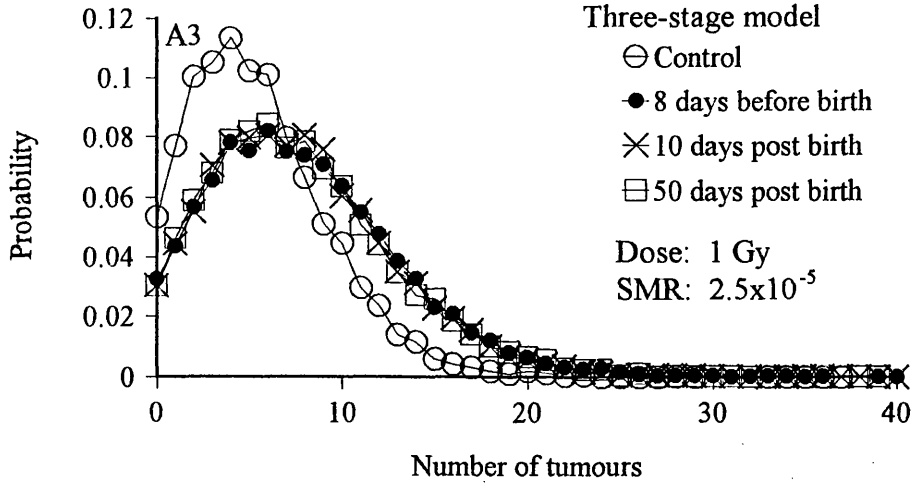


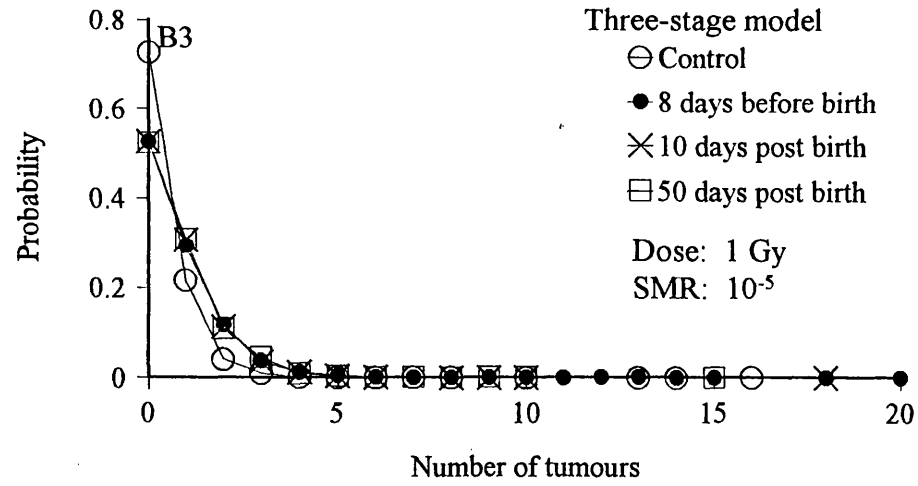


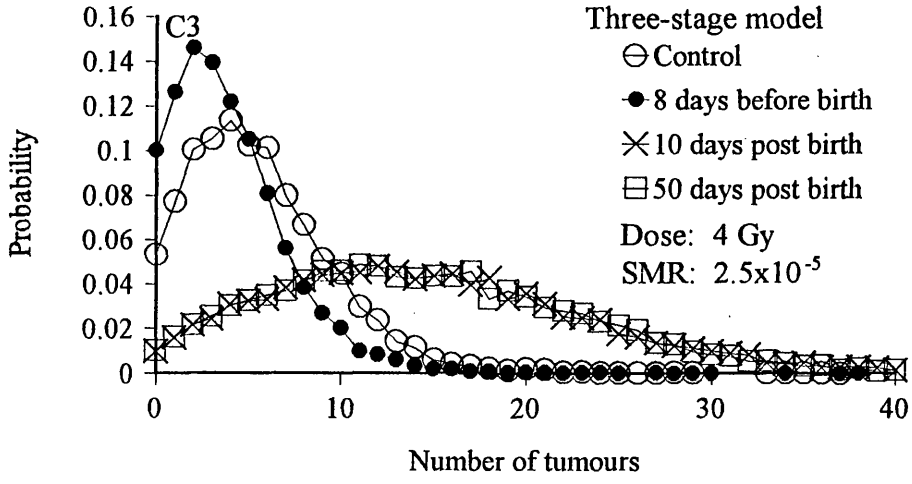


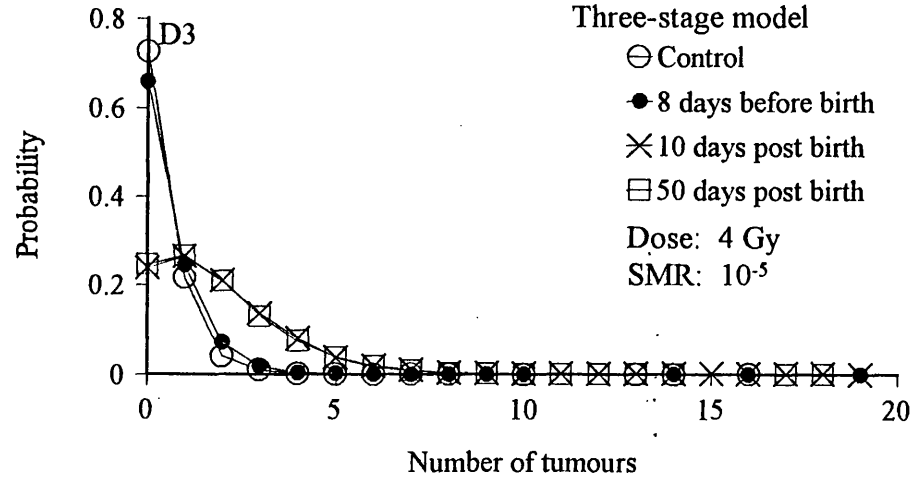


(Figure 6.3 to be continued)









(Figure 6.3 to be continued)

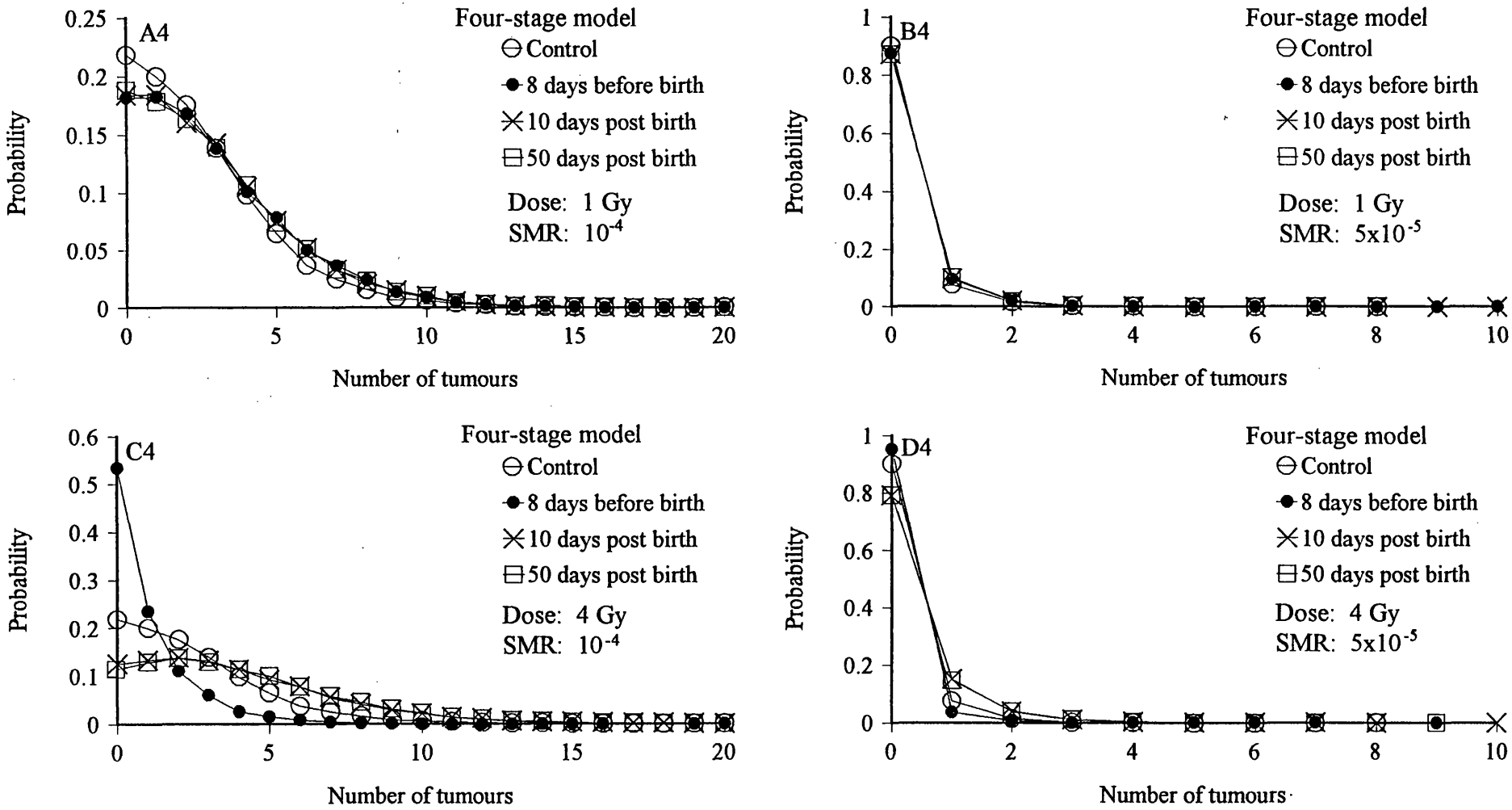
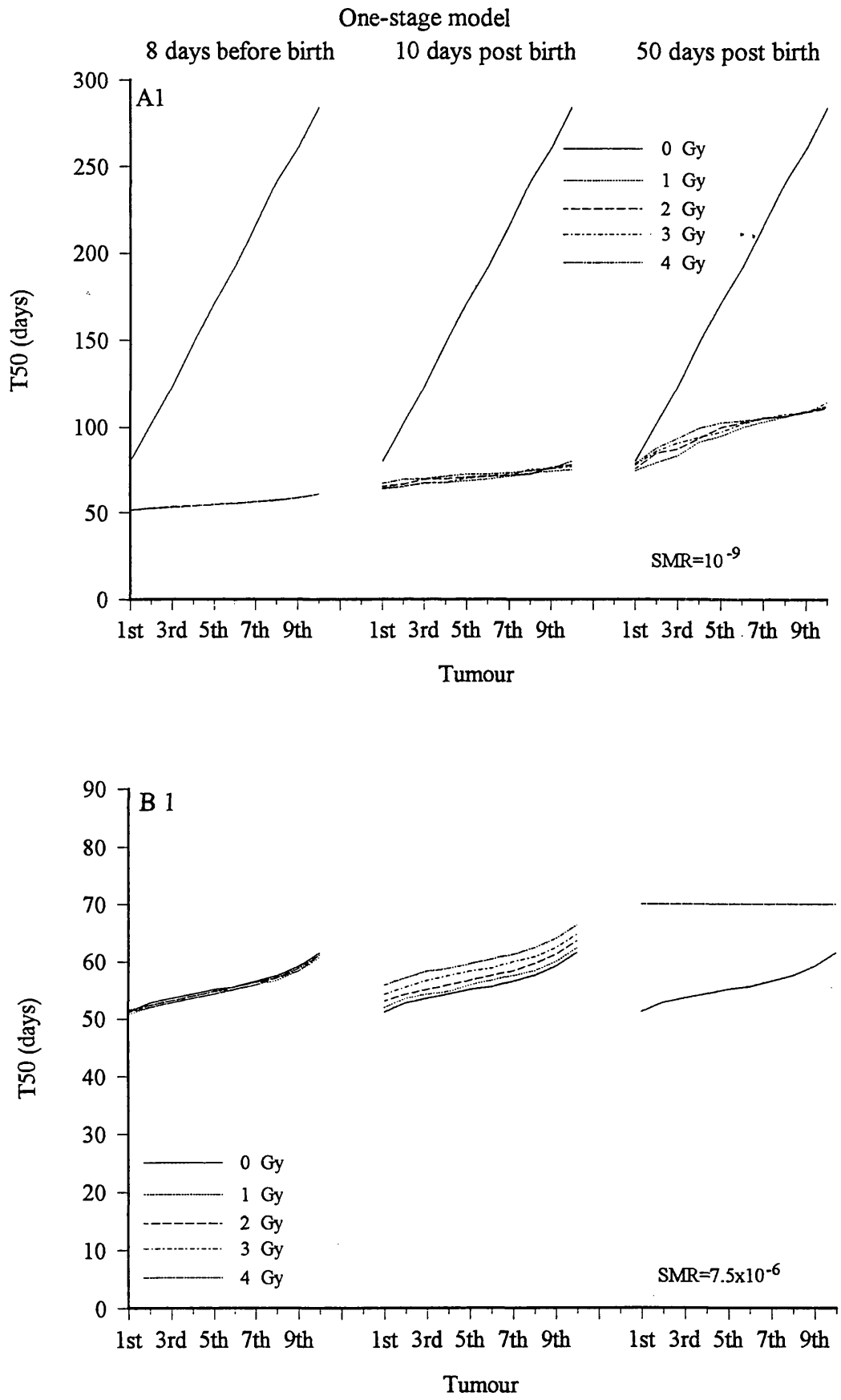
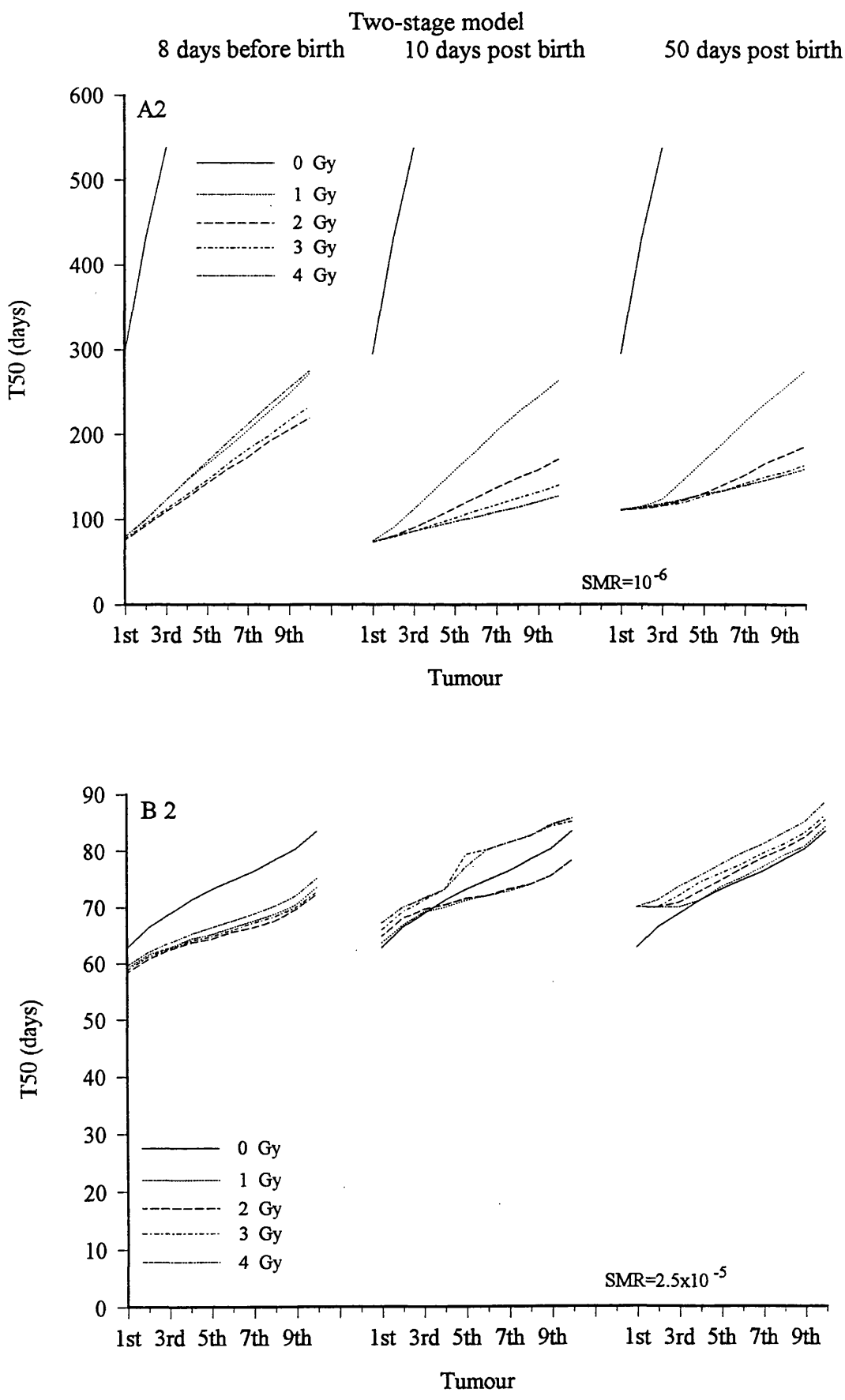


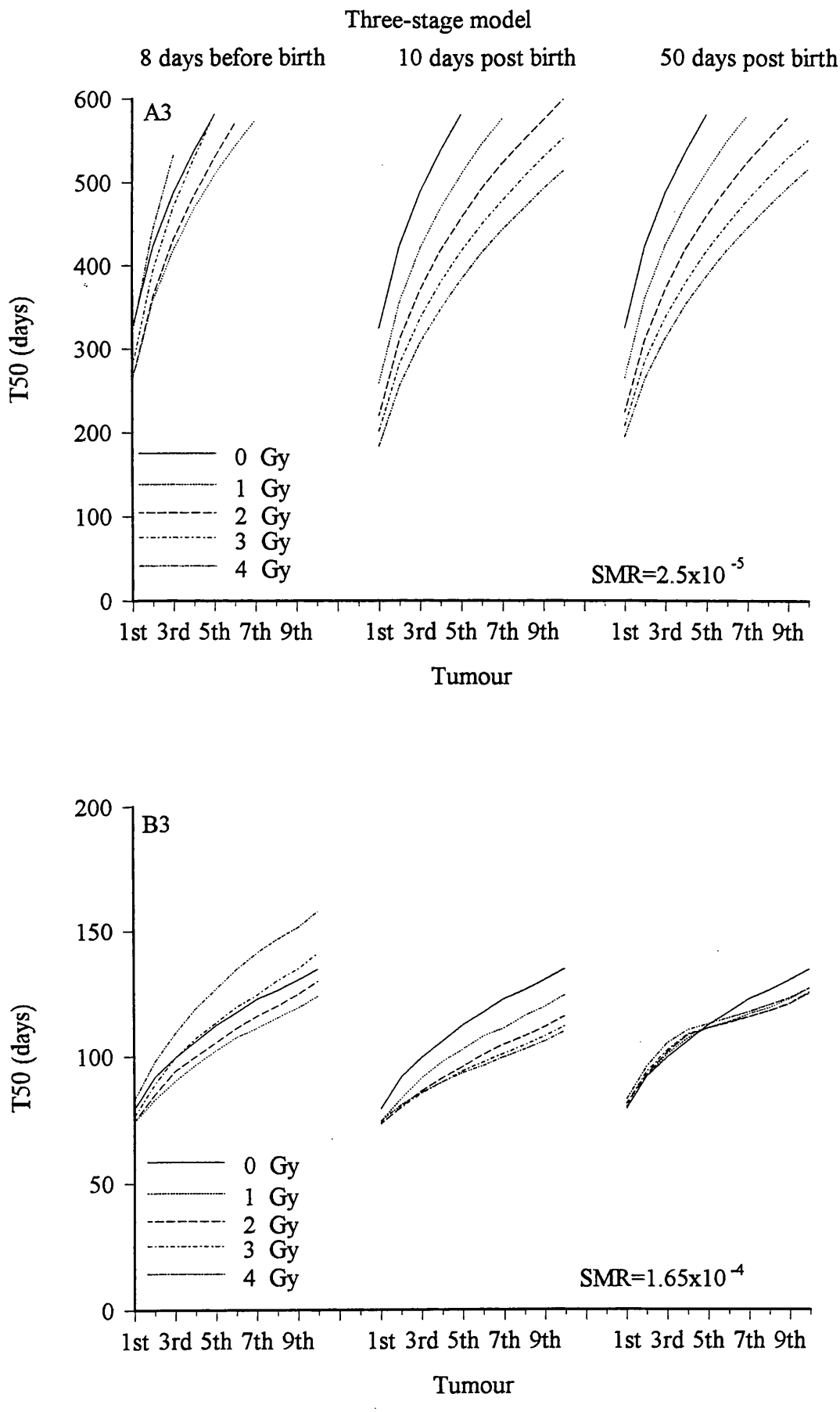
Figure 6.3 The probability density of the number of tumours prdicted by (A2 - D2) 2-stage model, (A3 - D3) 3-stage model, and (A4 - D4) 4-stage model.



(Figure 6.4 to be continued)



(Figure 6.4 to be continued)



(Figure 6.4 to be continued)

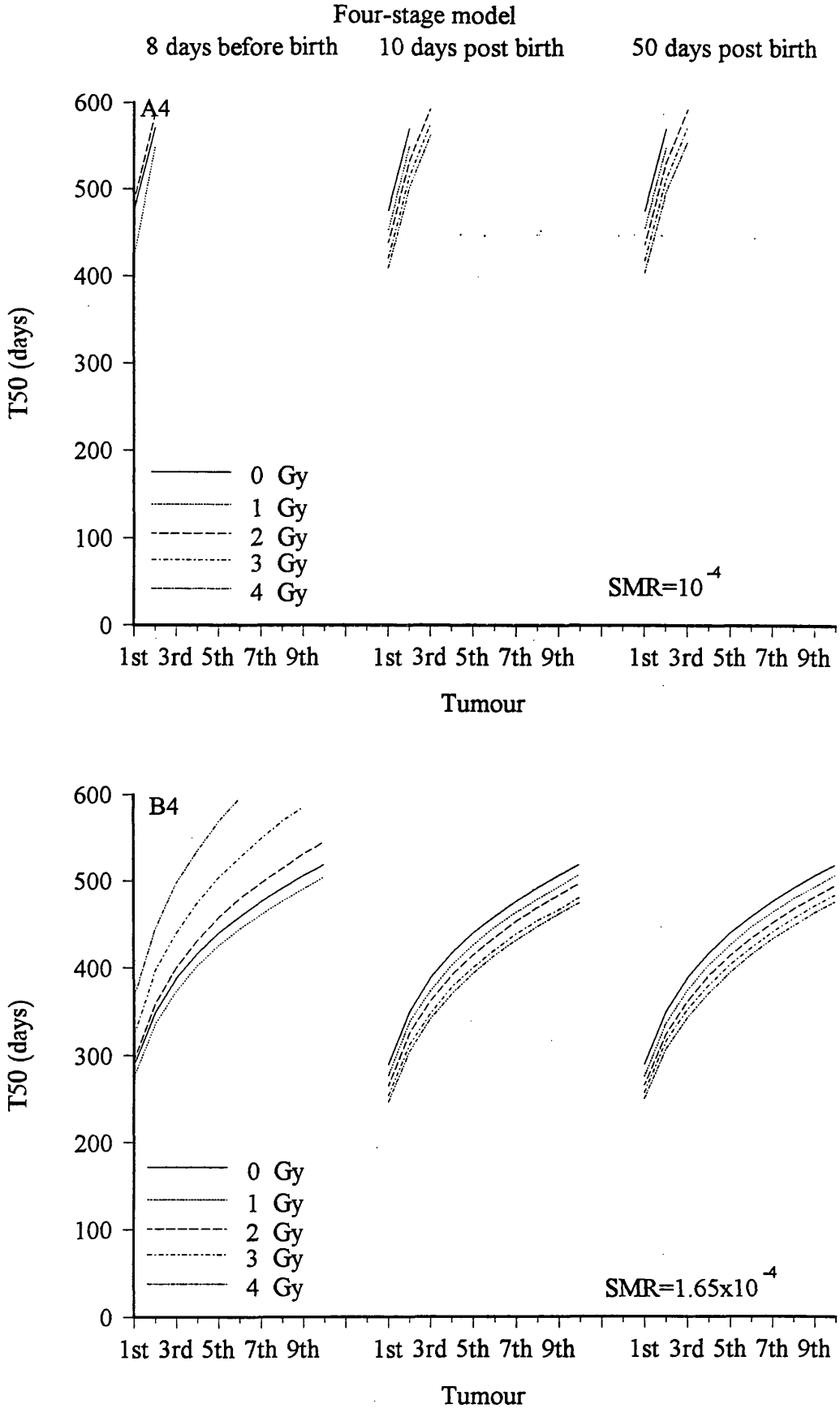


Figure 6.4 Time to appearance of 1st tumour, 2nd tumour, ..., 10th tumour in 50% mice (T50). (A1)and (B1) one-stage model; (A2) and (B2) two-stage model; (A3) and (B3) three-stage model; (A4) and (B4) four-stage model.

Surprisingly, with higher values of the spontaneous mutation rate in one and two-stage models, when the exposure to radiation occurs at a late age (eg 50 days), the latency of the first ten tumours is longer than in the spontaneous situation (Figure 6.4 B1 and B2). This is probably also due to the effect of radiation cell killing.

With higher number of mutational events and lower spontaneous mutation rate, the time between the first and subsequent tumours is also shortened by radiation. This relationship is also dependent on dose and age at exposure (Figure 6.4).

6.3.3 Spontaneous mutation rate

The spontaneous mutation rate is a very important biological factor for the effect of radiation on tumour incidence, multiplicity and latency. With higher values of mutation rate, when radiation is given very early or very late, the mean number of tumours/mouse is reduced (Figure 6.5), and the latency is prolonged by higher doses, especially for a small number of mutational events (Figure 6.2).

6.3.4 Number of mutational events

The number of mutational events required for malignant transformation is also a very important biological factor for the effect of radiation on tumour incidence, multiplicity and latency. With larger number of mutational events, the increase in tumour incidence and the mean number of tumours/mouse is not much increased by radiation (Figure 6.5), but the latency is shortened, especially when the spontaneous mutation rate is lower (Figure 6.2).

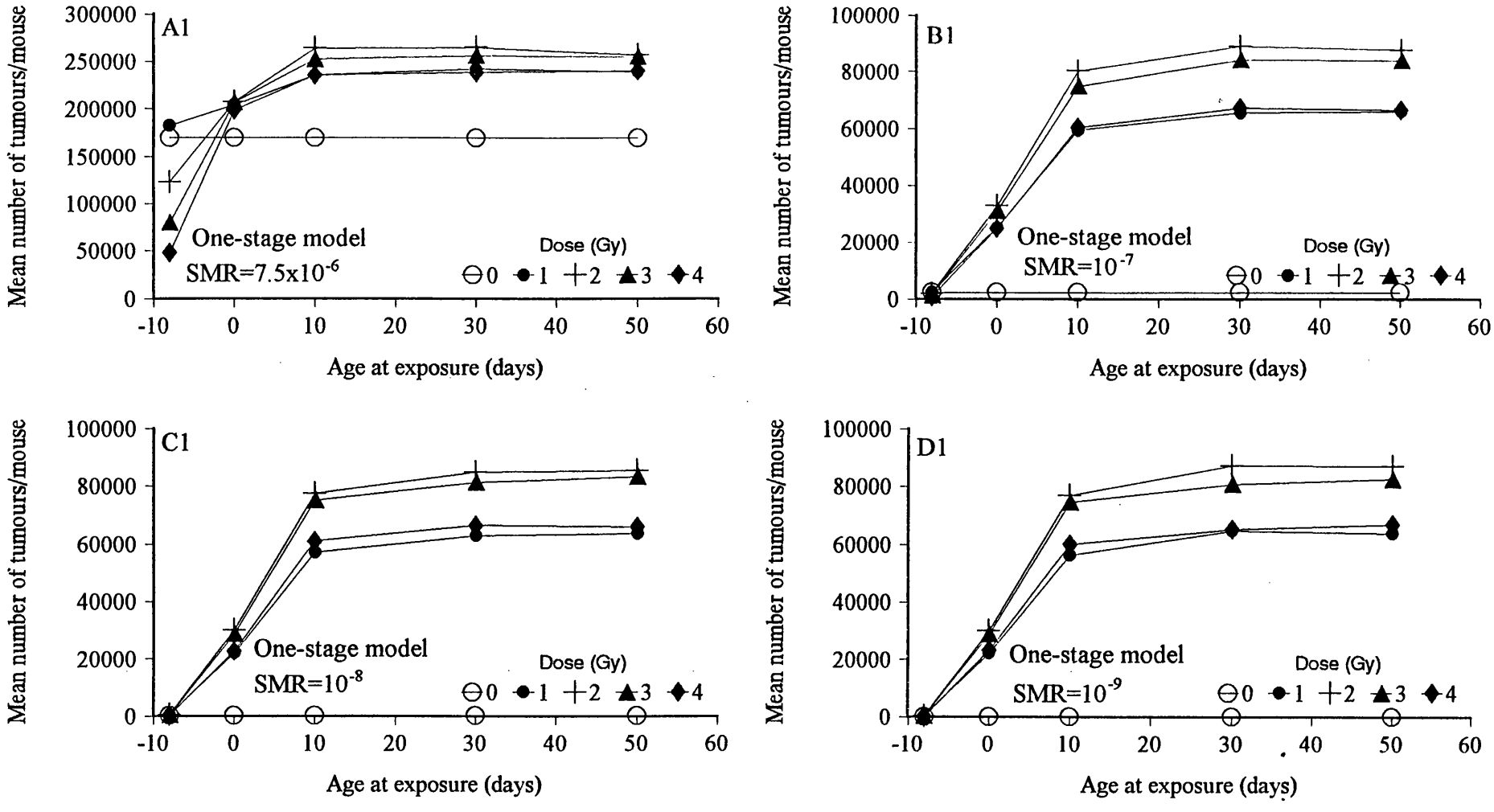
6.3.5 Age at exposure

Very interestingly, the effect of radiation on tumour multiplicity is very different between the cases of pre-natal and post-natal exposure (Figure 6.5). For single-dose pre-natal irradiation, the mean number of tumours per mouse decreases with dose, whereas when exposure is post-natal, the mean number of tumours per mouse increases with doses and does not reach the maximum by 4 Gy (except in the case of one-stage tumorigenesis) (Figure 6.2 (B), (C) and (D); and Figure 6.5).

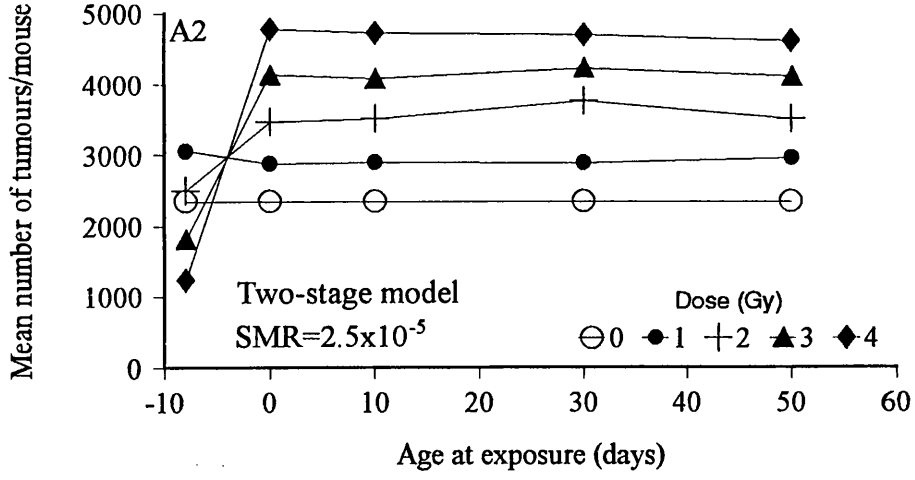
The other interesting thing is, (except for the one-stage model), the mean numbers of tumours per mouse do not change markedly with age at irradiation when the exposure is given post-natally (Figure 6.5 (A2-D2), (A3-D3) and (A4-D4)).

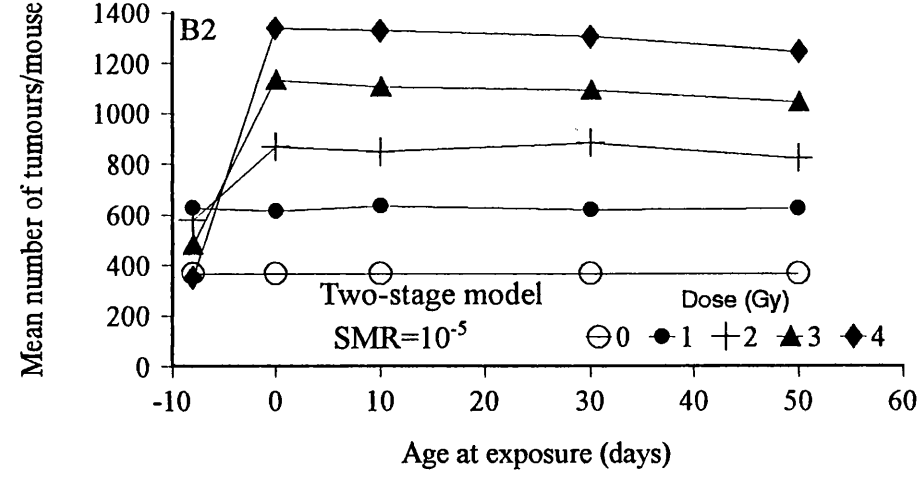
6.4 Tumour multiplicity in irradiated p53 deficient mice

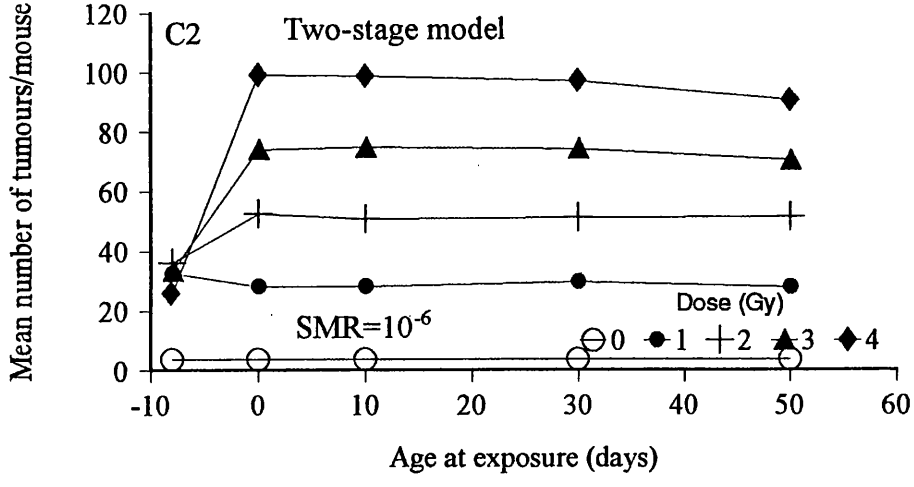
We also consider the situation where there is a certain background incidence (about 10% as previously discussed) in p53 wild type mice by 600 days and a single dose exposure is introduced. The corresponding tumour incidence for $p53^{+/-}$ and $p53^{-/-}$ mice then follows by subtracting 1 or 2 stages respectively without changing the spontaneous mutation rate and radiation sensitivity (i.e. ignoring any separate effect of p53 status on mutation rate and radiosensitivity).

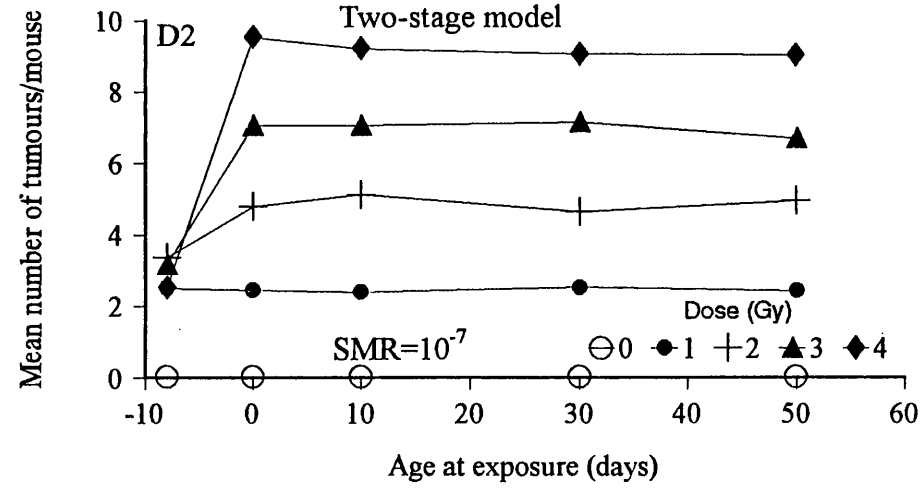


(Figure 6.5 to be continued)

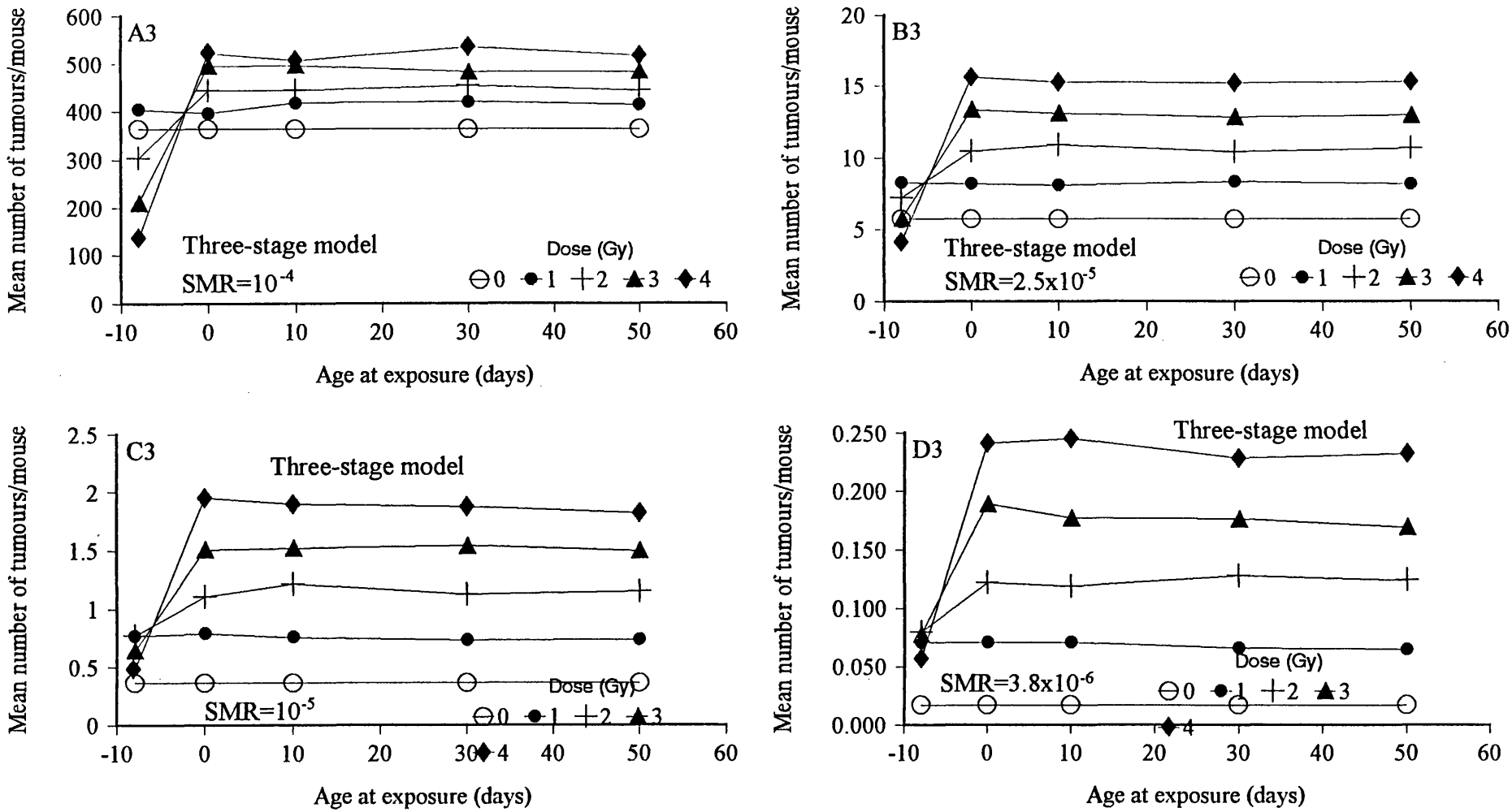








(Figure 6.5 to be continued)



(Figure 6.5 to be continued)

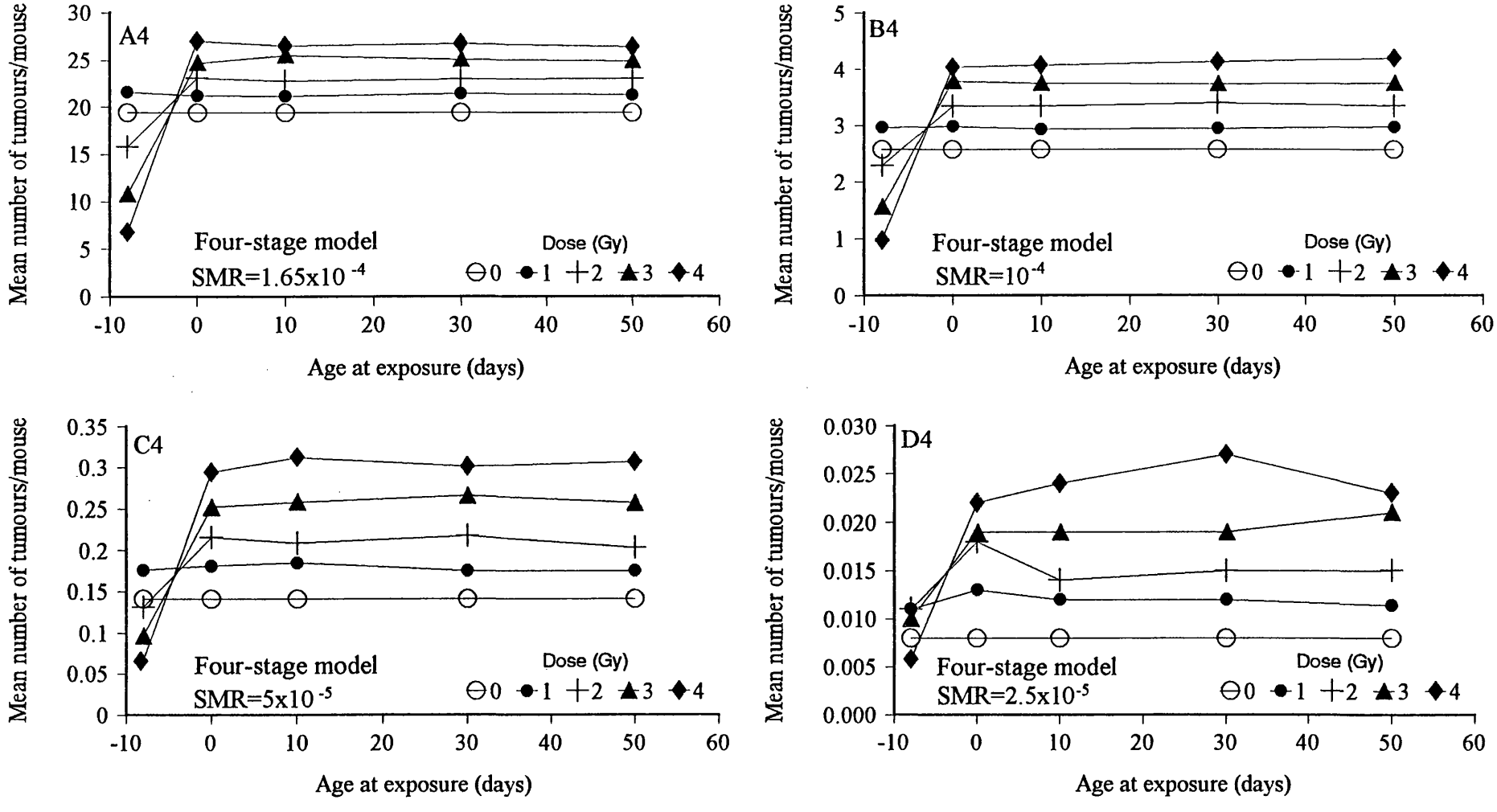


Figure 6.5 The relationship between the mean number of tumours per mouse and age at exposure predicted by (A1 - D1) 1-stage model, (A2 - D2) 2-stage model, (A3 - D3) 3-stage model, and (A4 - D4) 4-stage model

Figure 6.6 and 6.7 shows that 3, 4 and 5-stage models all predict the early development of large numbers of tumours in both irradiated $p53^{+/-}$ and $p53^{-/-}$ mice whatever the time of irradiation. Although the experimentally observed number of tumours will certainly be underestimates of the number destined to develop, it hardly seems possible that the predicted thousands of tumours could be reconciled with the observation of 1.623 tumours (23 tumours/14 mice) per $p53^{-/-}$ mouse irradiated with 4 Gy at age of 6 days reported by Kemp et al (1994). This discrepancy has been found to occur for all combinations of model parameters giving 10% lifetime background incidence of tumours in wild type mice, and it appears to be a robust feature of this class of model. Again, we have concluded that it is not possible to accommodate data on tumour incidence in wild type and p53 deficient mice by the classical multistage single-path model in which inactivation of each p53 allele is represented as a distinct stage in the tumorigenic process.

6.5 Discussion

The analysis has demonstrated that the effect of radiation on tumour multiplicity is strongly dependent on the number of mutational events required for malignant transformation, the spontaneous mutation rate, and age at exposure (summary in Table 6.1). Also, the shape of the dose-tumour-multiplicity curve is determined by these factors.

The age-associated change in tumour multiplicity may be attributable to two major factors: (1) the proliferative activity of the target cells during or post (or both) exposure to radiation, and (2) the number of cells at risk.

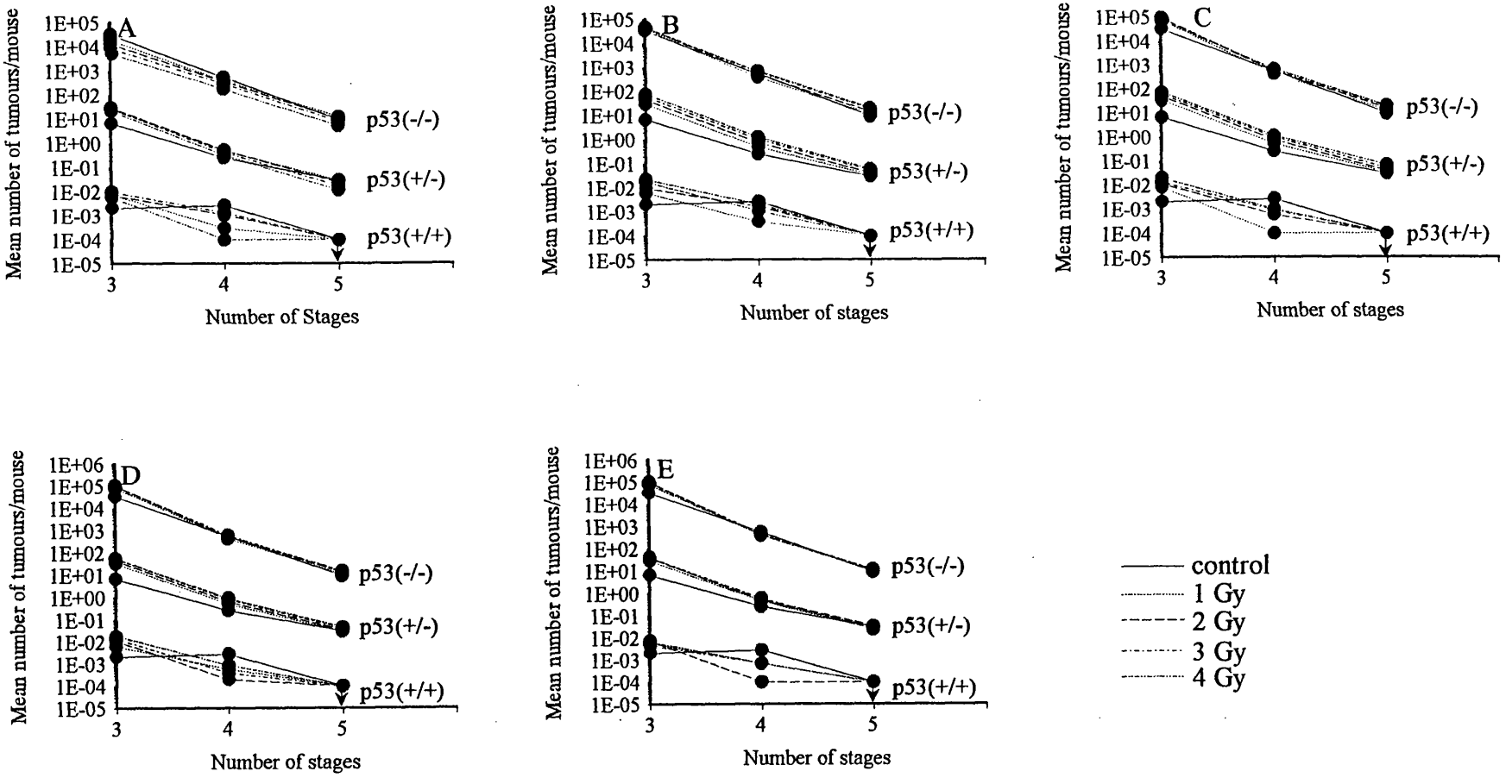


Figure 6.6 Mean number of tumours in irradiated wild type and p53 deficient mice predicted by 3-stage (SMR=7.5×10^{-6}), 4-stage (SMR=5×10^{-5}), and 5-stage (SMR=1.65×10^{-4}) models within 14 weeks. Age at exposure (A) 8 days before birth, (B) birthday, (C) 10 days post birth,(D) 30 days post birth, and (E) 50 days post birth. (↓ indicates the mean number of tumours less than 0.0001)

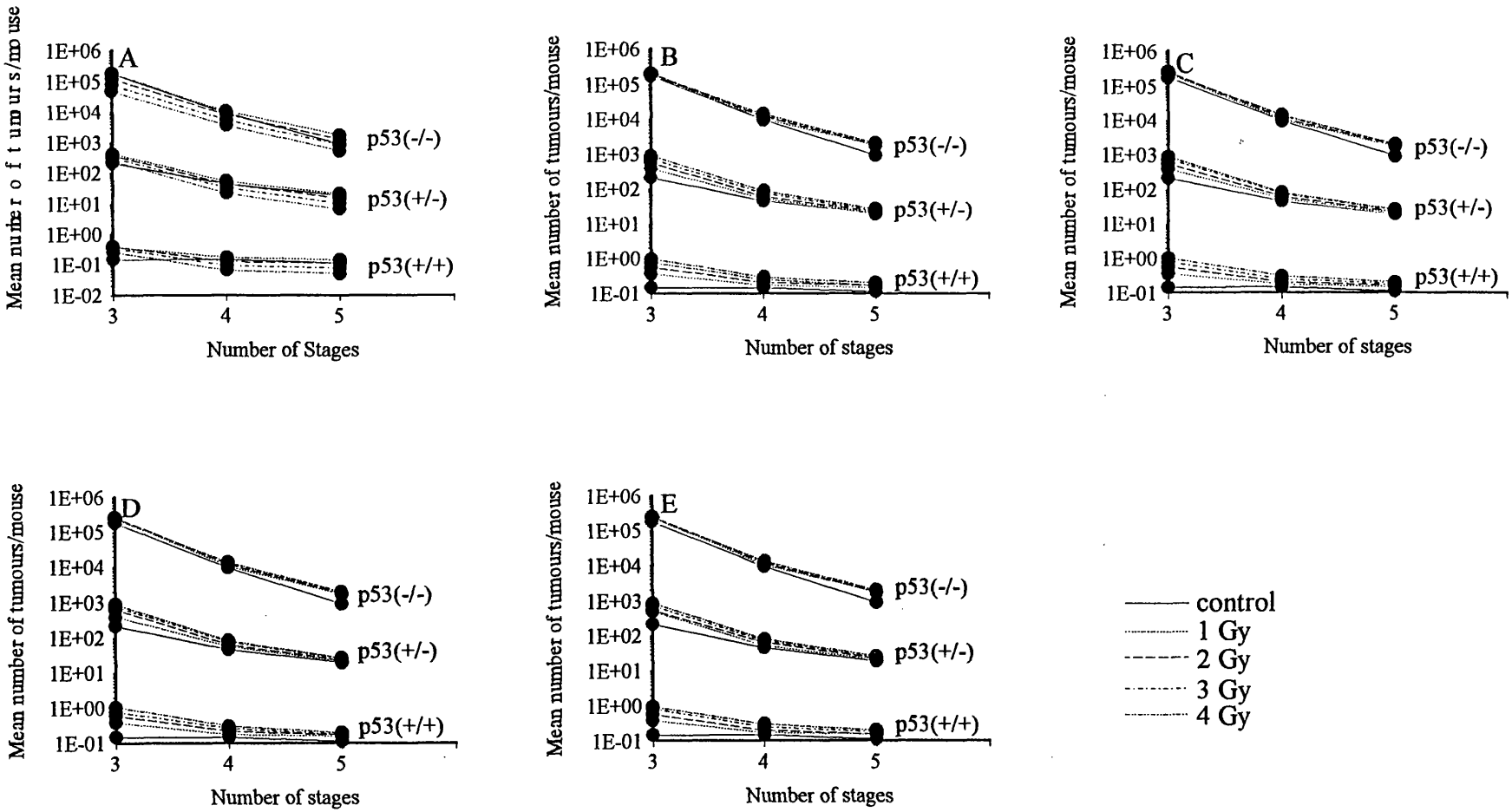


Figure 6.7 Mean number of tumours in irradiated wild type and p53 deficient mice predicted by 3-stage (SMR=7.5×10^{-6}), 4-stage (SMR=5×10^{-5}), and 5-stage (SMR=1.65×10^{-4}) models within 80 weeks. Age at exposure (A) 8 days before birth, (B) birthday, (C) 10 days post birth,(D) 30 days post birth, and (E) 50 days post birth.

Table 6.1 Effect of radiation (0-4 Gy) on tumour multiplicity

Age at exposure	Number of stages	Tumour multiplicity
Pre-natal	1	↗↘
	2	↗↘
	3	↗↘
	4	↗↘
Post-natal	1	↗↘
	2	↑
	3	↑
	4	↑

When the irradiation is given during the fetal period, the mean number of tumours in mice irradiated with higher doses is less than with lower doses. This result indicates that, when the number of cells at risk is smaller, with higher doses, more cells are killed, hence the likelihood of cells undergoing tumorigenic mutation is very small. On the contrary, with lower doses, fewer cells are killed, with the result that the likelihood of cells undergoing tumorigenic mutation is larger.

The latency of appearance of 1st, 2nd, 3rd, etc. tumour is also dependent on the number of mutational events, the spontaneous mutation rate, dose, and age at exposure (summary in Table 6.2). With a smaller number of mutational events and a higher value of the spontaneous mutation rate, when the radiation is given at older age, the time of appearance of the first few tumours is not shortened, but the time of the subsequent tumours is shortened. This could be because radiation kills some early spontaneous occurring tumours and induces the late occurring tumours. If true, this implies that these tumours which occur early in irradiated mice in this scenario are less likely to carry radiation-induced genomic

lesions than those tumours occurring later.

Table 6.2 Effect of radiation (0-4 Gy) on tumour latency

Age at exposure	Number of stages	Tumour latency	
		first few tumours	consequent tumours
Low spontaneous mutation rate			
Pre-natal	1	↓	↓
	2	↓	↓
	3	↓	↓
	4	↓	↓
Neonatal	1	↓	↓
	2	↓	↓
	3	↓	↓
	4	↓	↓
Adult	1	↓	↓
	2	↓	↓
	3	↓	↓
	4	↓	↓
High spontaneous mutation rate			
Pre-natal	1	↘↗	↘↗
	2	↘↗	↘↗
	3	↘↗	↘↗
	4	↘↗	↘↗
Neonatal	1	↑	↑
	2	↑	↘↗
	3	↓	↓
	4	↓	↓
Adult	1	↑	↑
	2	↑	↑
	3	↑	↓
	4	↓	↓

As in the previous chapter, the analysis has also demonstrated a fundamental problem in the application of the classic multistage model to radiation tumorigenesis in p53 deficient mice. On a single pathway multistage model of tumorigenesis, the reduction in stage number by one, without change of spontaneous mutation rate and radiation sensitivity, results in a marked increase in predicted tumour frequency, corresponding to a reduction in stage number by two, the number of tumours per mouse would be astronomical, no matter the time of exposure. In next two chapters, we will try to develop models which could account for this.

Chapter 7

Multiple Pathways Model for Multistage Tumorigenesis in p53 Deficient Mice

7.1 Introduction

In previous chapters, the models have been restricted to a single series of events leading to carcinogenesis, i.e., a single pathway, which have been found to predict a large number of tumours in p53 null heterozygous and null homozygous mice. In fact, for human cancer, p53 mutations do not occur in all cases of any one type of cancer (see Table 1.5.2 in chapter 1), and not all tumours developing spontaneously in p53 heterozygous mice show loss of the remaining p53 allele (Harvey et al, 1993; Kemp et al, 1994). This implies that there exist one or more p53 independent pathways.

Recently, several authors have considered an extension of the multistage model which allows for the development of any type of tumour by alternative multistage pathways involving different sets of genes (Tan 1991; Sherman and Portier 1994). In the present context, this means that a proportion of tumours in wild type mice developing by pathways which are independent of p53 status would have the same probability of occurrence in p53-deficient mice. In order to investigate this, a multiple pathway model for multistage tumorigenesis has been constructed. It was intended to explore the relationship between the fraction f of the tumours developing in wild-type mice by the p53 pathway and the tumour multiplicity in $p53^{+/-}$ and $p53^{-/-}$ mice and to determine whether the model is compatible with the experimental data or not.

7.2 Models and assumptions

Figure 7.1 displays a two-pathway multistage model of tumorigenesis. There are two

possible routes for a normal cell to be transformed into a malignant cell, i.e. the p53 mediated route and the p53-independent route. The essential features of each pathway are as described in section 3.2 of chapter 3.

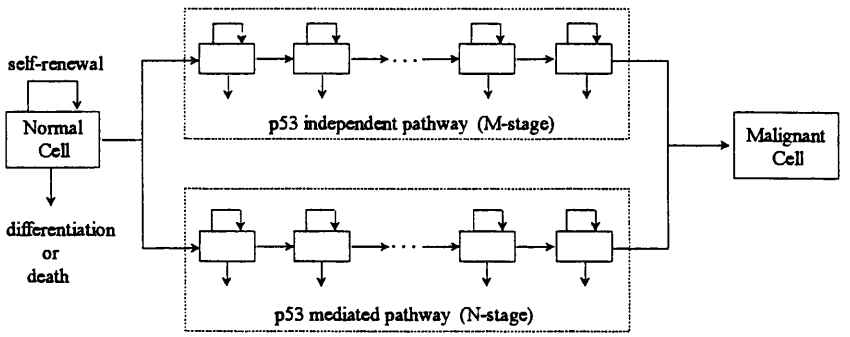


Figure 7.1 General structure of the multipath/multistage model with p53 mediated and p53 independent routes of tumorigenesis.

The completion of all mutational events in any either of two routes leads to malignant transformation. There exists competition between these two routes in tumour development. We assume that only a fraction f of the tumours developing in wild-type mice have arisen by the p53 mediated pathway. It is then only the p53 mediated tumours whose frequency is increased in the p53 deficient genotypes.

The non-p53-mediated path has unspecified structure and parameters. However, knock-out of p53 gene enhances only the p53-mediated tumour development. In $p53^{+/-}$ and $p53^{-/-}$ mice, the great majority of tumours develop by the p53-mediated route. For these reasons, the p53-mediated route has been the focus on in our studies, and we will not discuss further the non-p53-mediated path.

7.3 Simulation results

As in Chapter 5, we have simulated 3, 4 and 5 stage models for p53-mediated tumorigenesis in p53 wild-type mice with a range of f values (f=10 or 1%). As discussed in Chapter 5, the total incidence of tumorigenesis in wild-type mice is about 10%, and the incidence of the p53-mediated tumorigenesis will be about 10% times f. Therefore we have in each case chosen the mutation rate to give approximately 0.1f times the p53-mediated tumour incidence in wild type mice by 600 days. The corresponding p53-mediated tumour incidences for $p53^{+/-}$ and $p53^{-/-}$ mice then follow by subtracting 1 or 2 stages respectively without changing the mutation rate. For presentation, we have computed the number of p53-mediated tumours predicted to have appeared by 16 and 80 weeks.

For all models, it has been generally found that the number of tumours predicted in $p53^{+/-}$ and $p53^{-/-}$ genotypes decreases as the fraction f decreases (Table 7.1). This relationship is strongly dependent on the number of stages required for malignant transformation in p53 wild type mice.

Table 7.1 shows that 3 and 4-stage models still predict the early development of large number of tumours in $p53^{-/-}$ mice even when only 1% of all tumours in wild-type mice develop by the p53 mediated route. The model-predicted age-incidence distribution of tumours is shown in Figure 7.2 (A and B) and demonstrates much faster tumour development predicted by the model for $p53^{-/-}$ mice than occurs experimentally. These results indicate that f must be lower than 1% if the predicted number of tumours in $p53^{-/-}$ mice is to be compatible with experimental data. However the predicted number of

the number of tumours in $p53^{+/-}$ mice is already less than the experimentally observed number (Table 7.1). Furthermore, Figure 7.2(D) shows slower tumour development in $p53^{+/-}$ mice than occurs experimentally. The 3-stage model also predicts the early development of large numbers of tumours in $p53^{+/-}$ mice when f=1%. For compatibility with experimental data in $p53^{+/-}$ mice, it is required that the mutation rate is between 10^{-7} and 10^{-6}/cell division, but, tumour development predicted by the model in $p53^{-/-}$ mice is still faster than occurs experimentally. Therefore, for both the 3 and 4-stage models, it is impossible to find a value of f which makes the predicted number of tumours in both p53 deficient genotypes consistent with the experimental data.

Table 7.1 Predicted tumour development by the p53-mediated pathway in mice of different genotypes on 3, 4 and 5 stage models for p53 mediated tumorigenesis with 10% or 1% of all tumours in wild type mice developing by the p53 mediated route and 90% or 99% by p53 independent pathways.

Fraction	Genotype	3-stage model Tumours/mouse (SE)*		4-stage model Tumours/mouse (SE)*		5-stage model Tumours/mouse (SE)*	
		16 weeks	80 weeks	16 weeks	80 weeks	16 weeks	80 weeks
10%		(Mutation rate=3.8×10^{-6})		(Mutation rate=2.5×10^{-5})		(Mutation rate=5×10^{-5})	
	$p53^{+/+}$	0.0003 (0.0003)	0.018 (0.0016)	0.0017 (0.0006)	0.018 (0.002)	0 (0)	0.029 (0.003)
	$p53^{+/-}$	1.8 (0.17)	55.7 (0.89)	0.029 (0.002)	5.3 (0.03)	0.0028 (0.001)	0.2 (0.005)
	$p53^{-/-}$	1.7×10^{4} (79.2)	9.0×10^{4} (4.2×10^{2})	1.4×10^{2} (4.6)	2.4×10^{3} (15.7)	0.3 (0.02)	46.4 (0.55)
1%		(Mutation rate=2×10^{-6})		(Mutation rate=9×10^{-6})		(Mutation rate=10^{-5})	
	$p53^{+/+}$	0 (0)	0.0023 (0.0006)	0.0003 (0.0003)	0.0044 (0.001)	0 (0)	0.0036 (0.001)
	$p53^{+/-}$	0.5 (0.08)	15.6 (0.68)	0.0012 (0.0004)	0.3 (0.006)	0.0005 (0.0004)	0.0048 (0.001)
	$p53^{-/-}$	8.9×10^{3} (41.6)	4.7×10^{4} ($2.2\times10^{2)}$	11.3 (0.34)	3.1×10^{2} (2.7)	0.0011 (0.0003)	0.36 (0.007)

* SE = standard error of mean number of tumours per mouse

When f is less than 10%, the 5-stage model predicts fewer tumours in both p53 deficient genotypes (Table 7.1) and the age-incidence also shows slower development of tumours (Figure 7.2 (E) and (F)). Therefore we studied the 5-stage model extensively and observed compatability with the experimental data only for the 5 stage model with f = 20% (i.e 20% of all tumours in wild type mice developing by the p53 mediated pathway). For f > 20% an excess of tumours are predicted for $p53^{-/-}$ mice and for f < 20% a deficit of tumours is predicted in the $p53^{+/-}$ genotype (figure 7.3). The data for p53-mediated tumours for f = 0.2 are shown in table 7.2.

Table 7.2 Predicted tumour development by the p53 mediated pathway in wild type and p53 deficient mice on a 5 stage model for p53 mediated tumorigenesis with 20% of all tumours in wild type mice developing by the p53 mediated route and 80% by p53 independent pathways.

Genotype	**5-Stage Model** (Mutation rate = 7.5×10^{-5}) p53-mediated tumours /mouse (SE)*	
	16 weeks	80 weeks
$p53^{+/+}$	0 (0)	0.038 (0.003)
$p53^{+/-}$	0.006 (0.001)	0.83 (0.02)
$p53^{-/-}$	0.863 (0.028)	193 (1.5)

* SE = standard error of mean number of tumours per mouse

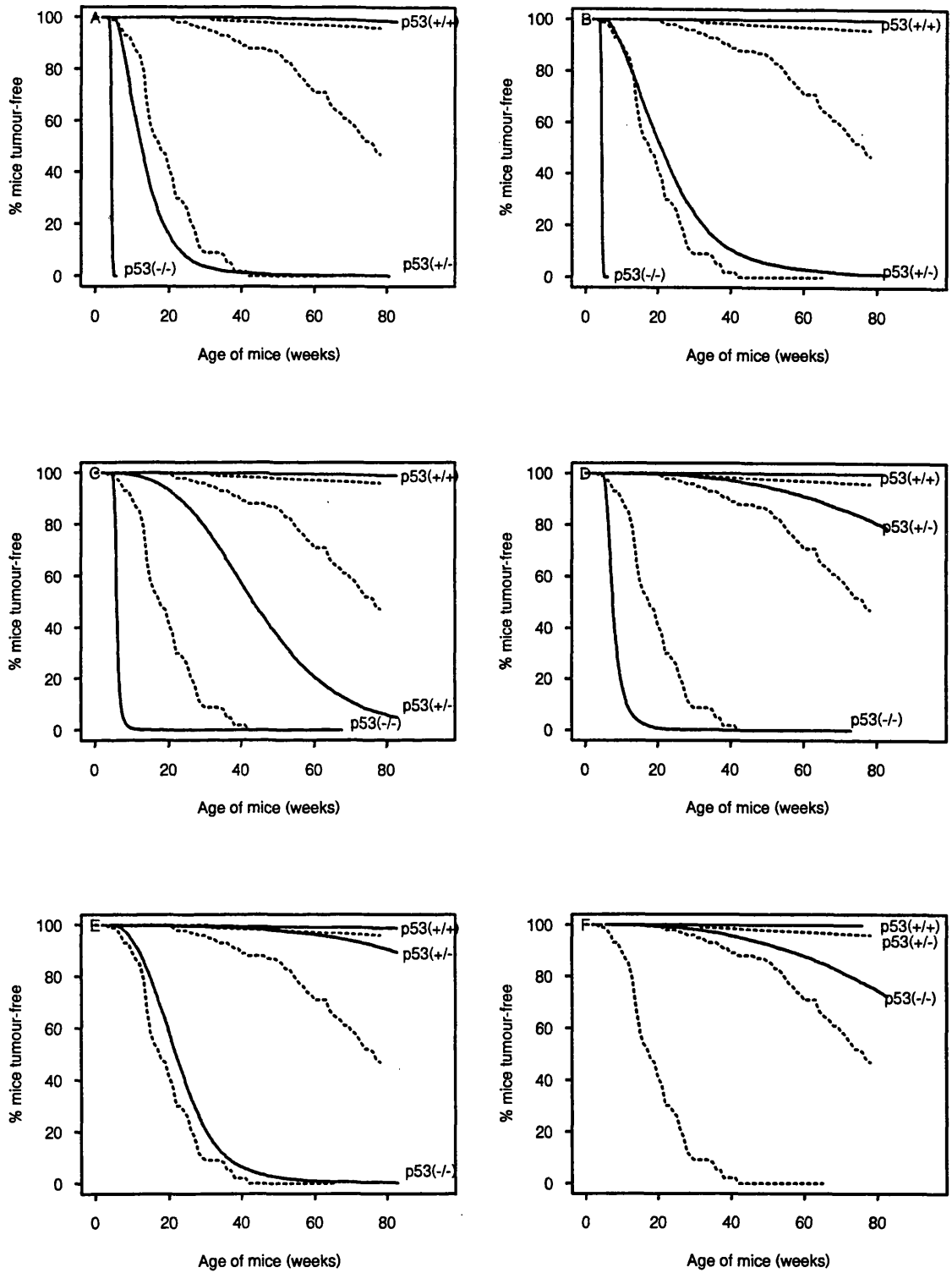


Figure 7.2 Predicted age-incidence pattern of p53-dependent tumour development for mice of three genotypes for the multipath multistage model with (A) 3-stage model and f=10%, (B) 3-stage model and f=1%, (C) 4-stage model and f=10%, (D) 4-stage model and f=1%, (E) 5-stage model and f=10%, (F) 5-stage model and f=1%. The broken lines in the diagrams show the incidence of experimental tumours (data from Donehower et al (1995), dashed line).

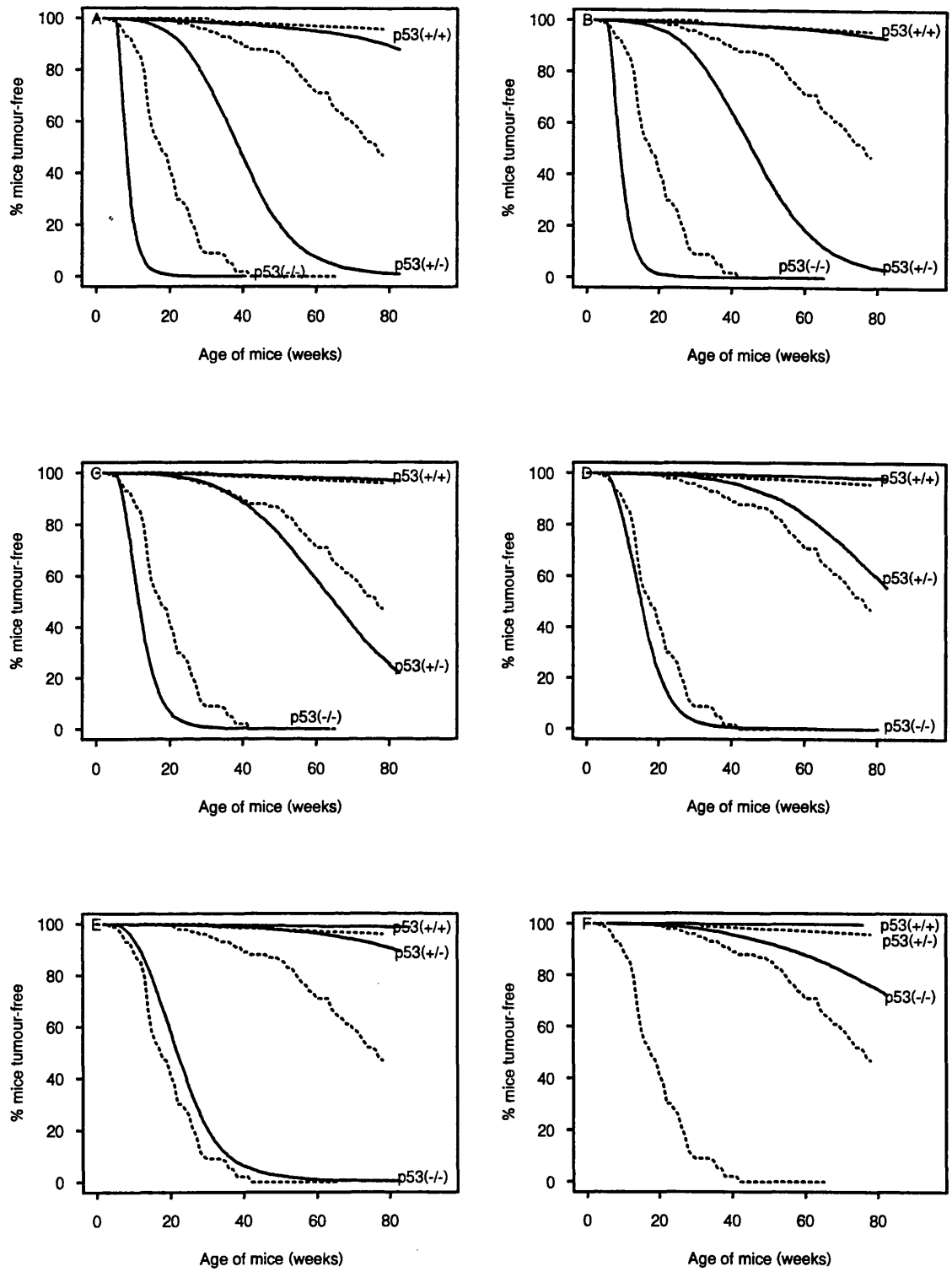


Figure 7.3 Predicted age-incidence pattern of p53-dependent tumour development for mice of three genotypes for the 5-stage multipath model with (A) 100, (B) 60, (C) 30, (D) 20, (E) 10, and (F) 1% of tumours in wild type mice arising by the p53 dependent route. The broken lines in the diagrams show the incidence of experimental tumours (data from Donehower et al (1995), dashed line).

7.3 Discussion

We have developed a multipath multistage model invoking a p53 independent pathway which exists in parallel with a p53 mediated route of tumorigenesis. Only the latter route is enhanced in p53 deficient genotypes. On this type of model, the mutation rates are independently fixed and these inherent rates are not changed by p53 inactivation, which only alters the number of stages required for malignant transformation by the p53-mediated route.

This model is supported by some experimental data. Heavly et al (1993) and Kemp et al (1994) have shown that the remaining p53 allele is not lost in all spontaneously-developing tumours in $p53^{+/-}$ mice. This means some tumour development in $p53^{+/-}$ mice may not involve p53 inactivation. Also, human cancer data has shown that only a proportion of developing malignancies involve p53 mutations, and this fraction varies with the type of cancer (see Table 1.5.2 in Chapter 1).

The disruption of the p53 gene accelerates p53-mediated tumour development. However, the mean number of tumours in $p53^{+/-}$ and $p53^{-/-}$ mice depends on the fraction f of all tumours in wild-type mice developing by the p53 mediated route. The present studies show that a higher value of f results in a larger number of tumours in $p53^{+/-}$ and $p53^{-/-}$ mice. This relationship is strongly influenced by the number of events required for malignant transformation by the p53-mediated route in wild type mice.

The detailed age-incidence kinetics of developing tumours cannot be computed on this

model since the process of tumour development is only defined mechanistically for the p53 mediated pathway - the non-p53-mediated path has unspecified structure and parameters. For this reason, only the tumours developing by the p53 mediated route are shown; in the $p53^{+/-}$ and $p53^{-/-}$ (but not wild type) genotypes these will be the great majority of tumours. It has been shown that more reasonable tumour numbers may be predicted for each of the genotypes by 5-stage p53 mediated pathway with f=20%. This means that the multipath multistage model does seem capable of being reconciled with most of the data.

However, reconciliation of the model with the data is only possible with a somewhat contrived choice of parameters, and with the p53 independent pathway a 'black box' for tumour development which is invoked as a means of reconciling the experimental data with model predictions, but does not have any given mechanistic structure of its own. In the next chapter, we will consider a class of models which seem able to account for the experimental findings in a more natural way.

Chapter 8

Multigate Model of Tumorigenesis with the p53 Gene as a Rate-Modifier of Gate-Pass Events

8.1 Introduction

In each of the preceding models discussed in the previous chapters, mutations occurred independently and the rate of mutation at any stage was unaffected by mutations having already occurred at other stages. However, it is a current hypothesis, termed by Lane (1992) the 'guardian of the genome' concept, that p53 inactivation results in generally increased mutation rates, i.e. wild-type p53 acts to confer genetic stability. We now wish to consider how this idea made be incorporated in stochastic modelling and how well the model accommodates the data. To do this, it may be useful to distinguish between mutations which are directly or indirectly tumorigenic.

Directly tumorigenic mutations, whether these are oncogene activations or inactivations of tumour suppressor genes, are 'enabling' or obligatory events which must accumulate to a minimum number, or possibly to one of several alternative configurations, for malignant transformation of the affected cell. We may consider that tumorigenesis requires a number of regulatory 'gates' to be passed and that a tumorigenic mutation of direct type alters a gateway gene and corresponds to a gate-pass event. The gate-pass events are the stages of the multistage model. However, we may conceive of a different class of mutations (indirect mutations) which are not enabling events in themselves but modifiers of the tumorigenic mutation rate. This leads to a multi-gate model of tumorigenesis with mutation rates under the control of rate-modifier genes. Mutated rate-modifier genes lead to altered mutation rates in gateway genes. A similar concept has been proposed by Loeb (1991), who has argued for the existence of a 'mutator phenotype' and by Sherman and Portier (1994), who have termed such a process a 'multi-hit' model to distinguish it from multi-stage. The

structure of a 3-gate model, associated with a 2-stage modifier (p53 inactivation) pathway is depicted in Figure 8.1. A more detailed breakdown of the structure is given in Figure 8.2. For simplicity, this new model is termed the multi-gate/multi-stage model.

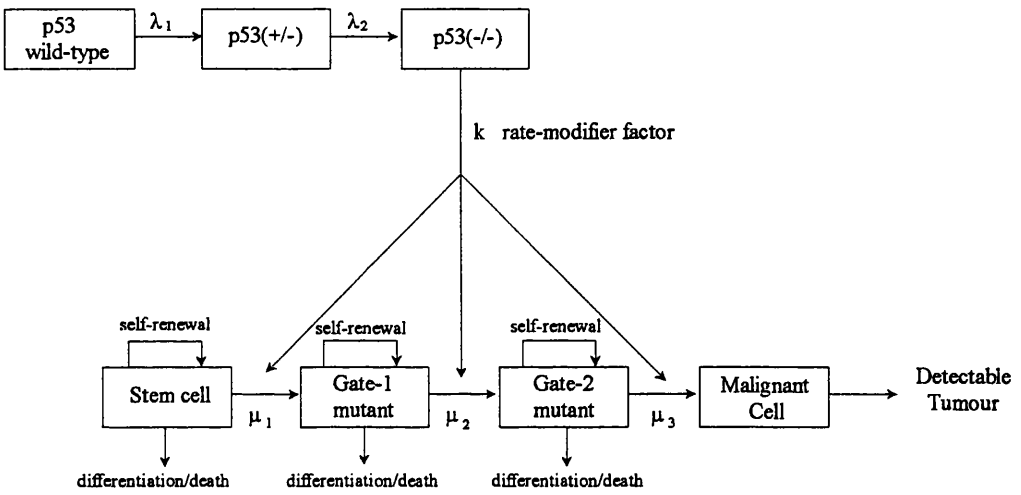


Figure 8.1 General structure of 3-gate/2-stage model for tumorigenesis.

A feature of the model is that mutation of the gateway genes alone (without modifier gene mutations) may lead to malignant transformation, whereas modifier mutations cannot achieve transformation without gate-pass events. Therefore, only a proportion of tumours developing by this gateway will be associated with modifier mutations. This proportion will depend on the numbers of genes involved in the gateway and in the modifier pathway, and on rates of mutation of modifier genes and of gateway genes, when modifier mutations have or have not occurred. Generally, the proportion of modifier-associated mutations will be low unless there are more gates than modifier stages or unless the modifier genes are

themselves more prone to mutation than the gateway genes. We now wish to establish whether this type of model can account for tumour incidence data in p53 deficient mice.

To apply these concepts to tumorigenesis in p53 deficient mice, we propose that p53 is a rate-modifier gene whose inactivation requires 2-stages in wild-type mice and 1-stage inactivation in p53 null heterozygous ($p53^{+/-}$) mice. Of course the gene is already inactivated in p53 null homozygous ($p53^{-/-}$) mice. In each genotype, the same number of gateway gene mutations is required. Gateway genes have a baseline mutation rate μ when p53 function is maintained, which is increased to mutation rate $k\mu$ when p53 function is lost. The parameter k will be termed the rate modifier factor. The mutation rate for two stage loss of p53 function is assigned the independent value λ. Computer simulations have been carried out for a range of values of the number of gates in the model, for a range of values of the mutation rates of the modifier genes and (independently) the mutation rates of the gate-pass genes, and a range of values of the modifying factor (the scaling factor for gate-pass mutation rate).

In this chapter, we intend to provide a quantitative approach to the understanding of the importance of rate-modifier genes in the onset of malignant tumours. Firstly, we will consider some quite general questions. It is intended to explore how the rate-modifier factor and the number of stages for the rate modifier process, the mutation rate of the rate-modified genes and the gate-pass genes, as well as the number of gates required for malignant transformation, affect tumorigenesis. How many tumours will arise by the gate-pass mutations only compared with the gate-pass plus modifier mutations? How does the proportion of tumours arising by the gate-pass plus modifier mutations change with age? After consideration of these generic questions, the model will be applied to p53 mediated tumorigenesis.

8.2 Three-gate/two-stage model of tumorigenesis

The detailed structure, expanded from Figure 8.1, of three-gate/two-stage model of tumorigenesis is represented in Figure 8.2. This expansion is necessary for mathematical development (see appendix 2). The essential features of this model are:

(1) Cells in each state (excluding the malignant state) are presumed to follow the Gomp-ex stochastic growth process described in Section 3.3 of Chapter 3. The malignant cells are assumed to follow linear birth-death stochastic growth.

(2) At each cell division, each daughter cell has a probability of experiencing any one mutation (either the gate-pass mutation or the modifier mutation). We do not consider the unlikely possibility that both mutations occur simultaneously in the same cell.

(3) The birth-death processes and the mutation processes are independent of one another and each cell goes through the above processes independently of other cells.

(4) We assume that the cell cycle time of the stem cells and mutants remains same and is not influenced by the mutational events.

Technically, these assumptions imply that the mathematical model portrays the process of tumorigenesis as a Markov process. A Markov process describes the fate of any cell at time t as depending only on the present state of the cell at time t and not on the past history of that cell.

The other multigate/multistage models have the same structure as the three-gate/two-stage model. These models differ only in the number of gates for malignant transformation and/or number of stages for rate-modifier process.

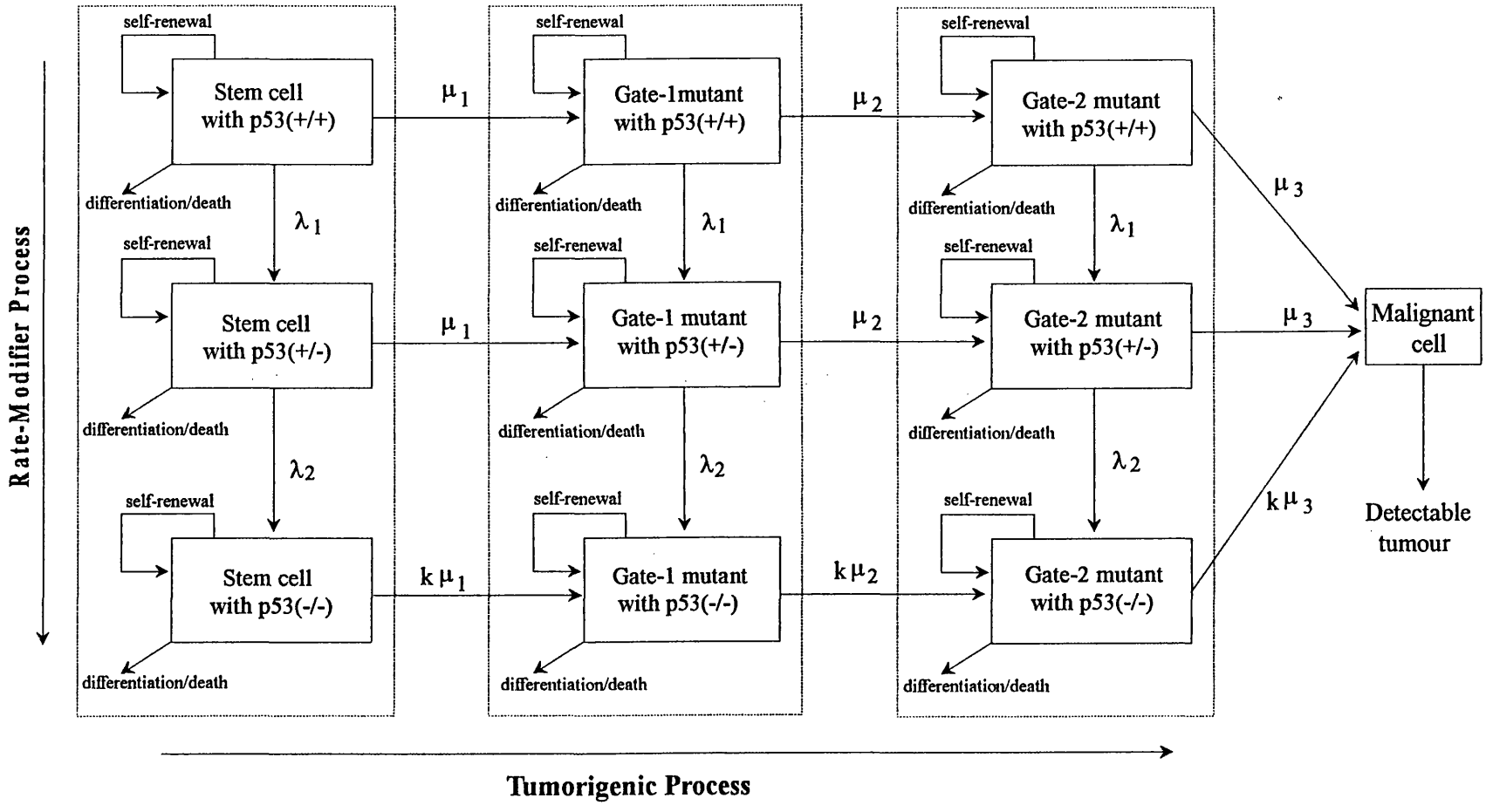


Figure 8.2 The detailed structure of 3-gate/2-stage model for tumorigenesis.

8.3 Simulation studies and results

The behaviour of a set of cells is followed using discrete event simulations (Maisel and Gnugnoli, 1972) in which the evolution of the population of cells is updated at each cell division. The simulation process has similar structure to that described in Section 3.4 of Chapter 3 (see appendix 3). It differs only in having the new possibility of rate-modifier mutations.

In our simulation studies, we have considered k-gate/2-stage and k-gate/1-stage (k=2, 3, or 4) models. The baseline parameter values have been chosen to be representative of cell kinetics in the developing mouse. The values used for the simulations refer to the section 3.4 of Chapter 3. At time zero, corresponding to fertilization, only a single normal stem cell (fertilized ovum) is present. 10^4 simulations were carried for each choice of the parameters in the models. Each simulation is terminated when the age of mouse reaches 600 days. The number of detectable tumours and the times of the first ten tumours are recorded. A tumour is considered to be detectable when its size reaches 10^6 cells.

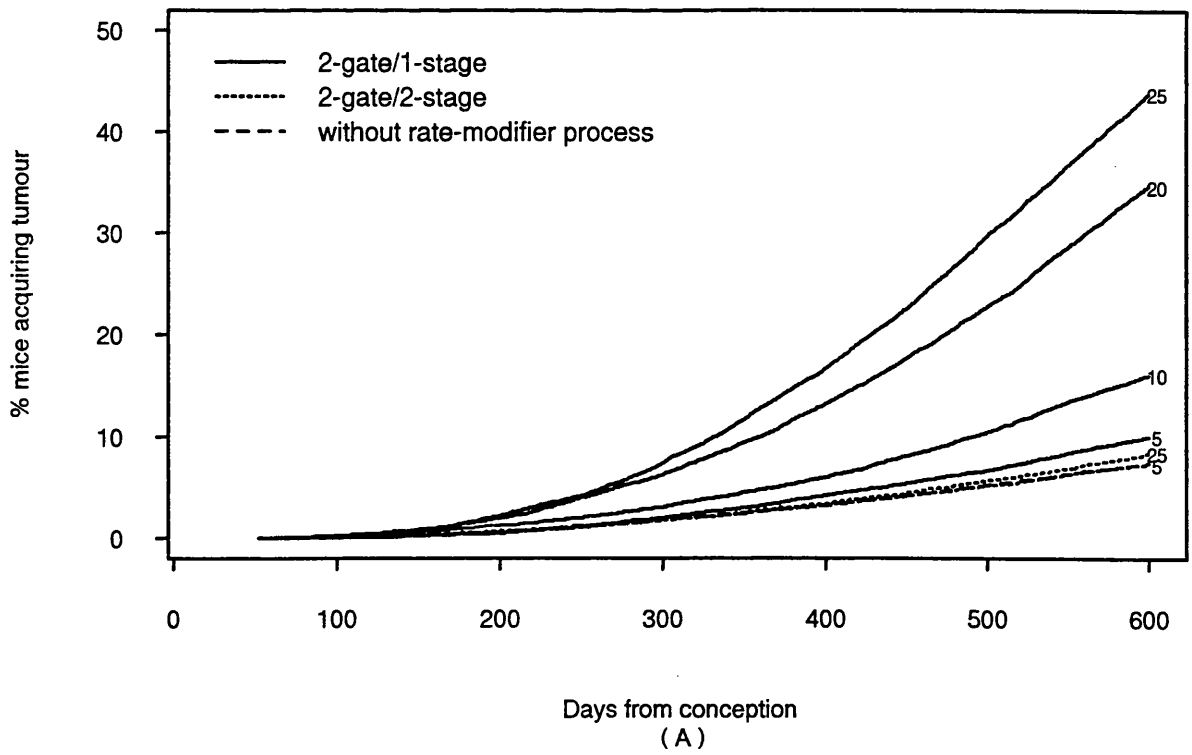
The ***relative risk***, is defined as the ratio of cumulative tumour incidence rate (by 600 days) with and without the rate-modifier process. The proportion of tumours which arise by gate-pass mutations only are used to evaluate the importance of the rate-modifier mutations in the onset of malignant tumours.

8.3.1 Kinetics of appearance of tumours

Figure 8.3 shows the predicted kinetics of appearance of tumours in mice using this new

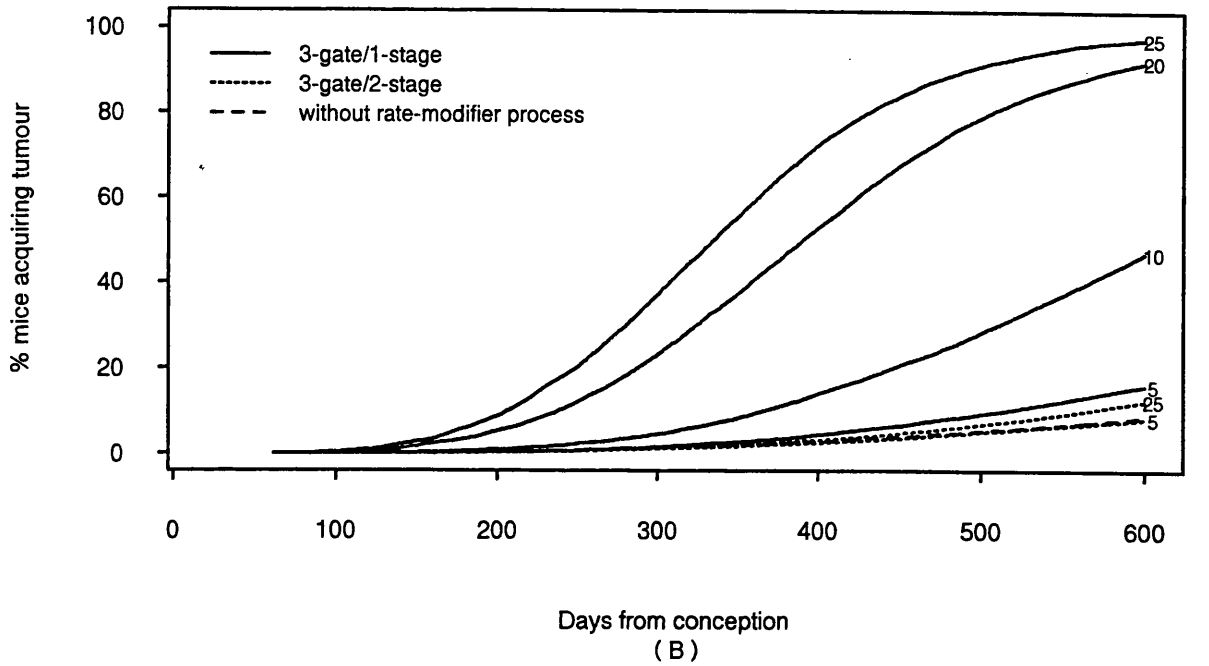
model. It is found that no matter how many gates are required for malignant transformation, the existence of the rate-modifier process means that tumour incidence is always increased relative to the same process in the absence of the rate-modification. However the magnitude of any such difference strongly depends on the rate-modifier factor, the number of stages and gates, and the mutation rate of gate-pass and modifier events. For example, with a two-stage rate-modifier process and lower gate-pass mutation rate, the difference is negligible when the rate-modifier factor is 5 or less (Figure 8.3).

Considering tumours which arise by gate-pass mutations only compared with the gate-pass plus modifier mutations, it is very interestingly found that tumours arising by gate-pass mutations only occur slightly earlier than those by the gate-pass plus modifier mutations although this difference is influenced by the number of gates and stages, the rate-modifier factor, and the mutation rate for gate-pass and modifier events (Figure 8.4).



(A)

(Figure 8.3 to be continued)



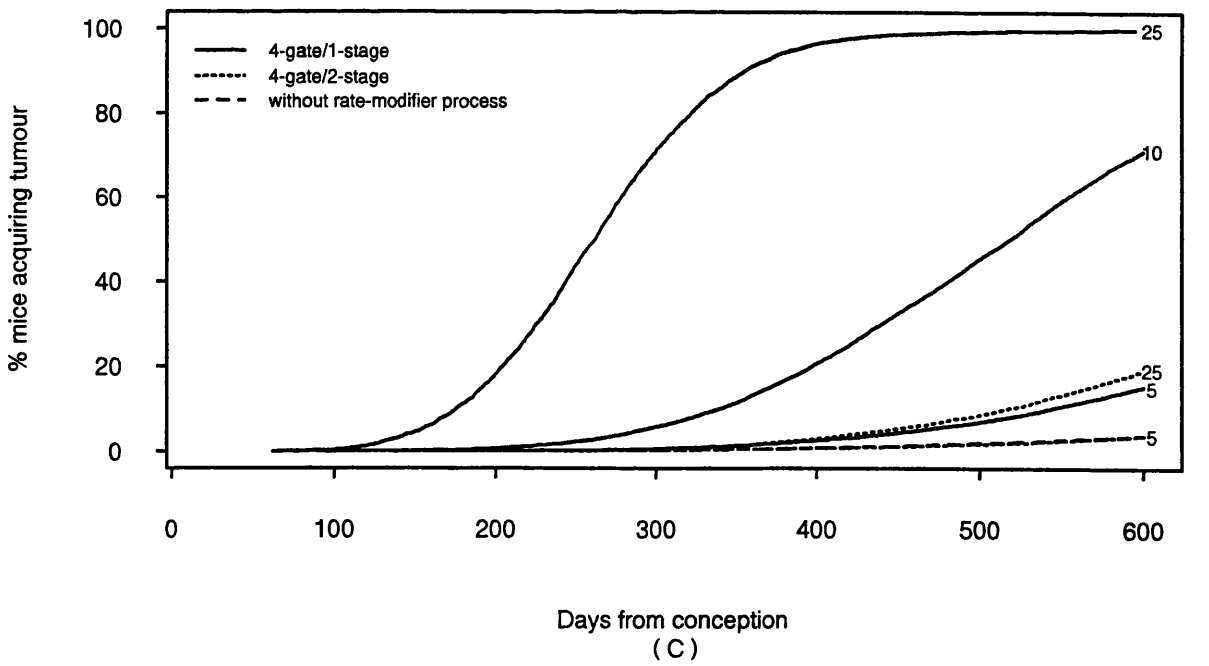
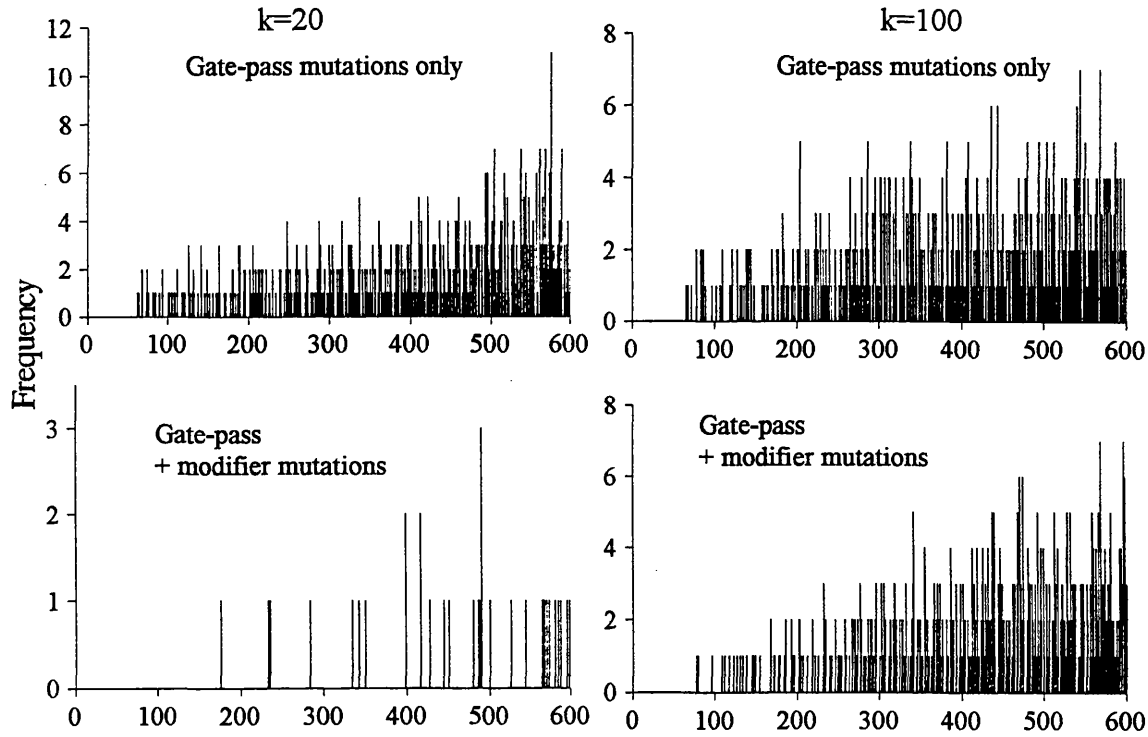
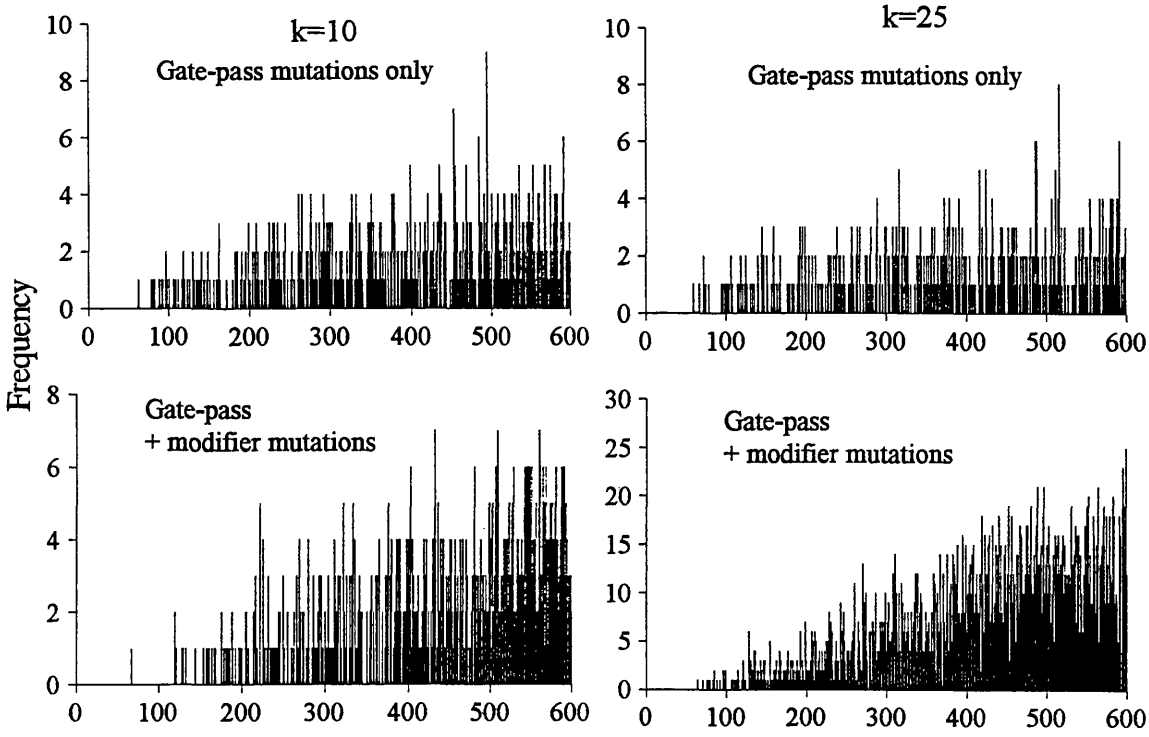


Figure 8.3 The kinetics of appearance of tumours in mice under the multigate model with rate-modifier process. The graphs are labelled by the value of rate-modifier factor k. (A) 2-gate/1 or 2-stage model with gate-pass mutation rate $3x10^{-7}$ and modifier mutation rate 10^{-4}; (B) 3-gate/1 or 2-stage model with gate-pass mutation rate 10^{-5} and modifier mutation rate 10^{-4}; (C) 4-gate/1 or 2-stage model with gate-pass mutation rate $5x10^{-5}$ and modifier mutation rate 10^{-4}.

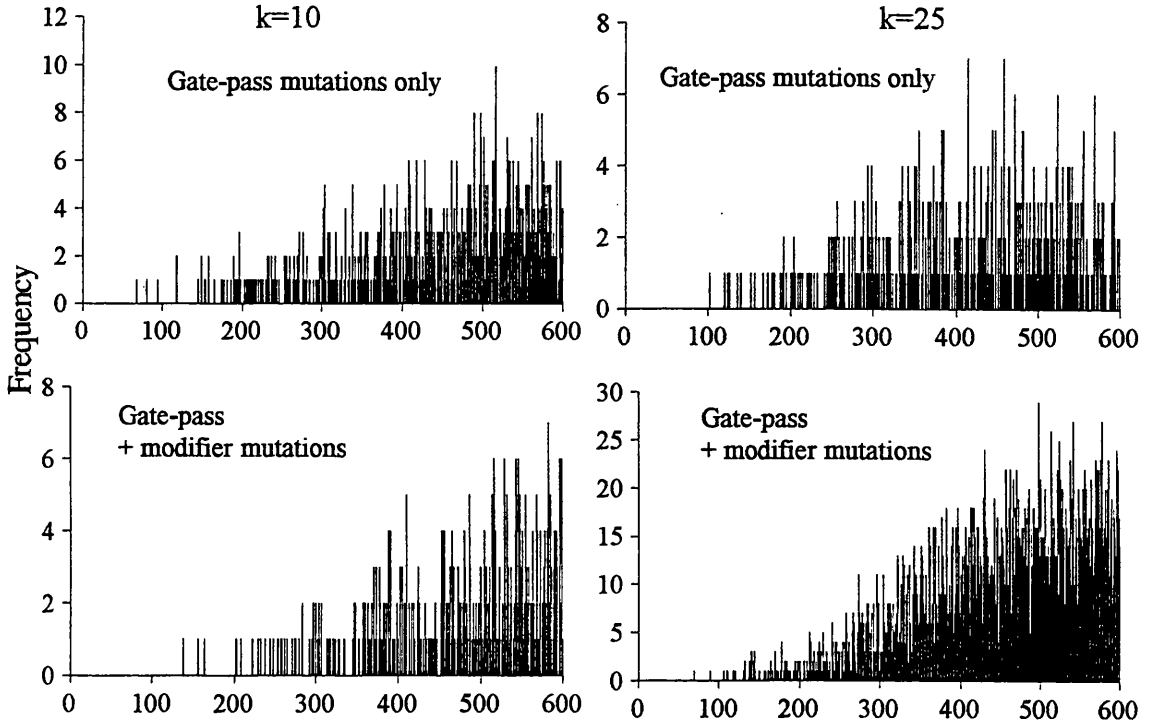


(A) 2-gate/1-stage model with similar mutation rate of gate-pass and modifier events

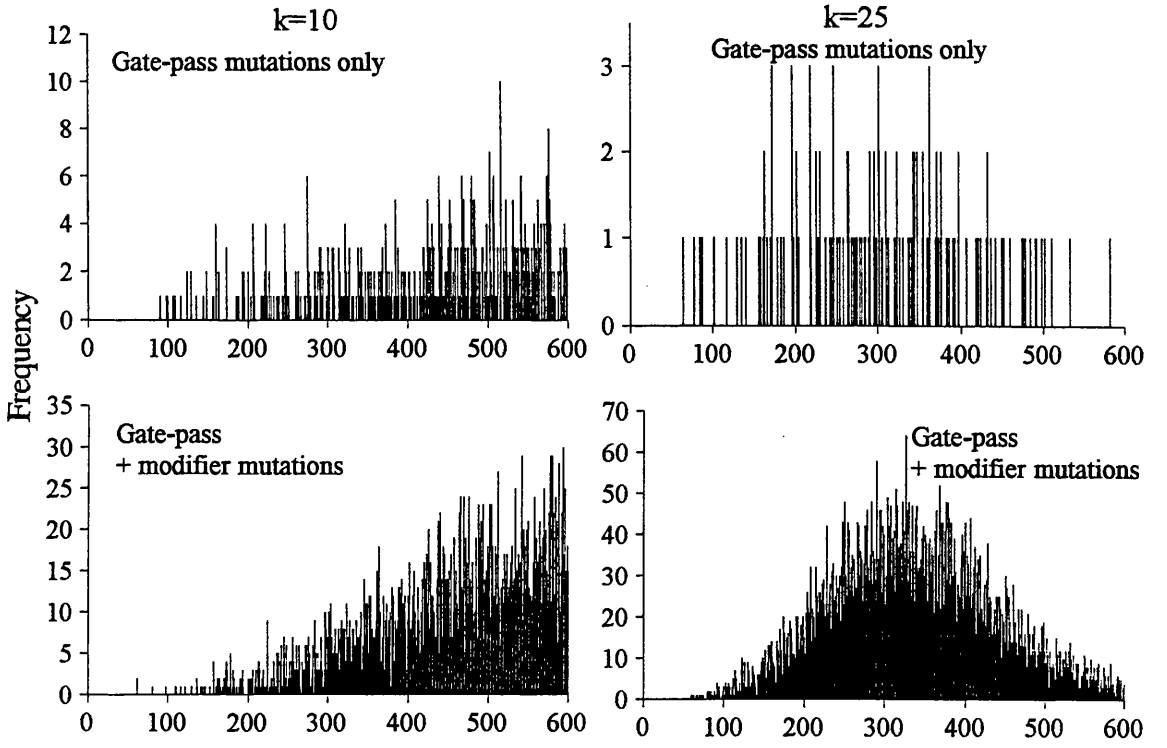


(B) 2-gate/1-stage model with higher mutation rate of modifier events than gate-pass events

(Figure 8.4 to be continued)

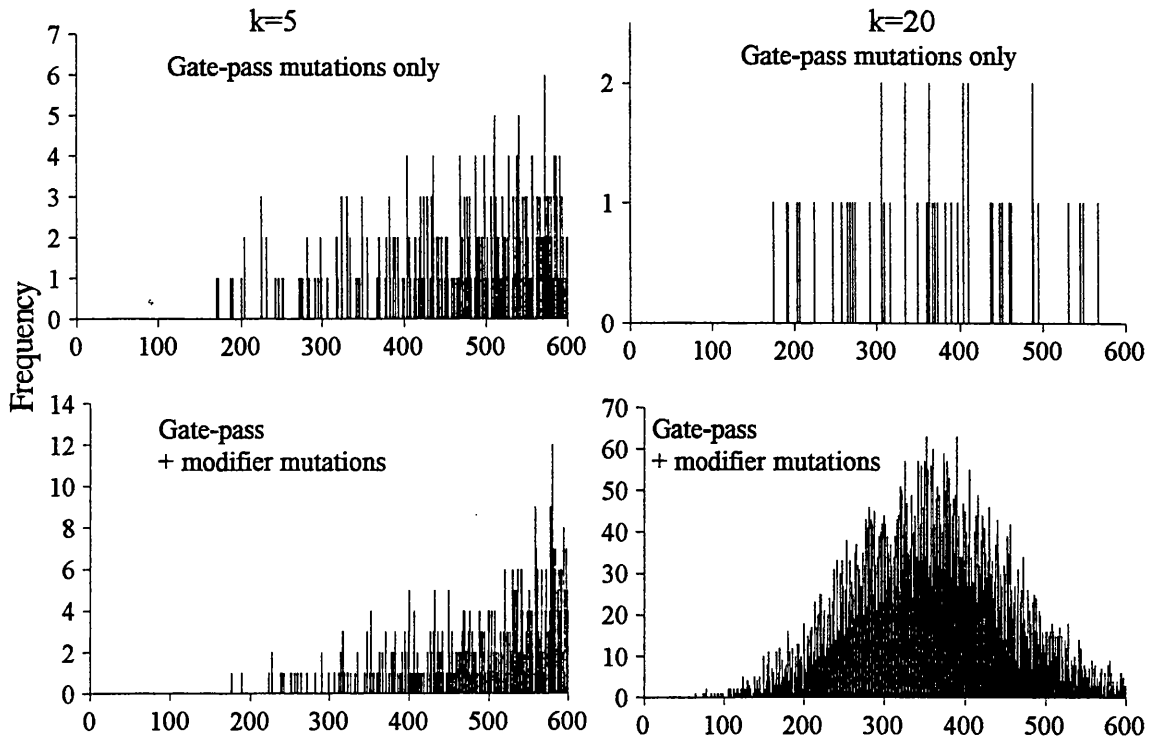


(C) 3-gate/1-stage model with same mutation rate of gate-pass and modifier events

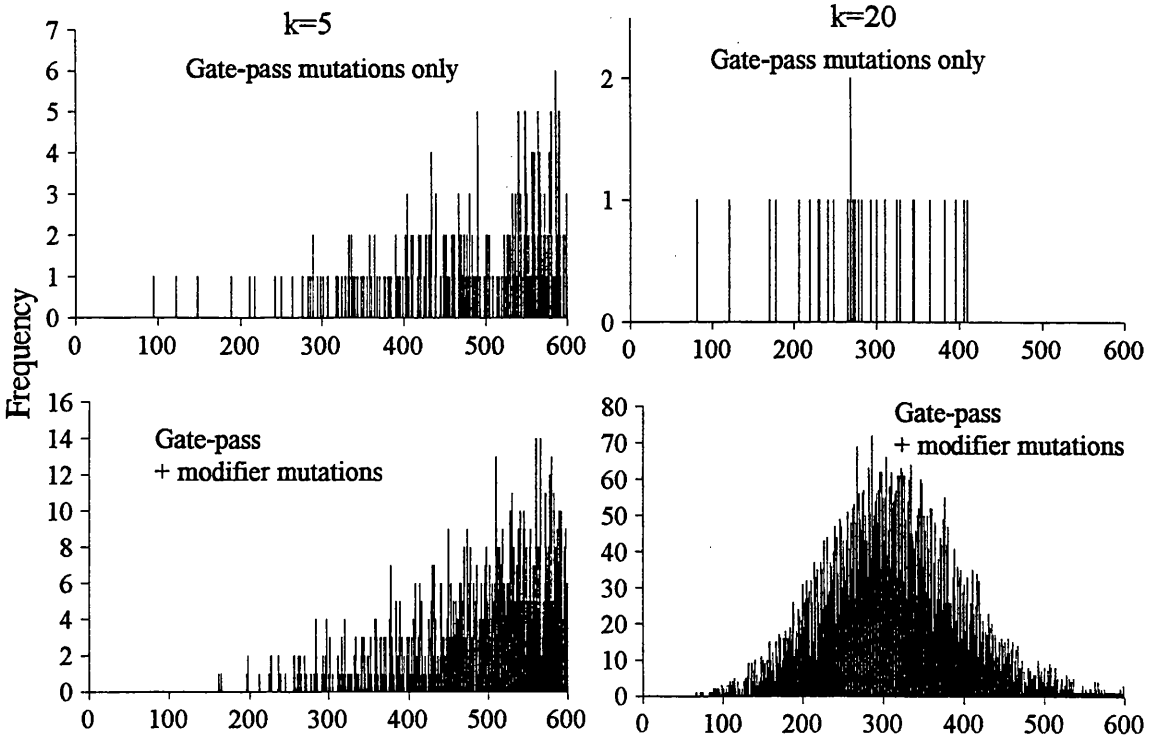


(D) 3-gate/1-stage model with higher mutation rate of modifier events than gate-pass events

(Figure 8.4 to be continued)

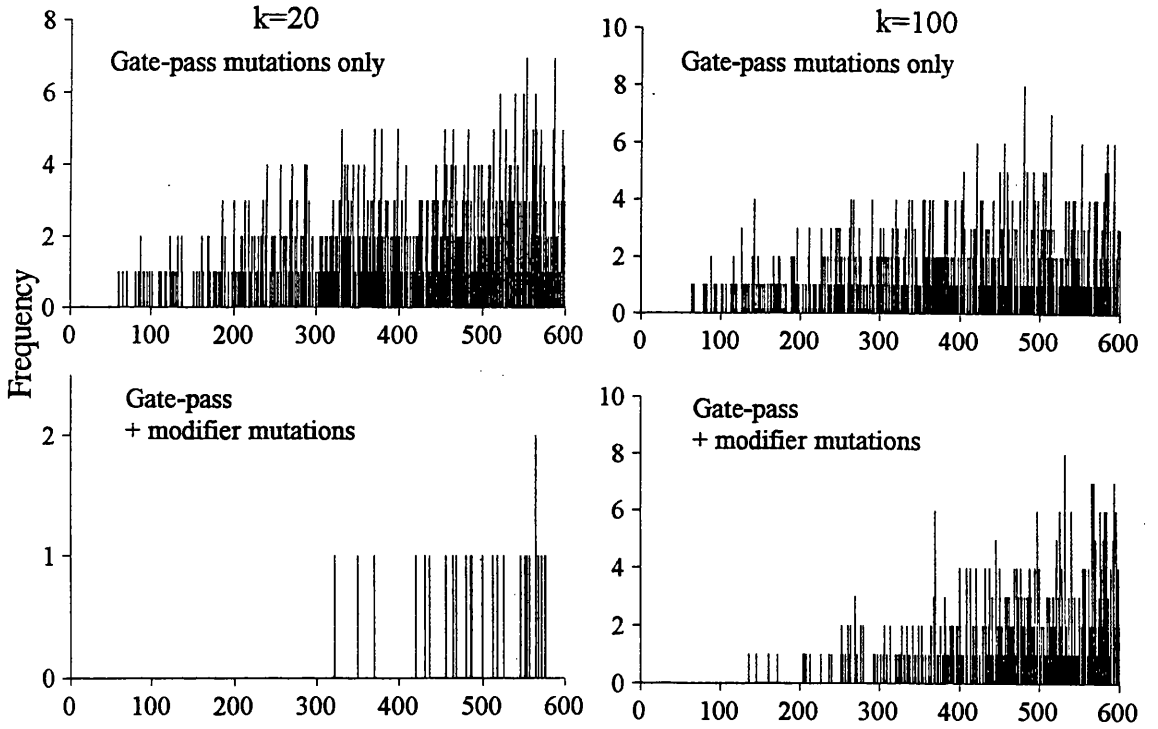


(E) 4-gate/1-stage model with same mutation rate of gate-pass and modifier events

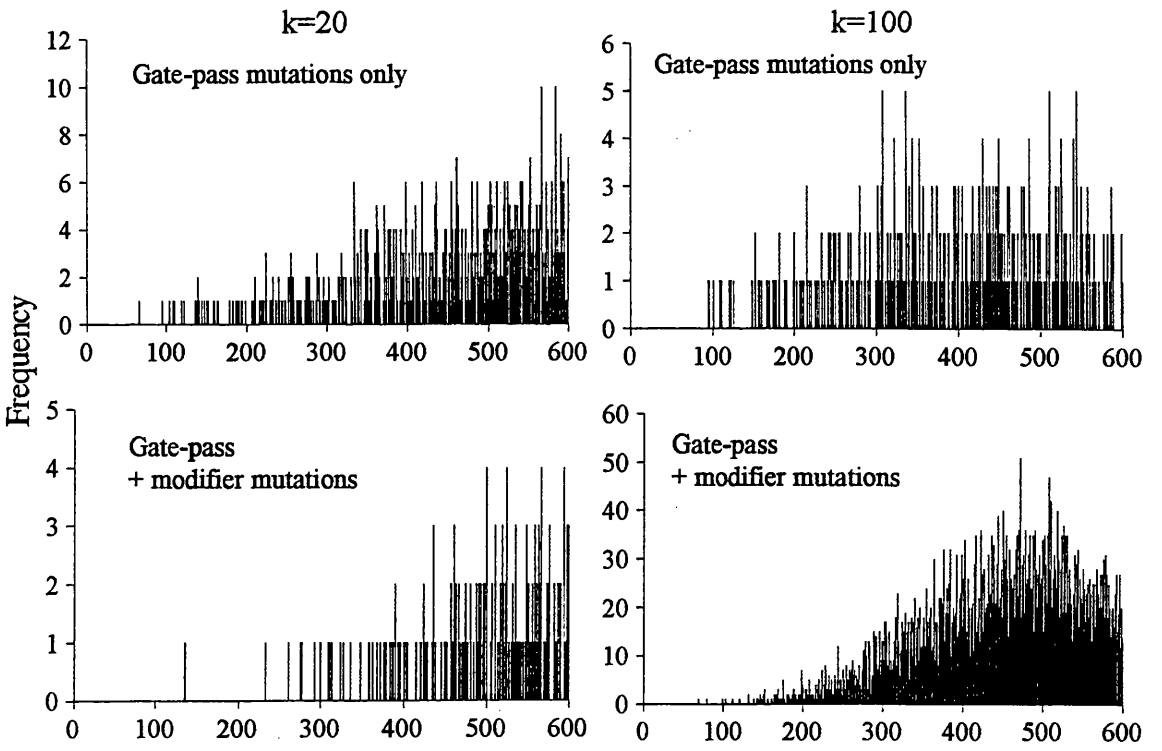


(F) 4-gate/1-stage model with higher mutation rate of modifier events than gate-pass events

(Figure 8.4 to be continued)

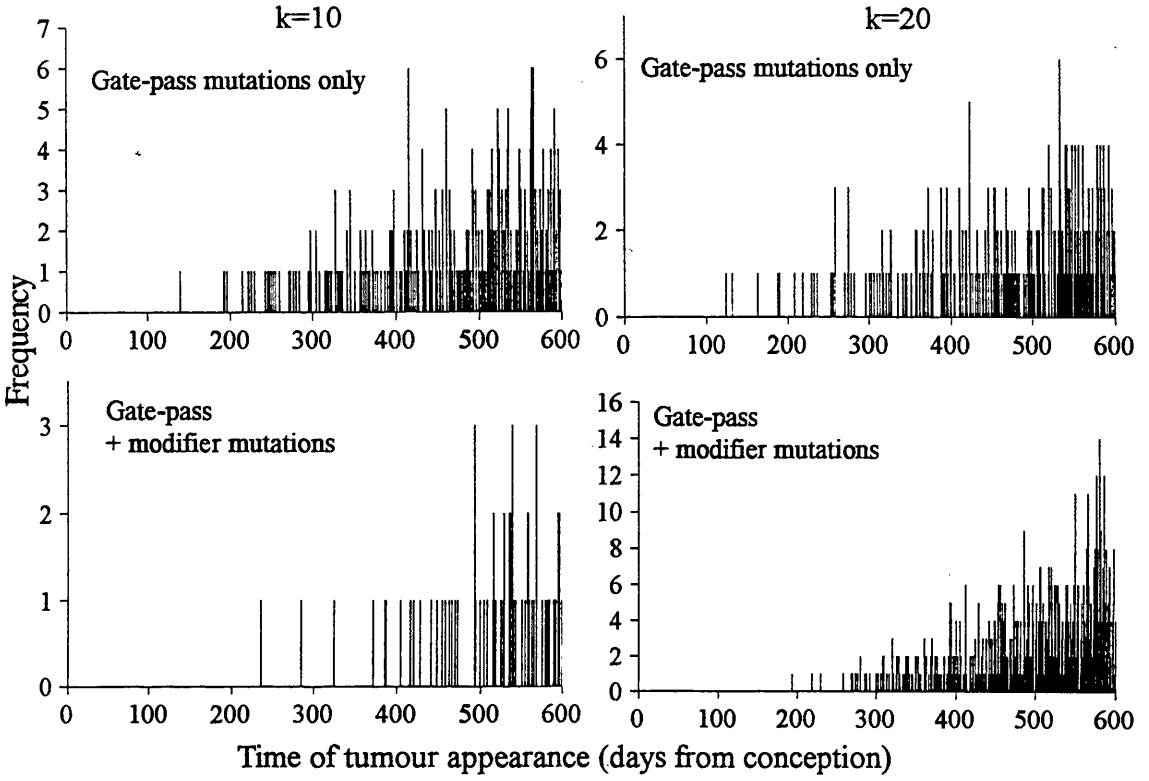


(G) 2-gate/2-stage model with higher mutation rate of modifier events than gate-pass events



(H) 3-gate/2-stage model with higher mutation rate of modifier events than gate-pass events

(Figure 8.4 to be continued)



(I) 4-gate/2-stage model with higher mutation rate of modifier events than gate-pass events

Figure 8.4 Comparison of latent period distributions of tumours arising by gate-pass mutations only with tumours arising by gate-pass plus modifier mutations. In each block, the upper lane shows the time of appearance of tumours arising by gate-pass mutations only, and the lower lane shows the time of appearance of tumours arising by gate-pass and modifier mutations. The value of the modifier factor k is labelled in each block. (A) 2-gate/1-stage model with gate-pass mutation rate $3x10^{-7}$ and modifier mutation rate 10^{-6}; (B) 2-gate/1-stage model with gate-pass mutation rate $3x10^{-7}$ and modifier mutation rate 10^{-4}; (C) 3-gate/1-stage model with gate-pass mutation rate 10^{-5} and modifier mutation rate 10^{-5}; (D) 3-gate/1-stage model with gate-pass mutation rate 10^{-5} and modifier mutation rate 10^{-4}; (E) 4-gate/1-stage model with gate-pass mutation rate $5x10^{-5}$ and modifier mutation rate $5x10^{-5}$; (F) 4-gate/1-stage model with gate-pass mutation rate $5x10^{-5}$ and modifier mutation rate 10^{-4}; (G) 2-gate/2-stage model with gate-pass mutation rate $3x10^{-7}$ and modifier mutation rate 10^{-4}; (H) 3-gate/2-stage model with gate-pass mutation rate 10^{-5} and modifier mutation rate 10^{-4}; (I) 4-gate/2-stage model with gate-pass mutation rate $5x10^{-5}$ and modifier mutation rate 10^{-4}.

8.3.2 Relative risk

For all models, the relative risk (ratio of accumulated tumour incidences in presence and absence of the rate-modifier process) increases with the rate-modifier factor (Figure 8.5). However, the steepness of this relationship is strongly dependent on the mutation rate of the gate-pass and modifier events, and the number of stages and gates (Figure 8.5). With fewer stages, and/or higher rate of modifier mutations, the relative risk changes rapidly with the rate-modifier factor (Figure 8.5). Surprisingly, for 2-gate/2-stage and 3-gate/2-stage models, when the modifier mutation rate is lower, even if the rate-modifier factor reaches 100, the relative risk are only 1.11 and 1.09, respectively.

8.3.3 Proportion of tumours arising by gate-pass mutations only

The proportion of tumours which arise by gate-pass mutations only reduces with increasing rate-modifier factor (Figure 8.6). However the details of this relationship are strongly dependent on the number of stages and gates, as well as the rate of the gate-pass and modifier mutations. Surprisingly, for the two-gate/two-stage model, even if the rate-modifier factor is high as 100, nearly all tumours still arise by gate-pass mutations only when the rate of modifier mutations is close to the rate of gate-pass mutations (see left part of Figure 8.6 (D)).

Very interestingly, the proportion of tumours arising by gate-pass mutations plus rate-modified gene mutation increases with age, implying that tumours with modifier mutations will be relatively over-represented amongst late-occurring tumours. However, this relationship is influenced by the rate-modifier factor, the number of stages and gates, and the rate of the gate-pass and modifier mutations (Figure 8.7).

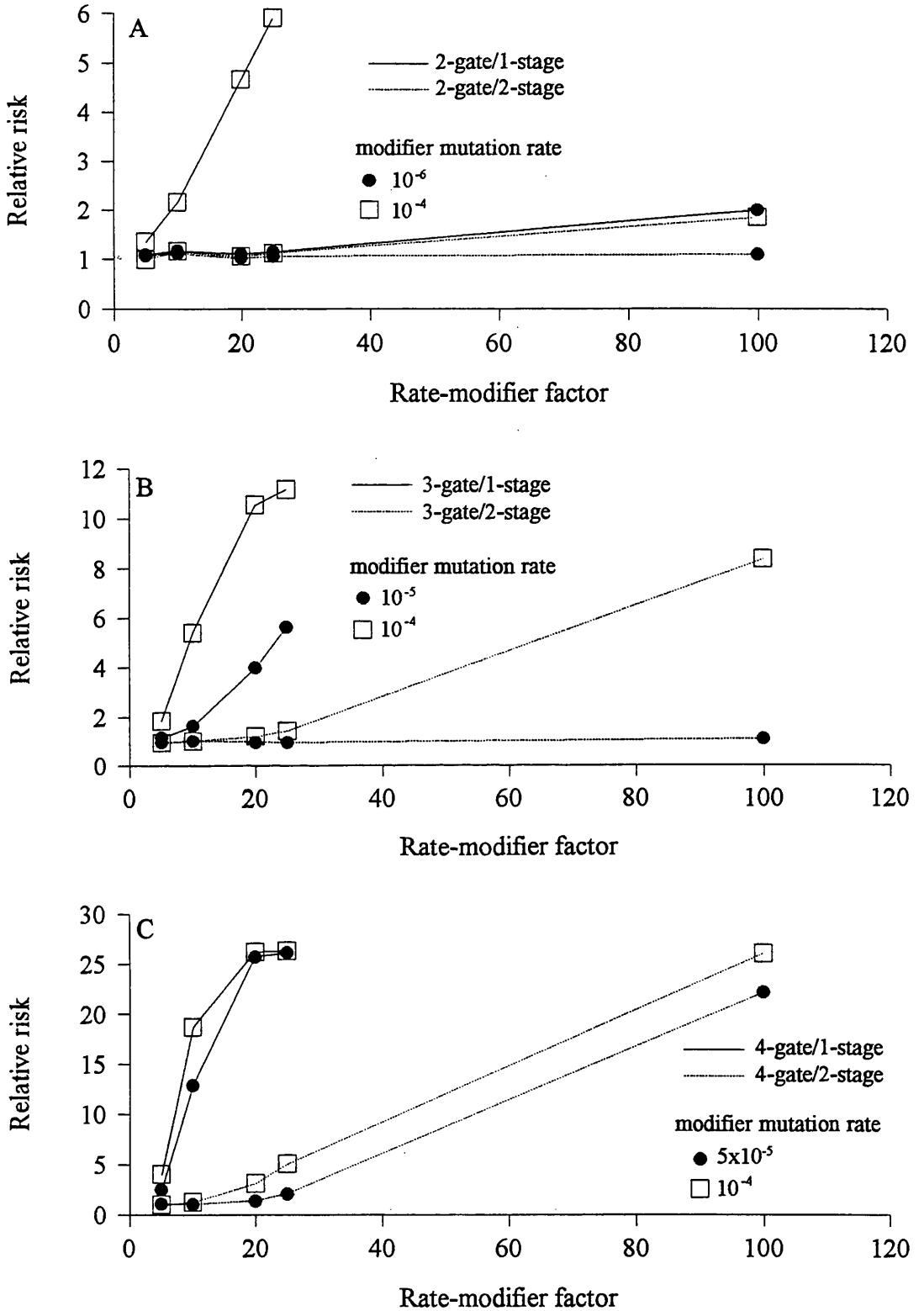
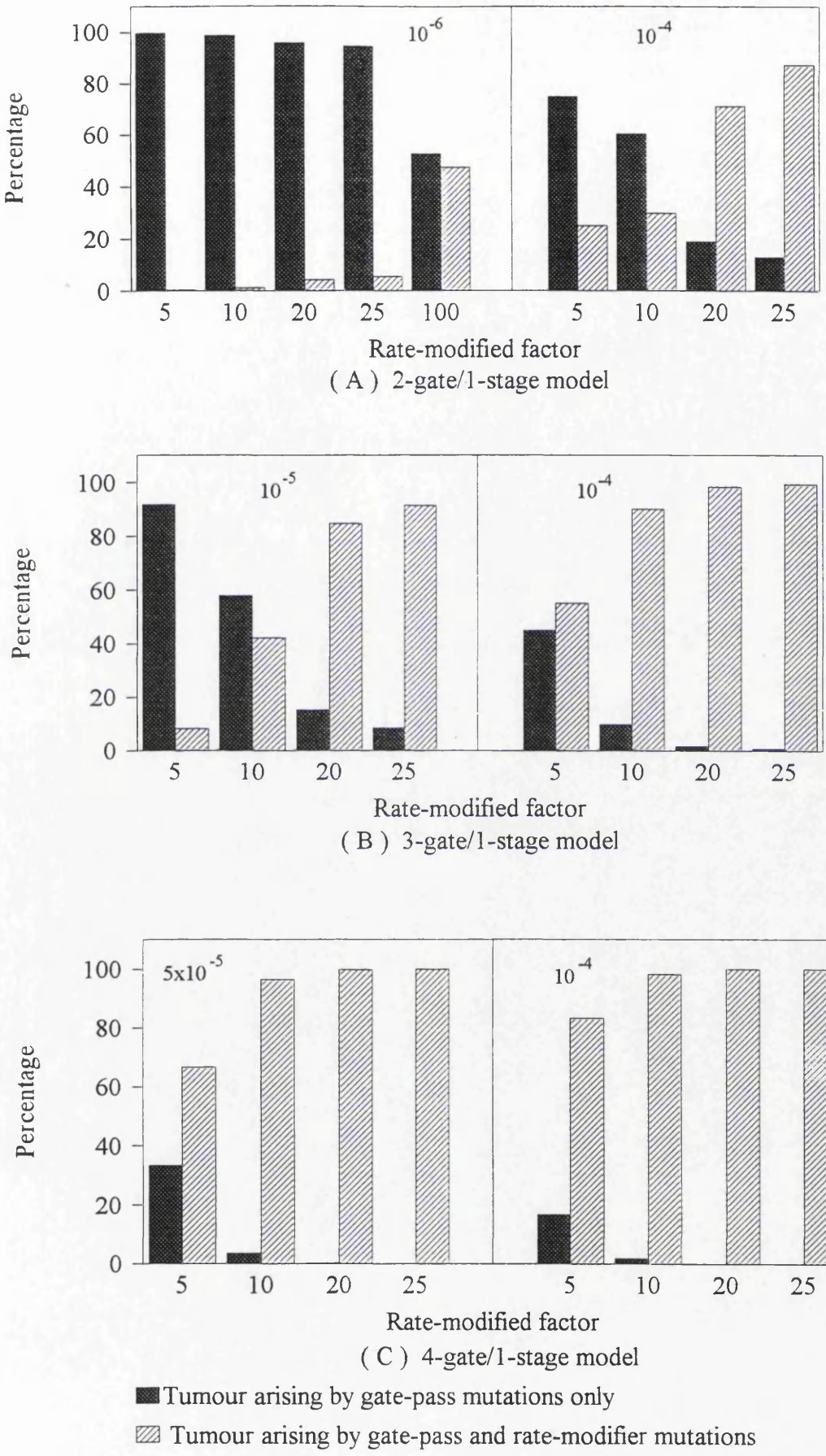


Figure 8.5 The relative risk of tumours under different models. (A) 2-gate/1 or 2-stage model with gate-pass mutation rate $3x10^{-7}$ and modifier mutation rate 10^{-6}or 10^{-4}; (B) 3-gate/1 or 2-stage model with gate-pass mutation rate 10^{-5} and modifier mutation rate 10^{-5} or 10^{-4}; (C) 4-gate/1 or 2-stage model with gate-pass mutation rate $5x10^{-5}$ and modifier mutation rate $5x10^{-5}$or 10^{-4}.



(Figure 8.6 to be continued)

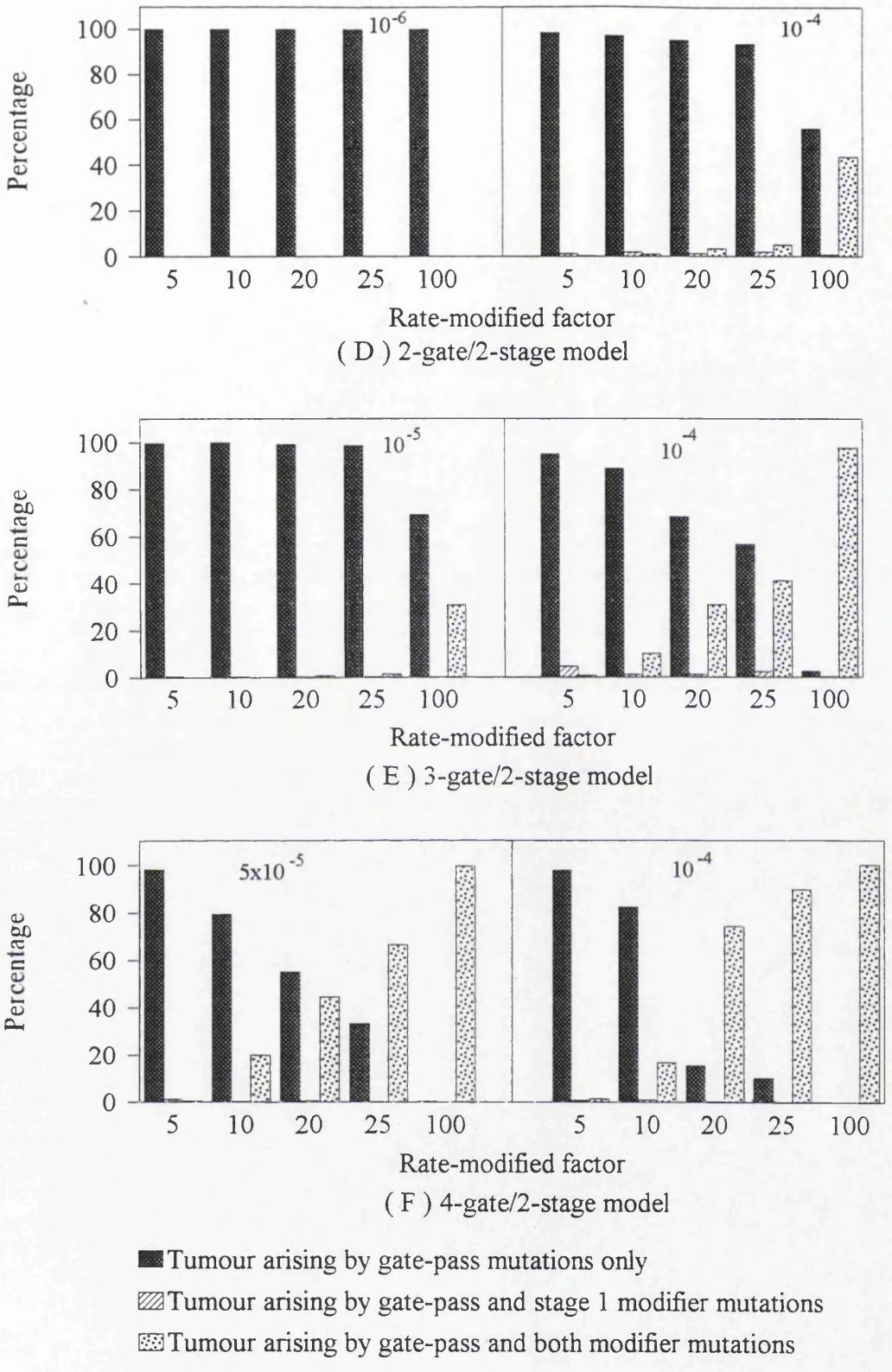


Figure 8.6 Predicted proportion of tumours arising from different routes by the multigate model with modifier process. The figure is labelled by the value of the modifier mutation rate. (A) 2-gate/1-stage model with gate-pass mutation rate $3x10^{-7}$; (B) 3-gate/1-stage model with gate-pass mutation rate 10^{-5} ; (C) 4-gate/1-stage model with gate-pass mutation rate $5x10^{-5}$; (D) 2-gate/2-stage model with gate-pass mutation rate $3x10^{-7}$; (E) 3-gate/2-stage model with gate-pass mutation rate 10^{-5} ; (F) 4-gate/2-stage model with gate-pass mutation rate $5x10^{-5}$.

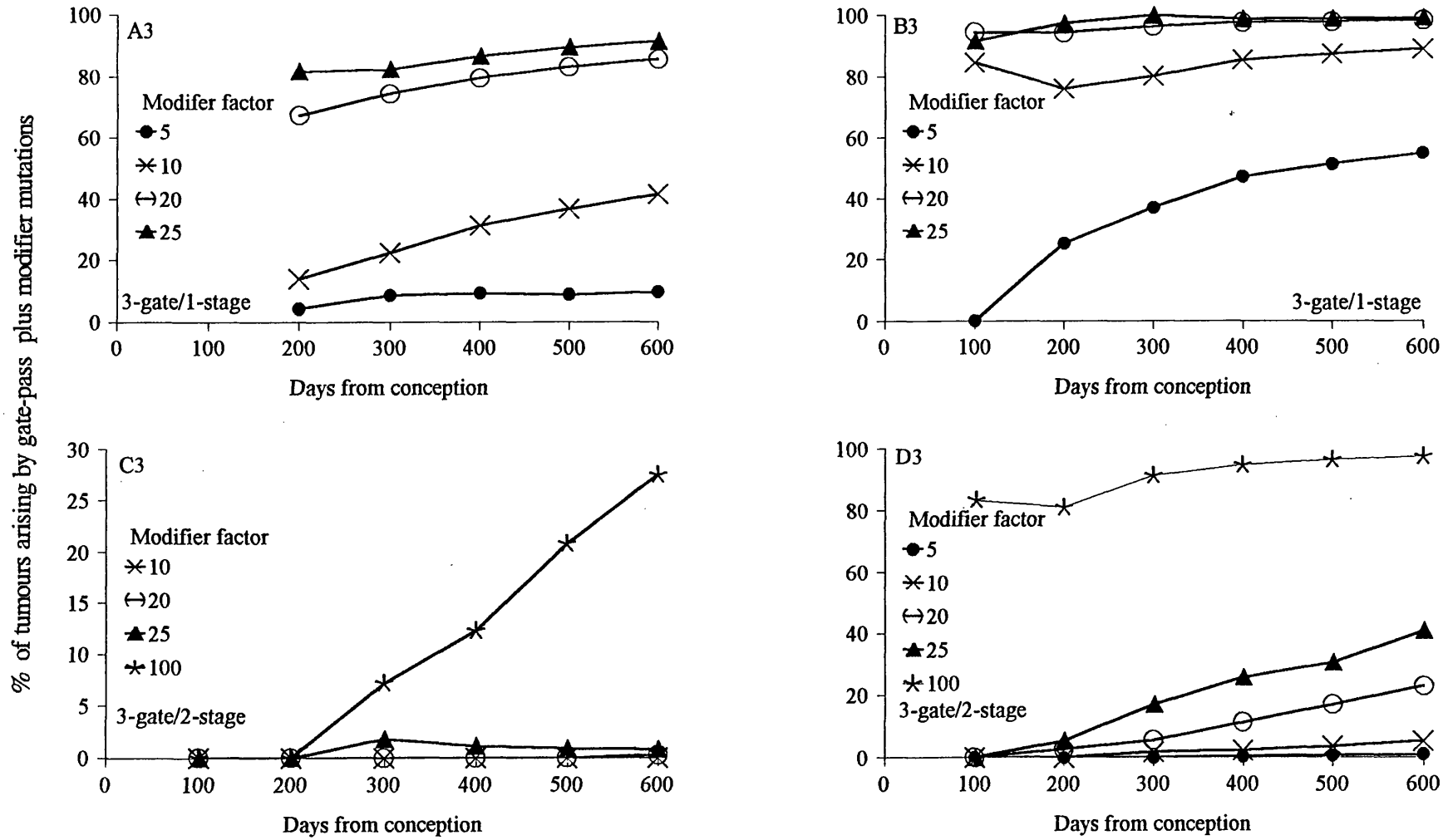


Figure 8.7 The changing proportion of tumours arising by gate-pass and modifier mutations as a function of age. The 3-gate model is represented here, other models are similar. (A3) $\mu_1 = \mu_2 = \mu_3 = 10^{-5}$, $\lambda_2 = 10^{-5}$ (B3) $\mu_1 = \mu_2 = \mu_3 = 10^{-5}$, $\lambda_2 = 10^{-4}$ (C3) $\mu_1 = \mu_2 = \mu_3 = 10^{-5}$, $\lambda_1 = \lambda_2 = 10^{-5}$ (D3) $\mu_1 = \mu_2 = \mu_3 = 10^{-5}$, $\lambda_1 = \lambda_2 = 10^{-4}$

8.3.4 Tumour multiplicity

The mean number of tumours per mouse increases with the rate-modifier factor (Figure 8.8). However, this relationship is influenced by the number of gates and stages and the mutation rate of the rate-modifier genes and the gate-pass genes (Figure 8.8).

8.4 Application to tumorigenesis in p53 deficient mice

To apply these new models to tumorigenesis in p53 deficient mice, we propose that p53 is a rate-modifier gene whose inactivation requires 2-stages in wild-type mice and 1-stage inactivation in $p53^{+/-}$ mice; of course the gene is already inactivated in $p53^{-/-}$ mice. In each genotype, the same number of gateway gene mutations is required. Gateway genes have a baseline mutation rate μ when p53 function is maintained, which is increased to mutation rate $k\mu$ when p53 function is lost. We have considered 2-gate, 3-gate and 4-gate models and have in each case chosen the mutation rate λ and μ, and rate-modifier factor k to match tumour incidence observed experimentally (Donehower et al, 1995) in wild type mice. However, with smaller value of the rate-modifier factor ($k < 25$), it was found that the tumour incidence in wild type mice does not change very much, so we have to assign the value of rate-modifier factor k by choosing k to match tumour incidence observed experimentally in $p53^{+/-}$ mice without changing the mutation rate. The corresponding tumour incidence for $p53^{-/-}$ mice then follows without changing the rate-modifier factor and mutation rate. For presentation, we have computed the number of tumours predicted to have appeared by 16 and 80 weeks, close to the observed median latency in $p53^{-/-}$ and $p53^{+/-}$ mice, in each of these situations.

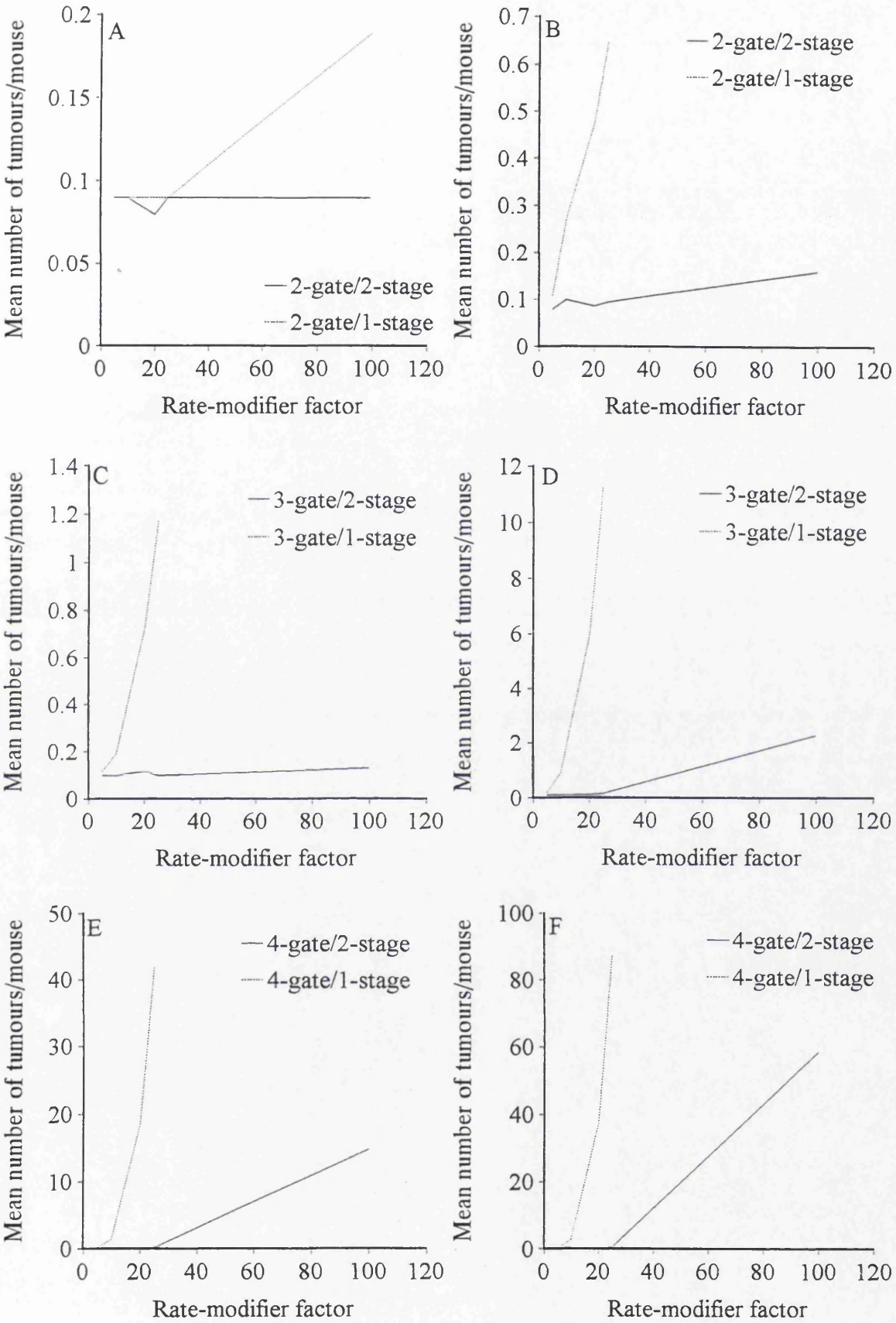


Figure 8.8 Relationship between tumour multiplicity and rate-modifier factor. (A) $\mu=3\times10^{-7}$ and $\lambda=10^{-6}$; (B) $\mu=3\times10^{-7}$ and $\lambda=10^{-4}$; (C) $\mu=10^{-5}$ and $\lambda=10^{-5}$; (D) $\mu=10^{-5}$ and $\lambda=10^{-4}$; (E) $\mu=5\times10^{-5}$ and $\lambda=5\times10^{-5}$; and (F) $\mu=5\times10^{-5}$ and $\lambda=10^{-4}$.

We have observed that better agreement with the experimental data is found when the mutation rate of the second p53 allele (second stage of modifier process) is higher than that of the gate-pass genes (Table 8.1). In support of this hypothesis, some evidence suggests that following loss of the first p53 allele, loss of the second allele occurs more easily (Harvey et al., 1993). Further experiments will be needed to confirm this possibility.

Interestingly, when we choose the appropriate value of parameters in the model, both two-gate and three-gate models can be compatible with the experimental data. However, it seems very difficult to find appropriate values of parameters in the 4-gate model to reconcile with the experimental data. Table 8.1 and figure 8.9 show predicted tumour incidence data for the three mouse genotypes by two-gate and three-gate model.

Table 8.1 Predicted tumour development in wild type and p53 deficient mice on 2-gate and 3-gate models with 2 stage modifier (p53 inactivation) pathway.

Genotype	2-gate model with 2-stage modifier ($\mu_1=\mu_2=3.7\times10^{-7}$; $\lambda_1=3.7\times10^{-7}$; $\lambda_2=5\times10^{-4}$; k=10)			3-gate model with 2-stage modifier ($\mu_1=\mu_2=\mu_3=10^{-5}$; $\lambda_1=10^{-5}$; $\lambda_2=1.5\times10^{-4}$; k=10)		
	Tumours/mouse		Proportion of tumours developing by 80 weeks with inactivated p53 (%)	Tumours/mouse		Proportion of tumours developing by 80 weeks with inactivated p53 (%)
	16 weeks	80 weeks		16 weeks	80 weeks	
p53(+/+)	0.003	0.14	0.1	0.0008	0.10	0.6
p53(+/-)	0.009	0.88	84.0	0.0021	1.29	89.4
p53(-/-)	0.95	33.91	100	0.56	375	100

Notice that the mean number of tumours/mouse is now near 1 for $p53^{+/-}$ and $p53^{-/-}$ mice by 80 and 16 weeks respectively, although large tumour numbers would still be predicted for $p53^{-/-}$ mice by 80 weeks on 3-gate model; however, in practice no such mice will survive to this time.

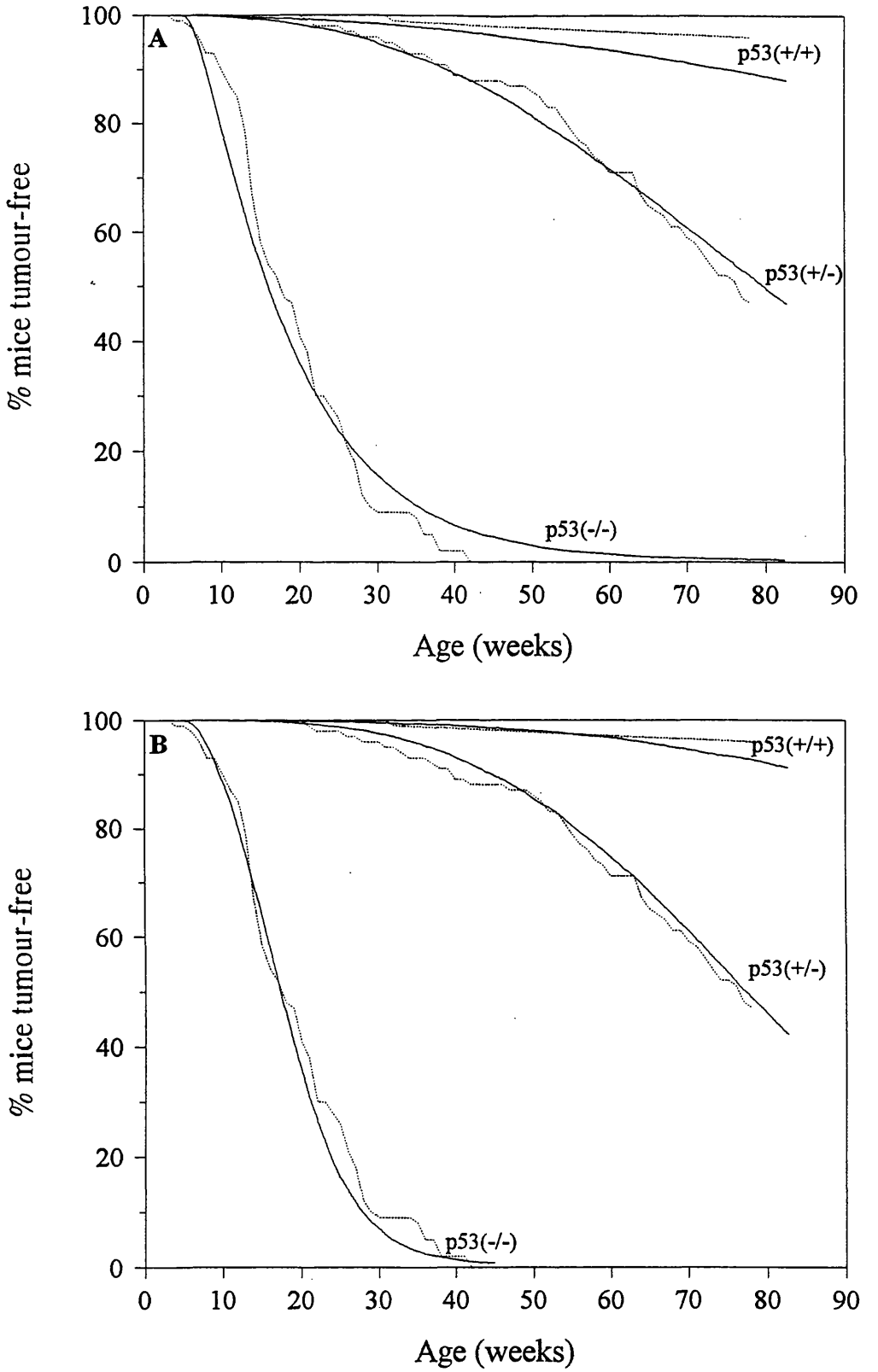


Figure 8.9 Predicted age-incidence pattern of tumour development for mice of three genotypes for the multi-gate model with 2 modifier stages. (A) 2-gate model with $\mu_1 = \mu_2 = 3.7\text{x}10^{-7}$, $\lambda_1 = 3.7\text{x}10^{-7}$, $\lambda_2 = 5\text{x}10^{-4}$, k = 10; (B) 3-gate model with $\mu_1 = \mu_2 = \mu_3 = 10^{-5}$, $\lambda_1 = 10^{-5}$, $\lambda_2 = 1.5\text{x}10^{-4}$, k=10. The broken lines in the diagrams show the incidence of experimental tumours (data from Donehower et al, 1995)

The model provides prediction of the proportions of tumours which occur in each genotype in association with p53 inactivation; these are shown for tumours accumulated by 80 weeks in table 8.1. We have also computed the proportion of p53 inactivation associated tumours as a function of mouse age in the wild-type and $p53^{+/-}$ genotypes and have observed that this proportion shows a tendency to increase with age (Figure 8.10), implying that p53 inactivated tumours will be relatively over-represented amongst late-occurring tumours.

8.5 Discussion

A new mathematical model has been developed for multistage tumorigenesis. Under this new model, tumorigenesis is viewed as passing several genetic gates with multistage rate-modifier of gate-pass events. The experimental evidence in support of this new model is found in the work of Holliday (1989) and Hartwell and Weinert (1991). Hartwell and Weinert pointed out that it is highly improbable that six successive spontaneous changes (as seems to be required on the multistage model for some tumours) could occur within a clone of cells in the mammalian organism to produce a tumour even after decades of selection when the spontaneous mutation rate is less than 10^{-5} mutation/gene/generation. One way out of this dilemma would be if the cell destined to produce the tumour acquired an increased mutation rate early in its history. As shown in chapter 3, the tumours only occur in very small numbers of mice even if only three changes are required when the spontaneous mutation rate is less than 10^{-5} mutation/gene/generation. Although the application of the multigate model theory has been principally to tumorigenesis in p53 deficient mice, it is possible that other rate-modifying pathways exist. For example,

Dietrich et al (1993) have recently reported that Mom-1 is a major modifier locus affecting Min-induced intestinal neoplasia in the mouse.

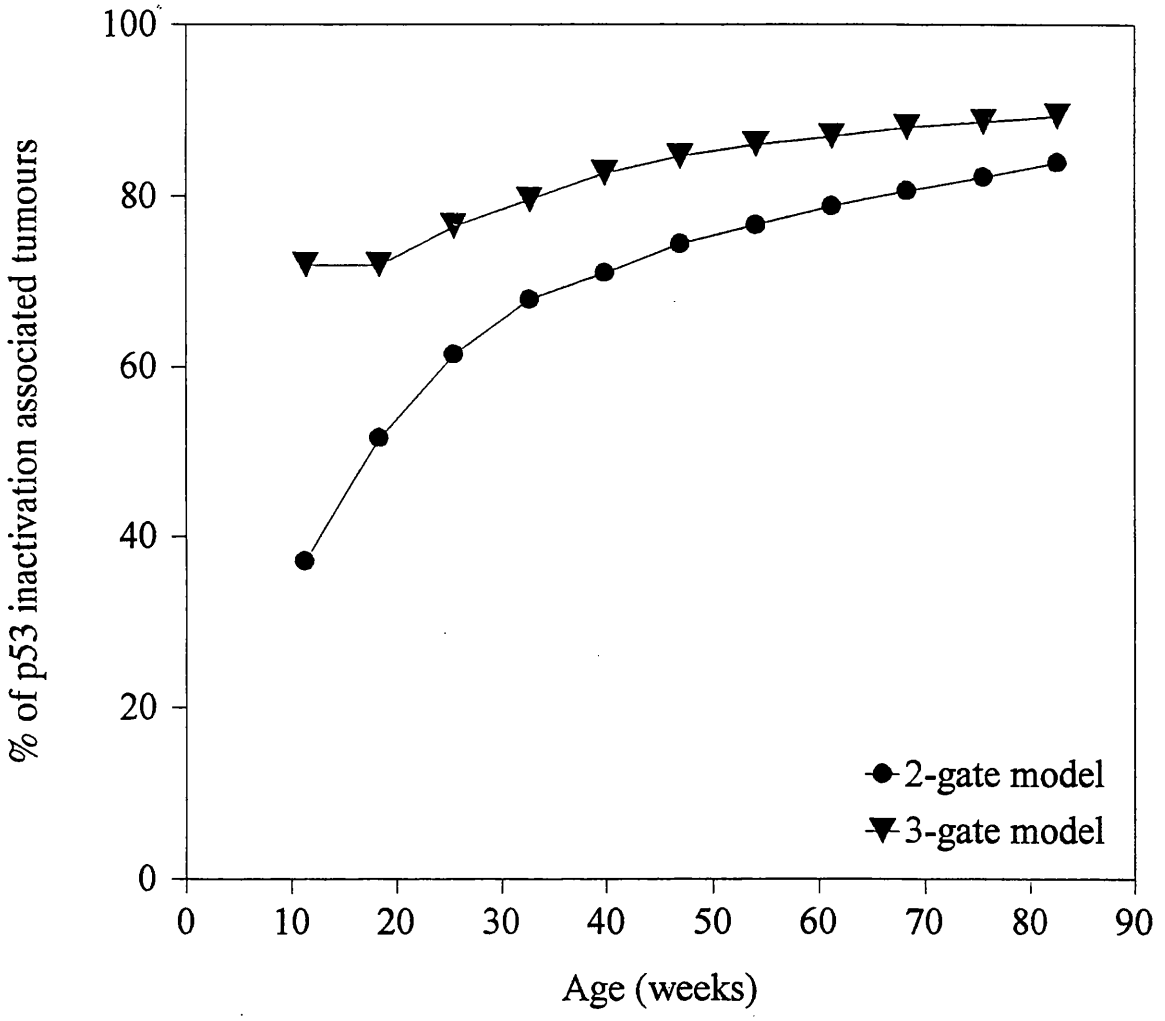


Figure 8.10 Relationship between the proportion of p53 inactivation associated tumours and mouse age in the $p53^{+/-}$ genotypes. It is difficult to describe this relationship in the p53 wild type due to very low proportion of p53 inactivation associated tumours by 80 weeks.

The importance of the rate-modifier process in tumorigenesis strongly depends on the number of stages and the mutation rate for the rate-modifier process, rate-modifier factor, and the number of gates required for malignant transformation. There are some possible

mechanisms which may increase the importance of the modifier pathway: (1) the modifier mutation rates may be much higher than the gate-pass mutation rates; (2) the number of stages for rate-modifier process may be much less than the number of gates; (3) the rate-modifier factor may be high, or any combination of these above. Otherwise, the rate-modifier process will not demonstrate any advantage. As shown, when the mutation rate of the gate-pass and modifier events is similar, analysis of the two-gate/two-stage model shows that almost all tumours arise by gate-pass mutation only even when the rate-modifier factor reaches 100.

The multigate model differs from the multistage/multipath model described in the previous chapter in postulating that p53 inactivation has a rate-modifying role in a tumorigenesis pathway which can nevertheless proceed independently of p53. An illustration of this possibility may be helpful. Here, consider a tumorigenic process in which the tumour suppressor gene Rb also plays a role. Suppose for example that inactivation of both Rb alleles are two enabling (gate-pass) genetic events in a particular tumour type. Then we expect that some tumours will be Rb doubly mutant (with intact p53) others will be Rb doubly mutant with inactivated p53. However, according to the multigate/multistage model, no tumours will be found to have suffered *only* p53 inactivation. The inactivation of p53 would therefore appear to be 'optional' in this mechanism of tumorigenesis. By contrast the multistage multipath model requires that if p53 is implicated in a tumorigenic pathway then any alternative pathway not involving p53 will instead have to involve some other genetic events (eg some genetic event additional to, or as an alternative, to the Rb inactivation considered in the example) ie p53 inactivation fulfils a role which is not 'optional' and would have to be replaced if not present. It is also a feature of the multigate model that p53 inactivation should precede at least one of the gate-pass events, whereas on the multistage model, p53 inactivation could just as easily come last.

We only analyse tumours in p53 deficient mice where p53 acts principally as a tumour suppressor gene. The case where p53 mutation acts to produce a dominant oncogene is not of major importance in these mouse genotypes and is not considered. Of course, it must be appreciated that such mutations do occur in human tumours, and that p53 mediated tumorigenesis will be more complex than discussed here.

Of course, the main difference between the models is that the multigate model is essentially a genetic instability model and requires that p53 inactivation be a destabilising event. Experimental evidence on this is not wholly consistent at present, with some workers reporting a significant increase in the mutation rate at a particular locus (Xia et al, 1995; Havre et al, 1995) and no difference being reported at other loci (Sands et al, 1995). Also, it is possible that the tumorigenic events which are enhanced by p53 inactivation correspond to chromosome abnormalities (Bouffler et al, 1995) or gene amplification (Livingstone et al, 1992; Yin et al, 1992) or other heritable events rather than traditional point mutations; also it is possible that the influence of p53 inactivation is confined to a restricted set of genes rather than being across the genome. It is also possible that the main impact of p53 inactivation will be in relation to the processing of DNA damage and that its role will be seen more clearly when mice of differing genotypes are subjected to graded doses of DNA-damaging agents. However, as we have emphasized at several points, the present model does not distinguish between increased production of genomic lesions in each cell and increased survival probability of lesion-bearing mutants. Bodmer and Thomlinson (1996) have recently argued for increased mutant survival, rather than increased production of primary genomic lesions, as the mechanism by which p53 inactivation leads to deteriorating genetic uniformity of a clonal cell population. The present model is compatible with this interpretation.

Chapter 9

Effect of Growth Patterns of Stem Cells on Tumour Spectrum and Multiplicity

9.1 Introduction

In previous chapters we have considered the development of tumours without distinguishing between the different tissue in which tumours may develop develop. We shall now see what happens when there are two or more distinct tissues. It is well known that the kinetics of growth of a tissue can strongly influence the age-specific incidence curve of cancer of this tissue type. Moolgavkar and his colleagues (1981 and 1986) have shown that the patterns of cancer incidence rate in human populations are explicable by the growth patterns of human tissues.

Recently, in the study of tumorigenesis in p53-deficient mice, differences in tumour spectra (i.e. tumours of differing pathological type) between the p53-deficient heterozygotes and homozygotes have been reported (Table 9.1; Donehower et al., 1995). These authors proposed as an explanatory hypothesis for the observed differences is that there are different temporal "windows of opportunity" for cancer development in different tissue compartments. It was thought that the greatest rates of cell division, cell numbers, and programmed genetic rearrangements may be in the lymphoid compartments during the neonatal stages. In the absence of wild-type p53, the large numbers of rapidly dividing cells in these lymphoid compartments may be more likely to incur further oncogenic lesions and progress to an early cancer. The presence of a single p53 allele in the p53 heterozygous mice may greatly reduce the likelihood of these lymphoid target cells' developing the requisite secondary oncogenic lessons, particularly after thymic involution, and this would open up "windows of cancer opportunity" in other tissue compartments.

To verify their hypothesis, the competition of tumour development in two different growth patterns of mouse tissues has been introduced into the tumorigenesis model, i.e. multistage models and multigate/multistage models. The computer simulation studies are used to show the proportion of the first-appearing tumours arising from two different tissues and tumour multiplicities in these tissues. It is intended to explore how the host factors, such as the spontaneous mutation rates and the number of stages, affect this proportion and tumour multiplicities.

Table 9.1 Tumour spectrum in C57BL/6 x 129/Sv p53 deficient mice (Donehower et al, 1995)

$p53^{-/-}$	$p53^{+/-}$
65% lymphomas	36% osteosarcomas
21% hemangiosarcomas	28% lymphomas
4% testicular tumours	9% hemangiosarcomas
4% undifferentiated sarcomas	7% rhabdomyosarcomas
2% osteosarcomas	4% fibrosarcomas
1% mammary adenocarcinoma	2% undifferentiated sarcomas
1% medulloblastoma	2% malignant schwannomas
1% malignant schwannomas	1% leiomyosarcoma
1% glioblastoma	1% myxosarcoma
	1% lung adenocarcinoma
	1% mammary adenocarcinoma
	1% squamous-cell carcinoma
	1% intestinal carcinoma
	1% salivary-gland myeloepithelioma
	1% harderian-gland carcinoma
	1% pituitary-gland adenoma
	1% preputial-gland carcinoma

9.2 Multistage models

Firstly, tumorigenesis is interpreted using the multistage model, in which there exists competition of tumour development in two mouse tissues with different growth patterns (Figure 9.1). For the sake of simplicity, we consider two tissues which differ only in the parameters describing cellular growth and death rates, i.e., with the same mutation rate and the same number of mutational events required for malignant transformation. In tissue I, the stem cells follow the Gomp-ex growth model described in Section 3.3 of Chapter 3. In tissue II, stem cells follow a Gamma growth model, which is described by the piecewise continuous equation

$$N(t) = \begin{cases} e^{c_1 t} & t \le 20 \ days \\ e^{20c_1} & 20 \le t \le 40 \ days \\ e^{20c_1} e^{-c_2(t-40)} & t \ge 40 \ days \end{cases}$$

for initial growth from single cell, where N(t) is the expected number of cell population in tissue II, and c_1 and c_2 are net growth rate. the growth pattern for tissue II corresponds to a stem cell population which peaks in early post-natal life and slowly declines thereafter. Such a pattern might be applicable to some classes of hemopoietic cells and to cell populations like retinoblasts which disappear in adult life. In our studies, for tissue I, the values of the parameters are those given in Chapter 3. For tissue II, c_1=0.9211/day, c_2=0.0065/day, and the death rate is 0.085/day. The expected growth curve of both tissues is shown in Figure 9.2.

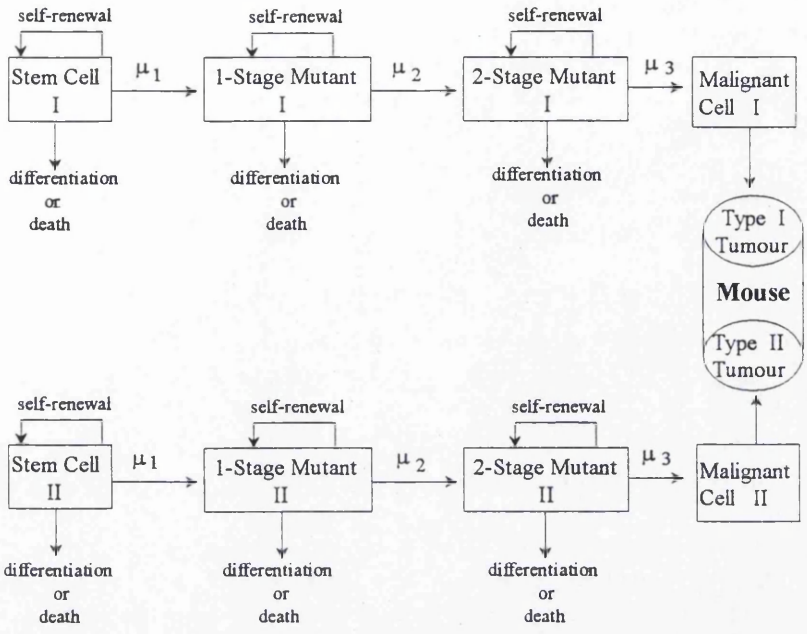


Figure 9.1 Multistage model of tumorigenesis with competition of tumour development in two different murine tissues.

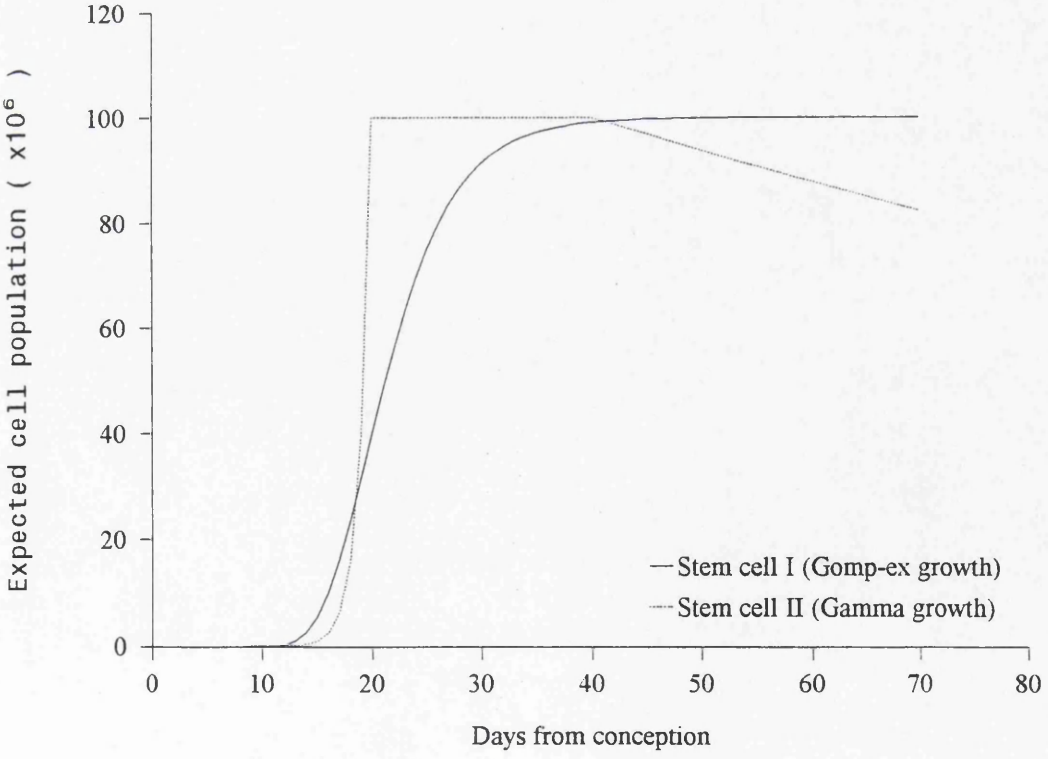


Figure 9.2 Postulated growth kinetics in two different tissues.

To explore the effect of the growth pattern of stem cells on the tumour spectra and multiplicity, computer simulation is used. The simulation process is quite similar to that described in Chapter 3, the only difference being that there are two tissues in this simulation. The simulation is terminated when the age of mouse reaches 600 days. The number of tumours in each tissues and the time of first ten tumours are recorded. For each set of parameters in the model, 10^4 simulations are repeated.

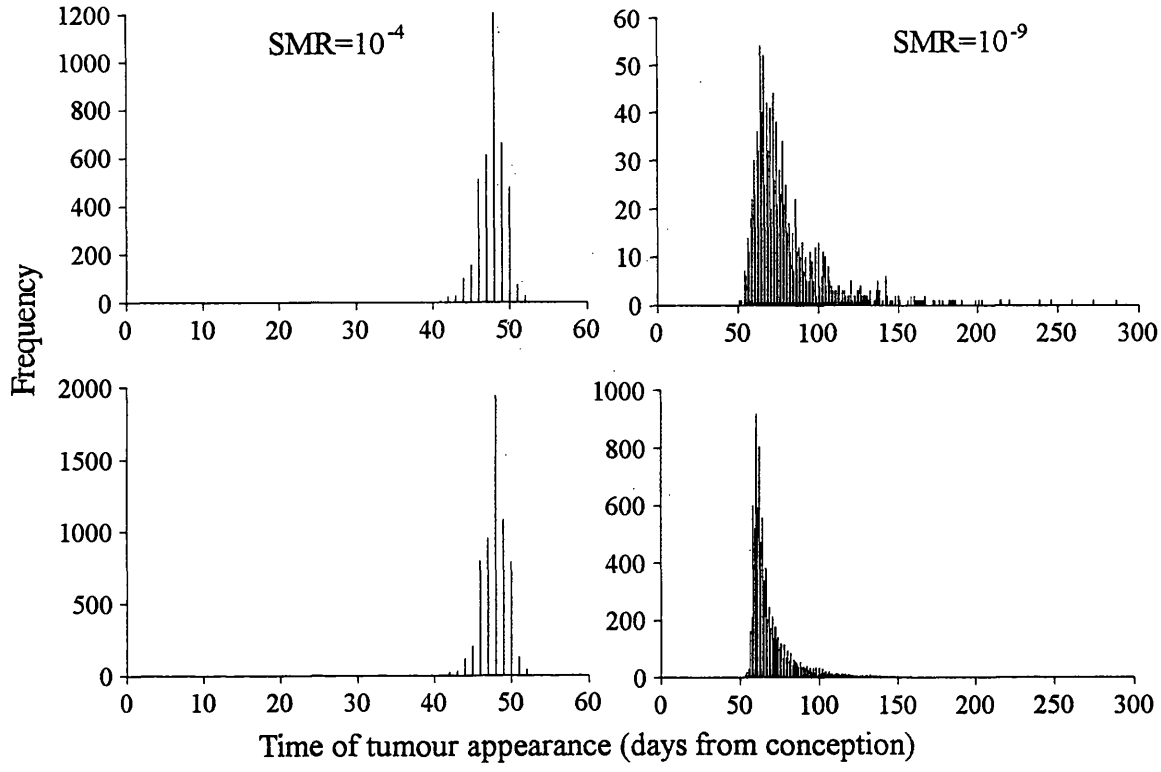
In our simulation studies, we suppose the growth and death rate of mutants are same as that of stem cells from which the mutants arose and the mutation rate at each stage are same.

9.2.1 Time of the first tumour appearance

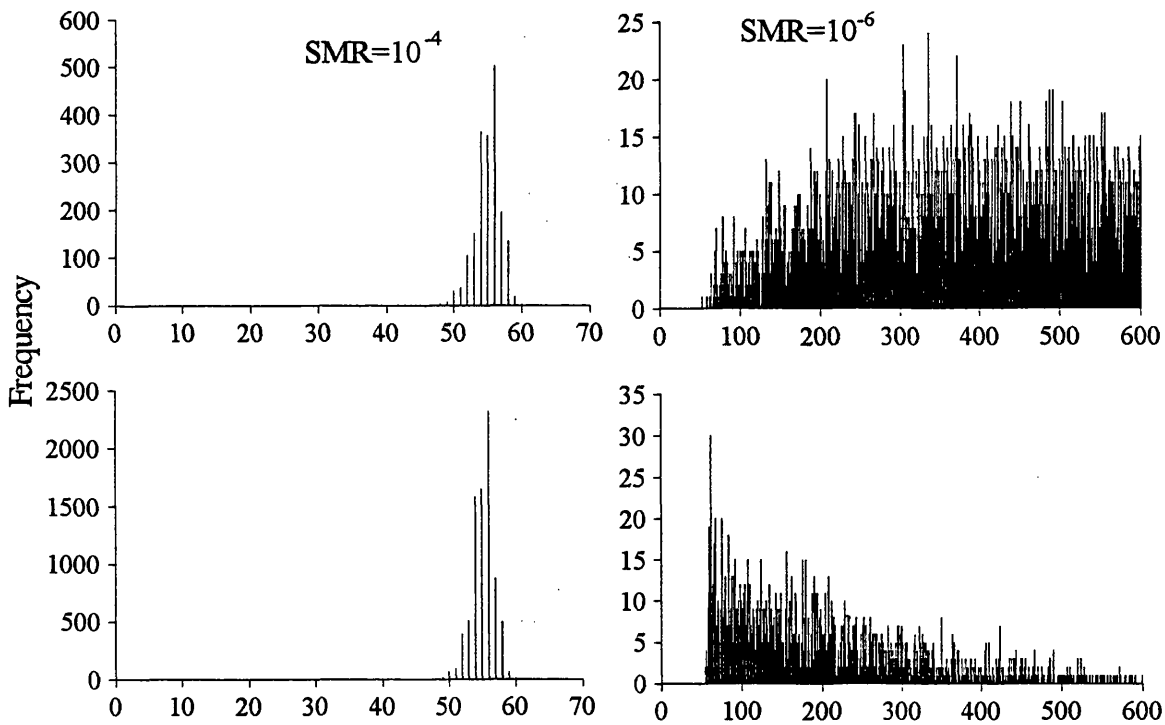
For all models, the type II tumours occur earlier than type I tumours (Figure 9.3), implying that earlier developing tissues (i.e., tissues having the greatest rates of cell division and greater number of cells occurring at earlier age), give rise to earlier appearance of tumours. This difference is influenced by the spontaneous mutation rate and the number of mutational events required for malignant transformation. With higher mutation rate and fewer number of stages, the difference is not marked (Figure 9.3).

9.2.2 Spectra of first-appearing tumours

For one-stage tumorigenesis, most of the first-appearing tumour are type II (Figure 9.4 (A)), whereas, for four-stage tumorigenesis, most are type I (Figure 9.4 (D)). However, for two- and three-stage tumorigenesis, with higher mutation rate, most are type II, but with lower mutation rate, most are type I (Figure 9.4 (B) and (C)).

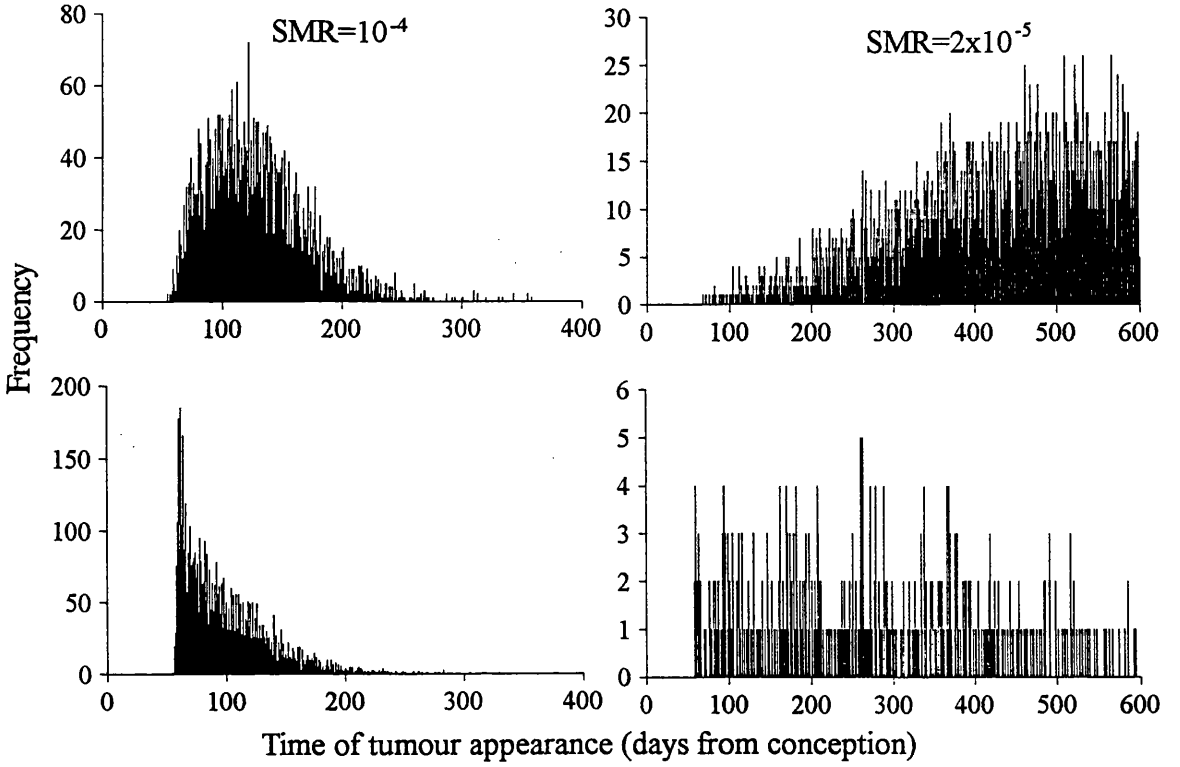


(A) 1-stage model

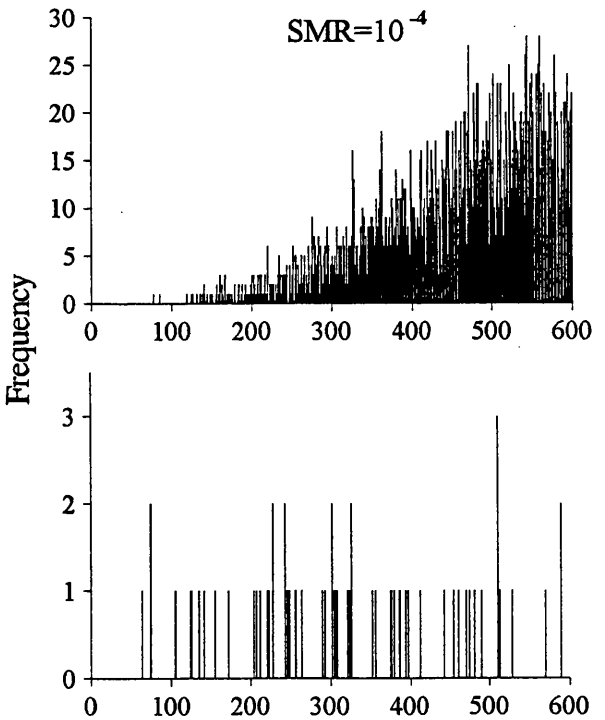


(B) 2--stage model

(Figure 9.3 to be continued)

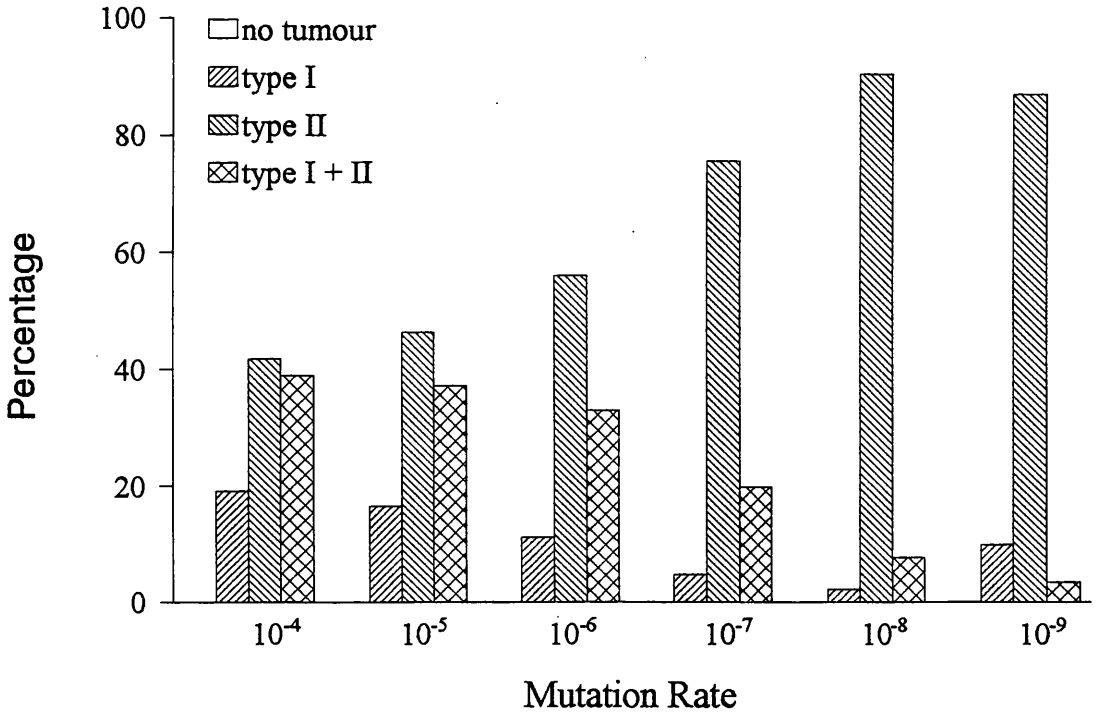


(C) 3-stage model

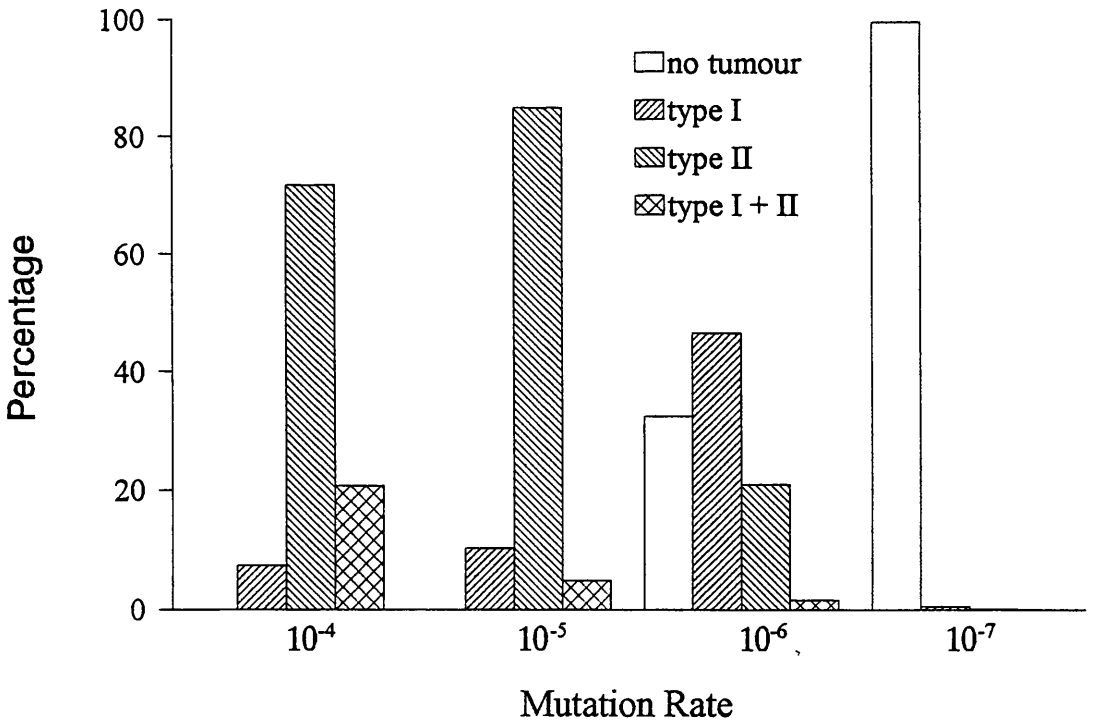


(D) 4-stage model

Figure 9.3 Comparison of time of appearance of tumours in two tissues. In each block, the upper lane shows time of appearance of type I tumours, and the lower lane shows time of appearance of type II tumours. (A) one-stage model; (B) two-stage model; (C) three-stage model; (D) four-stage model.

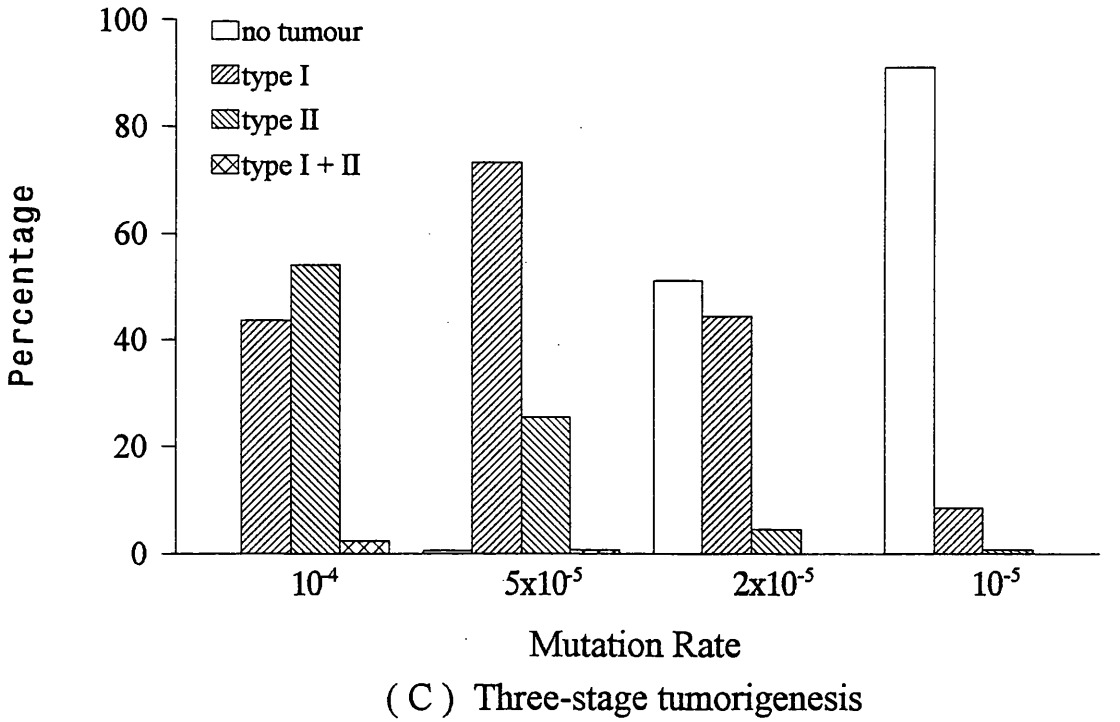


(A) One-stage tumorigenesis

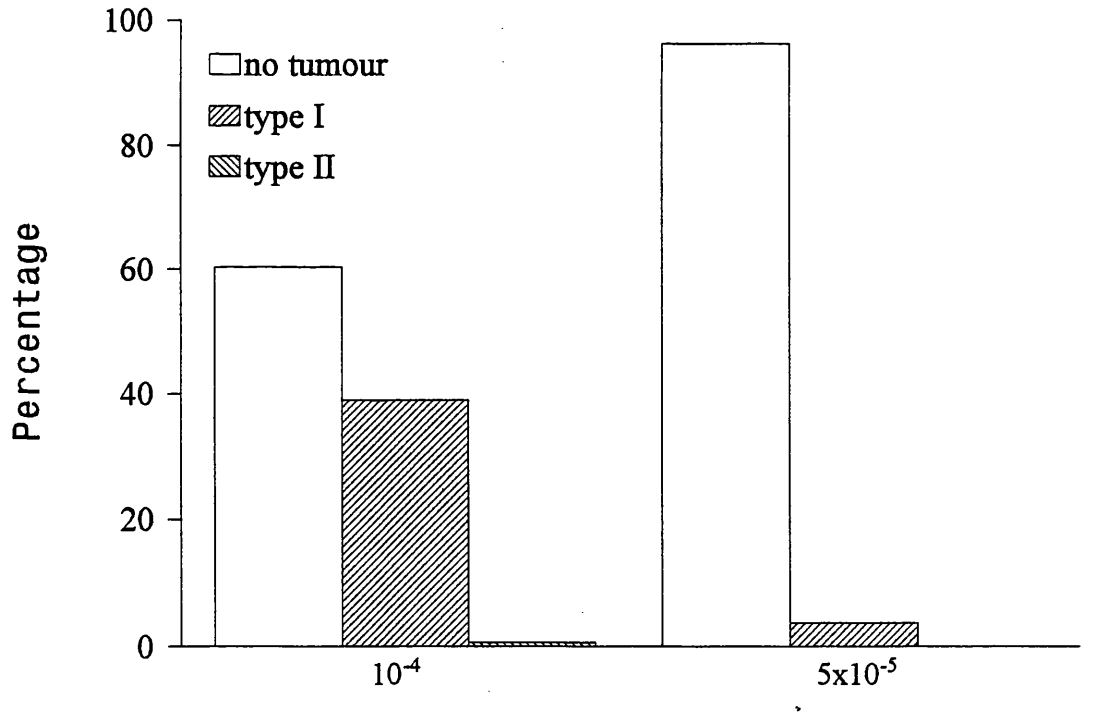


(B) Two-stage tumorigenesis

(Figure 9.4 to be continued)



(C) Three-stage tumorigenesis



(D) Four-stage tumourigenesis

Figure 9.4 Proportion of first-appearing tumours in two tissues. (A) one-stage tumorigenesis, (B) two-stage tumorigenesis, (C) three-stage tumorigenesis, and (D) four-stage tumorigenesis. When the difference of tumour appearing time between type I and II is less than 1 day, we say type I and II tumour appears together.

Figure 9.4 also shows that the proportion of type II tumours in the first-appearing tumours decreases with increasing number of stages required for malignant transformation and with decreasing spontaneous mutation rates (The one-stage model is anomalous; this is due to a 'cross-over' between the Gomp-ex and Gamma growth curves at early times).

We have also computed the proportion of type II tumours as a function of mouse age and have observed that this proportion tends to decrease with age (Figure 9.5), implying that type II tumours will be relatively over-represented amongst early-occurring tumours whereas type I tumours will be relatively over-represented amongst late-occurring tumours. However, this relationship is influenced by the spontaneous mutation rate and the number of mutational events. With smaller number of mutational events and higher value of the spontaneous mutation rate, this relationship tends to flatten out.

9.2.3 Multiplicity analysis of type I and II tumours

Interestingly, for all models, by 120 days, the mean number of type II tumours per mouse is greater than type I, however, by 600 days, the mean number of type II tumours is less than type I (Table 9.2). This indicates, in tissue II, most tumours appear in younger ages, whereas in tissue I, most tumours appear in older age group. Unfortunately, in the experiments, such a result may not be observable because the first tumour may cause mouse death (or humane sacrifice).

Table 9.2 also shows that the mean number of both types of tumours decreases with decrease in the spontaneous mutation rates, and with increase in the number of stages required for malignant transformation. This is consistent with the previous observations.

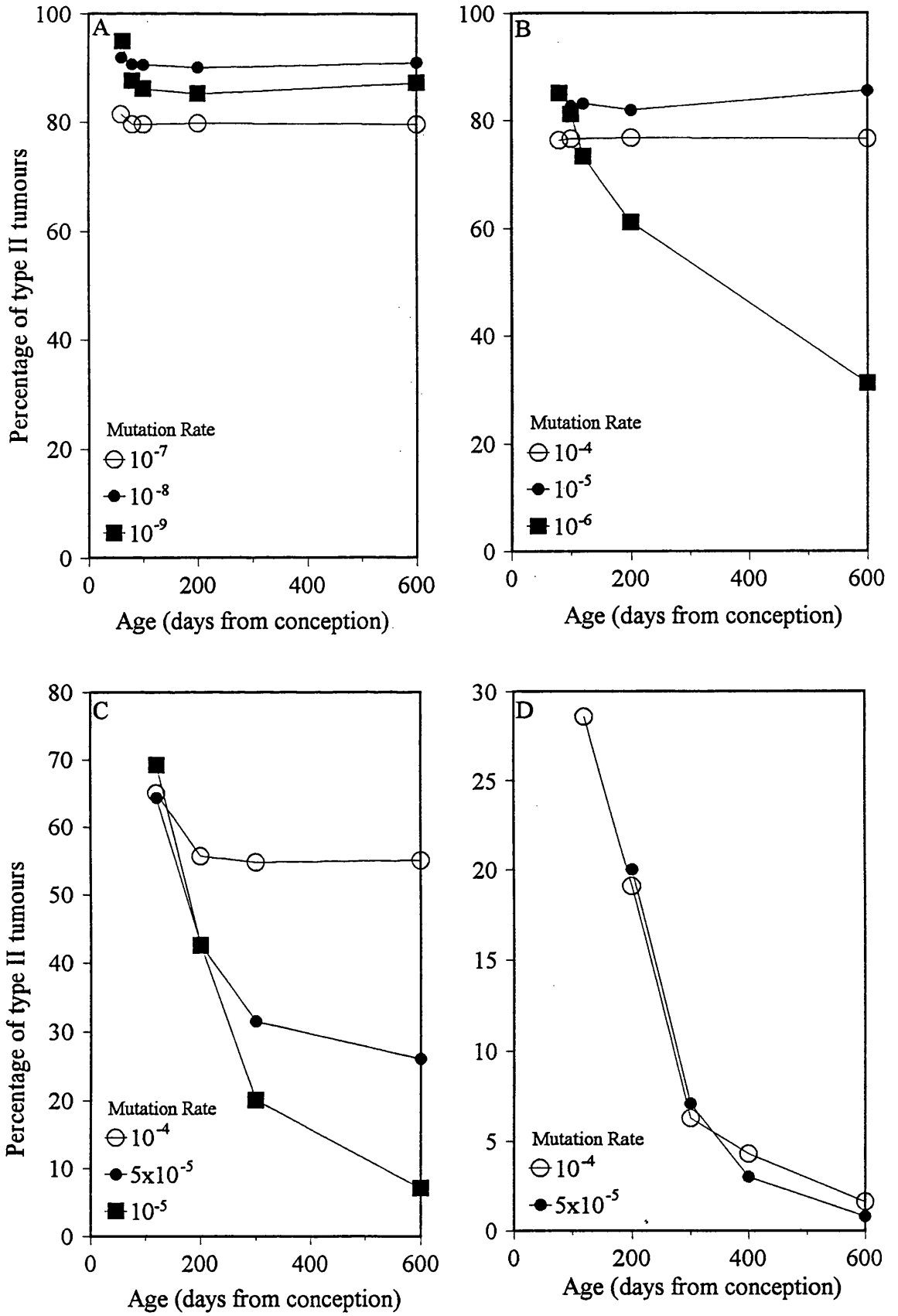


Figure 9.5 The relationship between the percentage of type II tumours and age. (A) one-stage model, (B) two-stage model, (C) three-stage model, and (D) four-stage model.

Table 9.2 Mean number of tumours per mouse predicted by the multistage model

Number of stages	Spontaneous mutation rate	Mean number of type I tumours		Mean number of type II tumours	
		120 days	600 days	120 days	600 days
1	10^{-4}	6.95×10^{4}	2.35×10^{6}	3.74×10^{5}	9.91×10^{5}
	10^{-5}	6.98×10^{3}	2.44×10^{5}	3.77×10^{4}	1.00×10^{5}
	10^{-6}	6.96×10^{2}	2.42×10^{4}	3.77×10^{3}	9.96×10^{3}
	10^{-7}	6.97×10^{1}	2.38×10^{3}	3.79×10^{2}	9.97×10^{2}
	10^{-8}	6.95×10^{0}	2.41×10^{2}	3.78×10^{1}	9.93×10^{1}
	10^{-9}	6.80×10^{-1}	2.37×10^{1}	3.72×10^{0}	9.91×10^{0}
2	10^{-4}	2.77×10^{2}	3.74×10^{4}	1.01×10^{3}	3.95×10^{3}
	10^{-5}	3.06×10^{0}	3.67×10^{2}	9.53×10^{0}	3.65×10^{1}
	10^{-6}	3.40×10^{-2}	3.63×10^{0}	1.14×10^{-1}	3.60×10^{-1}
	10^{-7}	0	2.80×10^{-2}	4.00×10^{-4}	4.00×10^{-4}
3	10^{-4}	5.57×10^{-1}	3.75×10^{2}	1.20×10^{0}	7.78×10^{0}
	5×10^{-5}	6.10×10^{-2}	4.64×10^{1}	1.79×10^{-1}	1.02×10^{0}
	10^{-5}	5.00×10^{-4}	3.63×10^{-1}	3.40×10^{-3}	6.70×10^{-3}
4	10^{-4}	5.00×10^{-4}	2.49×10^{0}	1.00×10^{-3}	1.00×10^{-2}
	5×10^{-5}	0	1.50×10^{-1}	0	4.00×10^{-4}
	10^{-5}	0	2.00×10^{-3}	0	0

9.3 Multigate/multistage models

We now consider the multigate/multistage model to provide interpretation of tissue-specific competitive tumorigenesis (Figure 9.6). The assumptions regarding the two tissues are as before. Computer simulations have been carried out for a range of values of the number

of gates in the model, for a range of values of the mutation rates of the modifier genes and (independently) the mutation rate of the gate-pass genes, and a range of values of the modifier factor.

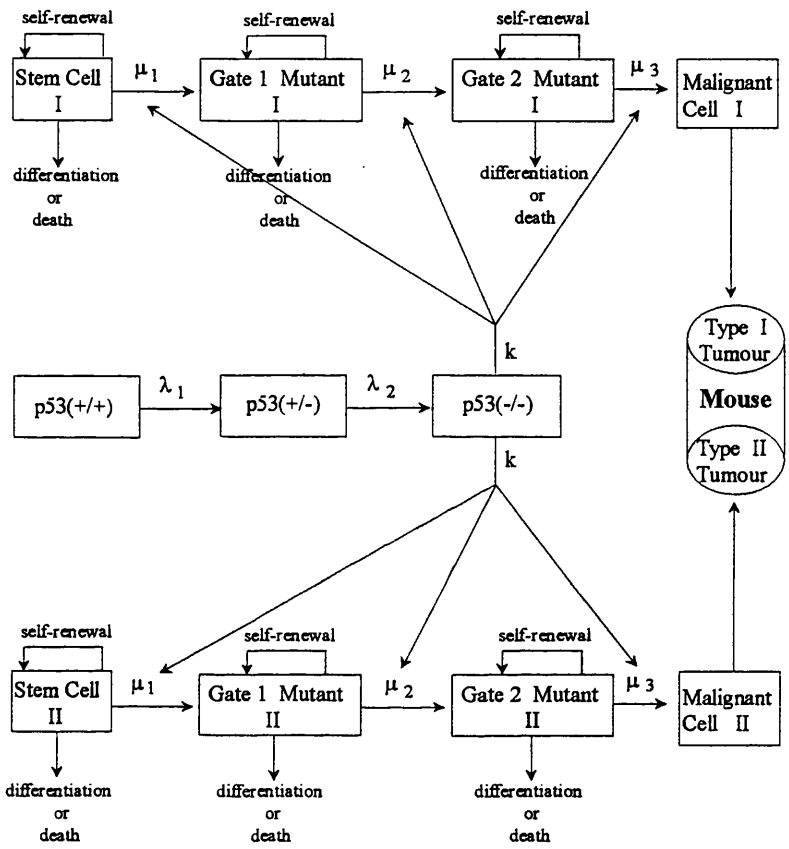


Figure 9.6 Multigate/multistage model of tumorigenesis with competition of tumour development in two different tissues.

9.3.1 Time of the first tumour appearing

It is very interestingly found that type II tumours still occur earlier than type I tumours under the multigate/multistage model although this difference is influenced by the rates of the gate-pass and modifier mutation, number of gates, and modifier factor (Figure 9.7).

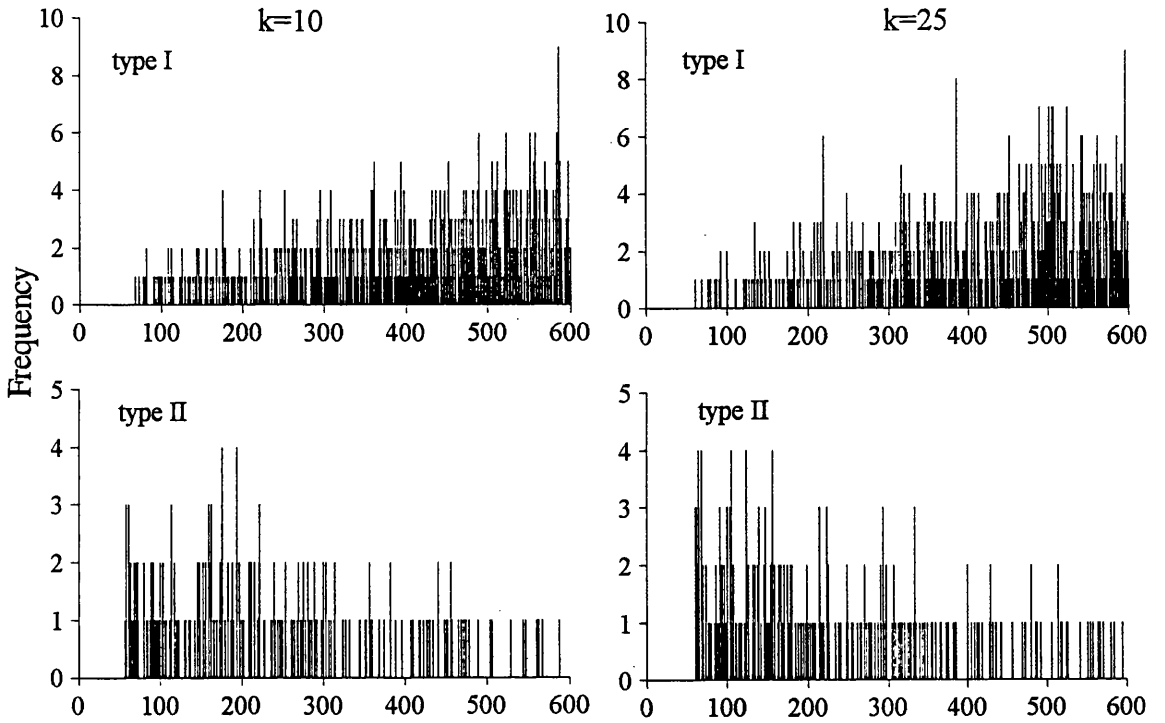
9.3.2 Proportion of type II tumours as a function of the modifier factor

Surprisingly, the relationship between the proportion of type II tumours and the modifier factor seems to follow no simple rules for the multigate model with 2-stage rate-modifier process (Figure 9.8 (B1), (B2) and (B3)). However, for the multigate model with 1-stage rate-modifier process, this proportion decreases with the modifier factor down to a minimum, with an increase following that minimum (Figure 9.8 (A1), (A2) and (A3)). This relationship is influenced by the rate of the gate-pass and modifier mutations, and the number of gates (Figure 9.8).

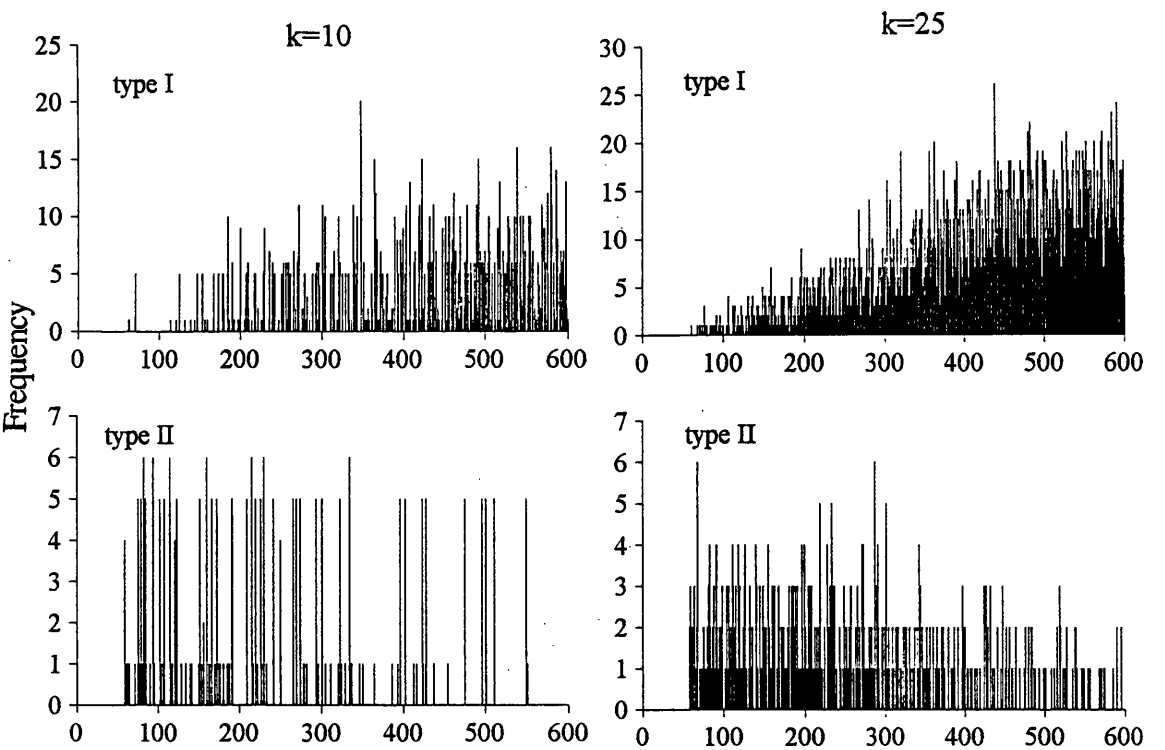
9.4 Proportions of type I and II tumours in p53 deficient mice

Tumorigenesis in p53 deficient mice can be interpreted by the multistage multipath model or by the multigate/multistage model as described in the two previous chapters. As shown above, the proportion of type I and II tumours in $p53^{+/-}$ and $p53^{-/-}$ can be predicted by the multigate/multistage model. Table 9.2 shows how this proportion changes with age. By 120 days, in both genotypes, the proportion of type II tumours is higher than that of type I, however, by 300 or 600 days, in $p53^{+/-}$, the proportion of type II tumours is lower, whereas, in $p53^{-/-}$, the proportion of type II tumours is still higher.

With the multistage multi-path model, because an unknown route for tumour development exists, it seems difficult to predict the proportion of type I and II tumours.

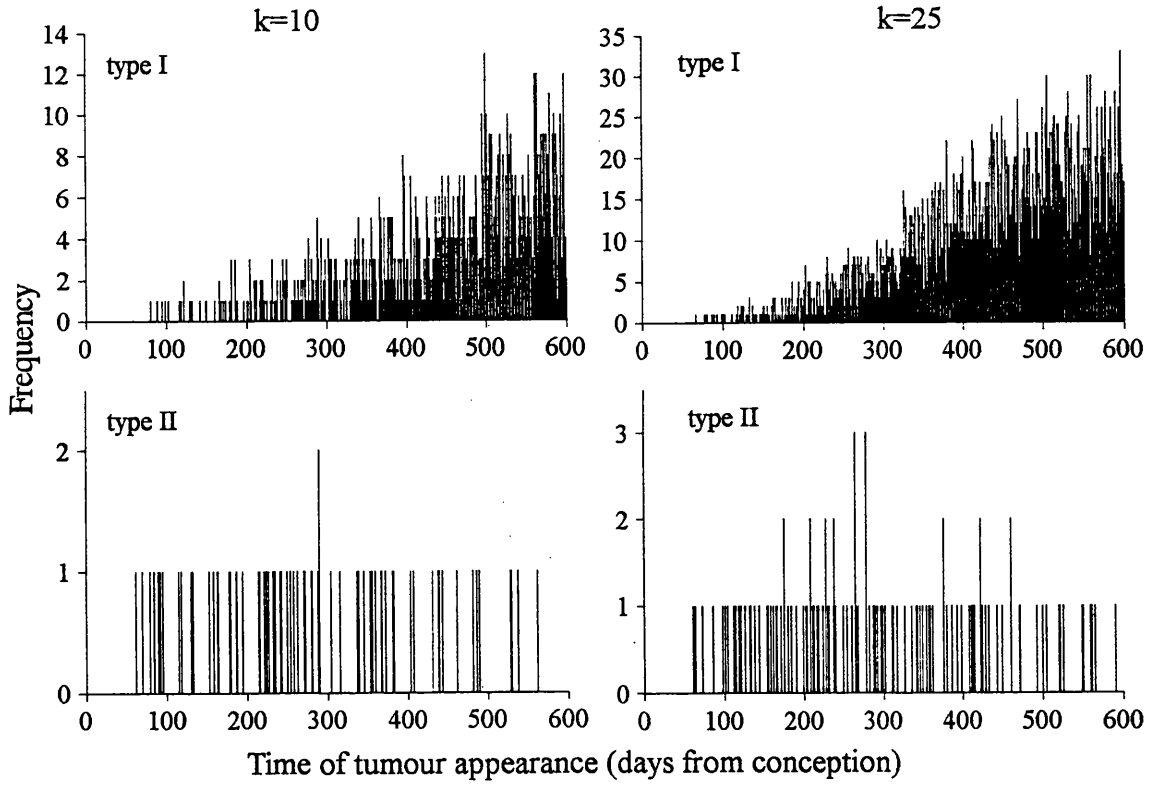


(A) 2-gate/1-stage model with similar mutation rate of gate-pass and modifier events

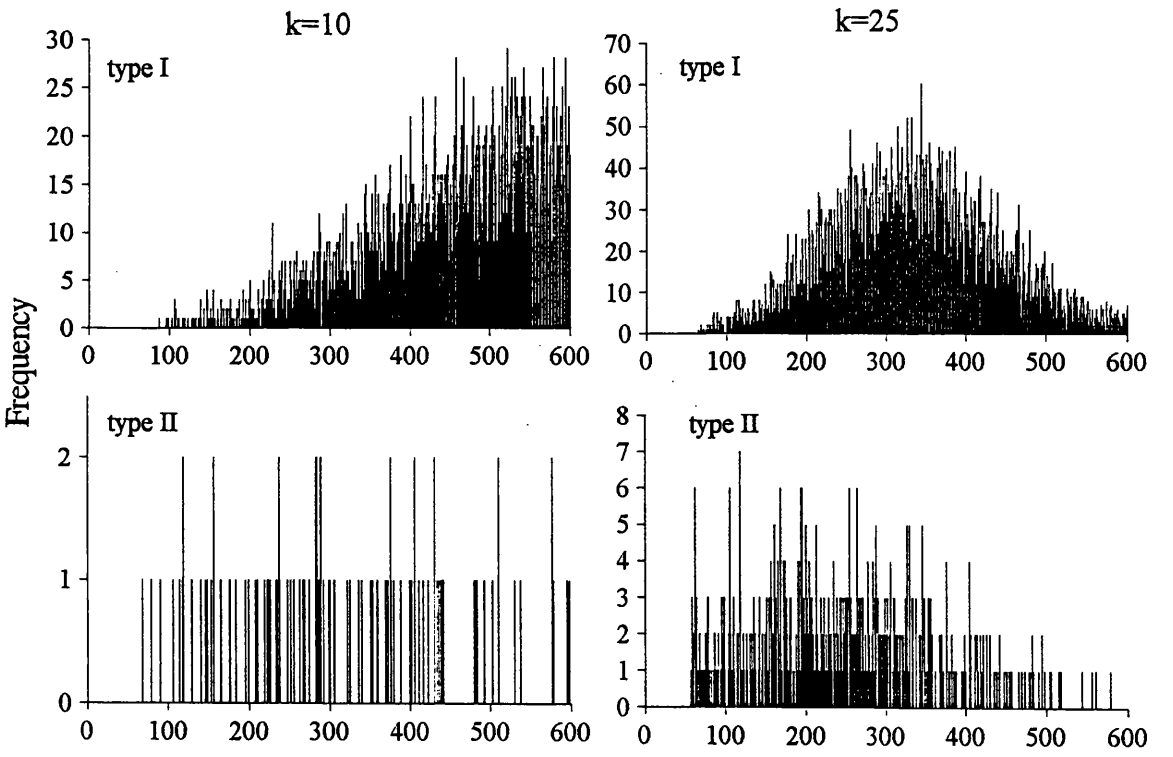


(B) 2-gate/1-stage model with higher mutation rate of modifier events than gate-pass events

(Figure 9.7 to be continued)

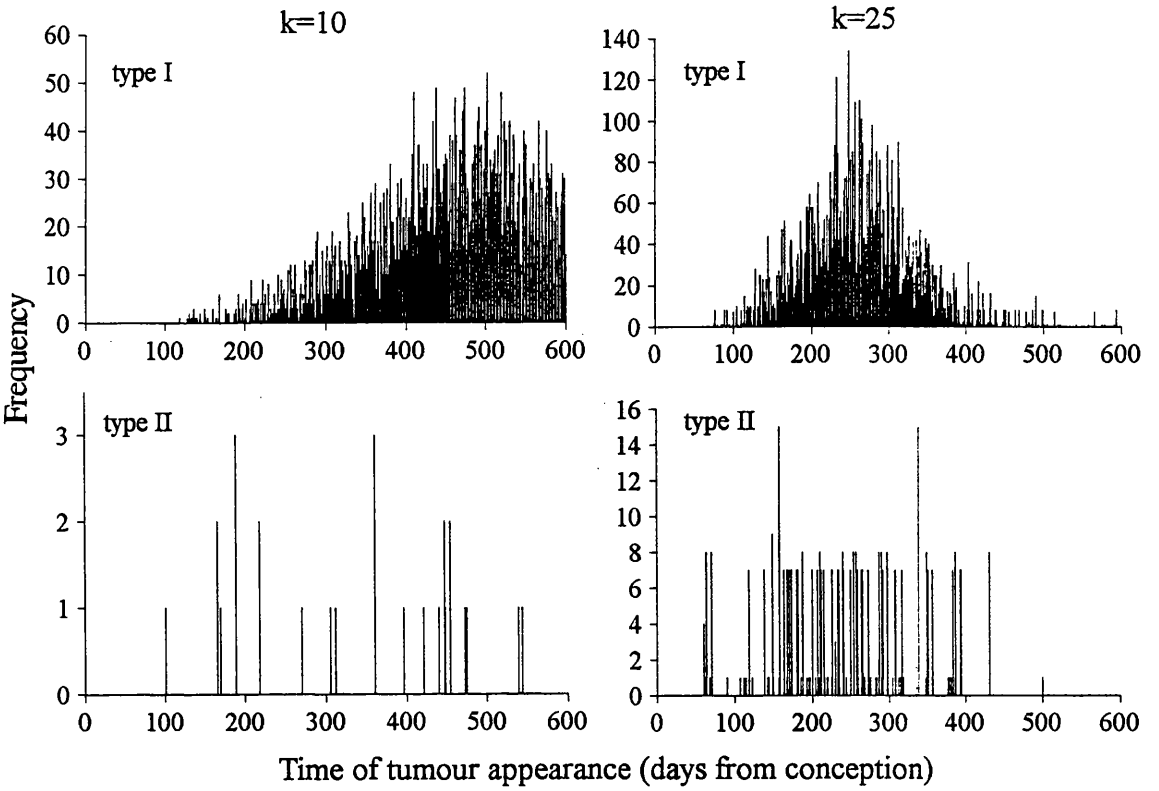


(C) 3-gate/1-stage model with same mutation rate of gate-pass and modifier events



(D) 3-gate/1-stage model with higher mutation rate of modifier events than gate-pass events

(Figure 9.7 to be continued)



(E) 4-gate/1-stage model with higher mutation rate of modifier events than gate-pass events

Figure 9.7 Comparison of time of appearance of tumours in two tissues. In each block, the upper lane shows the time of type I tumours appearing, and the lower lane shows the time of type II tumours appearing. (A) 2-gate/1-stage model with gate-pass mutation rate $3x10^{-7}$ and modifier mutation rate 10^{-6}; (B) 2-gate/1-stage model with gate-pass mutation rate $3x10^{-7}$ and modifier mutation rate 10^{-4}; (C) 3-gate/1-stage model with gate-pass mutation rate 10^{-5} and modifier mutation rate 10^{-5}; (D) 3-gate/1-stage model with gate-pass mutation rate 10^{-5} and modifier mutation rate 10^{-4}; (E) 4-gate/1-stage model with gate-pass mutation rate $5x10^{-5}$ and modifier mutation rate 10^{-4}.

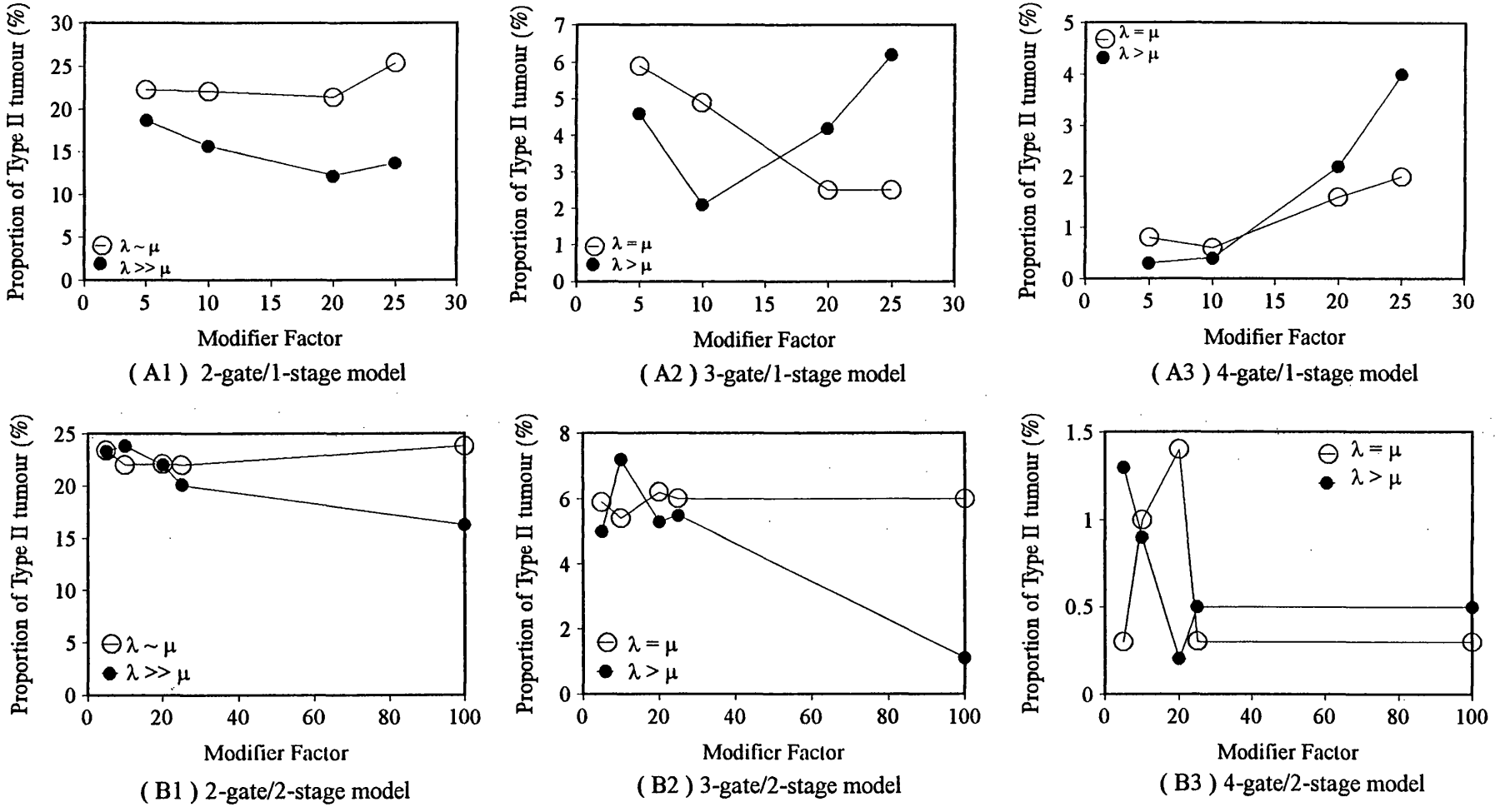


Figure 9.8 Relationship between proportion of type II tumours and the rate-modifier factor. (A1) 2-gate/1-stage model with $\mu = 3\times10^{-7}$ and $\lambda = 10^{-6}$ or 10^{-4}; (A2) 3-gate/1-stage model with $\mu = 10^{-5}$ and $\lambda = 10^{-5}$ or 10^{-4}; (A3) 4-gate/1-stage model with $\mu = 5\times10^{-5}$ and $\lambda = 5\times10^{-5}$ or 10^{-4}; (B1) 2-gate/2-stage model with $\mu = 3\times10^{-7}$ and $\lambda = 10^{-6}$ or 10^{-4} ; (B2) 3-gate/2-stage model with $\mu = 10^{-5}$ and $\lambda = 10^{-5}$ or 10^{-4} ; (B3) 4-gate/2-stage model with $\mu = 5\times10^{-5}$ and $\lambda = 5\times10^{-5}$ or 10^{-4} .

Table 9.3 Proportion of type I and II tumours in p53 deficient mice as predicted by multigate/multistage model

Number of gates	Tumour type	$p53^{+/-}$ Proportion (%)			$p53^{-/-}$ Proportion (%)		
		120 days	300 days	600 days	120 days	300 days	600 days
2	I	30.6	66.1	86.4	17.0	29.5	29.0
	II	69.0	33.7	13.3	69.1	65.0	65.1
	I+II	0.4	0.2	0.3	14.1	5.5	5.9
	total incidence	**2.3**	**15.3**	**56.3**	**65.1**	**98.6**	**100**
3	I	35.7	89.7	97.2	31.7	41.0	43.6
	II	64.3	10.3	2.6	63.3	51.3	54.1
	I+II	0	0	0.2	5.1	7.7	2.3
	total incidence	**0.42**	**7.2**	**59.3**	**62.7**	**99.9**	**100**

9.5 Discussion

Competition of tumour development in two mouse tissues showing different growth pattern has been studied by using multistage and multigate/multistage models. As result of this competition, the tumour spectra will be different in the different genotypes. However, the effect of tissue growth pattern on tumour spectra clearly depends on host factors, such as the mutation rate, the number of mutational events required for malignant transformation, etc.

Both multistage and multigate/multistage models demonstrate that tumours occur at younger age in the tissues that show a sudden burst of growth in early life. Physically, this may be because the earlier great rate of cell division results in the earlier accumulation of mutations for malignant transformation.

On the multistage tumorigenesis models, our studies have shown the "windows of opportunity" for cancer development in different tissue compartments. With fewer stages and higher mutation rate, most first-appearing tumours will arise from the tissue in which the greatest rates of cell division occur at an earlier age.

Biologically, the growth of osteocytes (giving rise to bone sarcomas) can be reasonably well represented by the Gomp-ex growth curve, whereas growth of lymphoid tissue (giving rise to malignant lymphomas) can be presented by a gamma growth curve. Thus, the difference in tumour spectra between $p53^{+/-}$ and $p53^{-/-}$ mice can be shown by the multigate/multistage model. Although, we have here supposed that the number of gates (i.e., mutational events) required for malignant transformation in two tissues and baseline mutation rate in each gate are same, this findings may be still not difficult to rationalize with Donehower's hypothesis, i.e. the existence of 'windows of opportunity' for tumour development.

In fact, in different tissues, the number of mutational events may be different (Renan, 1993). Further, the mutation rates for different mutational events (different genes) may be also different. As shown in Chapter 3, the number of mutational events and the mutation rates determine the time of tumour appearance. At present the exact number of mutations required for malignant transformation and the rate of mutations occurring in each tissue remain unknown.

Chapter 10

Experimental Studies on an *In Vivo* Model System to Determine the Effect of p53 Deficiency on Cellular Mutation Frequency

10.1 Introduction

This chapter describes an experimental approach to a central problem of p53-mediated tumorigenesis: how to measure the effect of p53 inactivation on mutation frequencies at other genetic loci. It is known that the p53 gene is mutated or inactivated at a high frequency in a wide variety of human cancers (Nigro et al., 1989; Harris and Hollstein, 1993). The physiological functions of the p53 protein are not fully understood, but one well-known hypothesis states that p53 acts as the 'guardian of genome', i.e., it is a key component of a system activated by DNA damage that either arrests the cell cycle in G1 to allow DNA repair, or initiates apoptosis if the damage is severe (Lane, 1992; Donehower, 1994; other references cited in Chapter 1). This is of course consistent with the 'modifier' role for p53 suggested in the multigate/multistage model (Chapter 8).

If the 'guardian of the genome' hypothesis is correct, lack of normal p53 function should increase the mutation rate elsewhere in the genome. In support of this, some workers have reported a significant increase in the mutation rate at particular loci (Havre et al, 1995; Xia et al, 1995; Yuan et al, 1995), increase of chromosome abnormalities (Harvey et al, 1993; Bouffler et al, 1995), or of gene amplification (Livingstone et al, 1992; Yin et al, 1992). However, these data are all demonstrated using *in vitro* systems. In order to investigate this question *in vivo*, two groups (Nishino et al, 1995; Sands et al, 1995) have used a transgenic mouse system (Big Blue) to examine the effect of the presence or absence of the functional p53 gene on the rate and pattern of background somatic mutation of the *LacI* transgene *in vivo*. Surprisingly, no difference in the frequencies of *LacI* point mutations between p53 wild-type and p53 nullizygous mice was found by both groups.

One limitation of Big Blue transgenic mouse mutation detection system, which uses chromosomally integrated bacteriophage lambda shuttle vectors containing the *LacI* gene as a target for mutagenesis (Kohler et al, 1991; Mirsalis et al, 1994), is that deletions larger than 5 kb or insertions larger than 8 kb package inefficiently because of the size of constraint on genomic DNA that could be packaged into phage particles. To improve this, we have used an alternative transgenic mouse model (ROSA) in which the transgene, *LacZ*, is expressed ubiquitously. By crossbreeding with p53 knock-out mice according to classic mouse genetics, we should be able to obtain a *LacZ* transgenic mouse having heterozygous or homozygous p53 null background. *LacZ* gene mutations can be detected by X-gal staining. In an organ section, cells which express functional *LacZ* gene product, β-galactosidase, will stain blue in colour. Therefore, any mutation affecting the enzymic activity of β-galactosidase will turn the cells from blue to white. We set out to measure the spontaneous and radiation-induced mutation frequencies of *LacZ* gene in different organs, such as skin, liver, spleen, intestine, brain etc.

10.2 Materials and methods

10.2.1 Animals

LacZ transgenic mice (ROSA mice) which were derived by electroporating C57BL/6J ES cells with ROSAβ-geo trap carrying a reporter gene *LacZ* were obtained from Friedrich and Soriano (1991), and mice lacking one or both alleles of p53 which were derived by gene targeting of 129/Sv ES cells were obtained from Donehower et al (1992).

10.2.2 Genotyping

Genotypes were determined by PCR for the p53 gene. Separate primer pairs were used for PCR genotyping each p53 allele.

Primers for the wild type p53 allele were as follows:

WT1: 5' GTG TTT CAT TAG TTC CCC ACC TTG AC 3'

WT2: 5' CTG TCT TCC AGA TAC TCG GGA TAC 3'

Primers for the null p53 allele were as follows:

M1: 5' GGG ACA GCC AAG TCT GTT ATG TGC 3'

M2: 5' TTT ACG GAG CCC TGG CGC TCG ATG 3'

The PCR reactions for each allele were performed separately and then mixed before running on an agarose gel. The PCR reaction for both the wild-type and null alleles were performed using the following standard conditions: 2 µl of sample, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 µM of each primer oligonucleotide (see above), and 0.02 u/µl Taq polymerase (Perkin-Elmer) in a 50 µl reaction volume. A total of 35 cycles were used after originally denaturing at 94°C for two minutes. In each cycle of the first 34 cycles, the protocol was as follows:

30 seconds denaturing at 91°C
30 seconds annealing at 55°C
30 seconds elongation at 72°C,

followed by one cycle of 30 seconds denaturing at 91°C, 30 seconds annealing at 55°C and four minutes elongation at 72°C.

X-gal staining of the mouse ear punch was used to determine the mouse genotype for *LacZ* (see section 10.2.4).

10.2.3 Preparation of genomic DNA from mouse tail

Genomic DNA was extracted from the mouse tail according to a standard protocol (Laird et al, 1991). Mice were humanely sacrificed before tails were cut, minced in small pieces and placed in a microfuge tube containing 0.5 ml lysis buffer (100 mM Tris.HCl pH8.5, 5mM EDTA, 0.2% SDS, 200 mM NaCl). Proteinase K was added at a final concentration of 100 μg/ml and incubated at 55°C overnight. Following complete lysis, the tubes were vortexed. Tubes were then spun in a centrifuge for 10 minutes. The supernatant was then poured into prelabeled tubes, each containing 0.5 ml of isopropanol. The samples were swirled by hand until precipitation was complete. The DNA was recovered by lifting the aggregated precipitate from solution using a disposable yellow tip. Excess liquid is dabbed off and the DNA was dissolved in TE buffer (10 mM Tris.HCl, 0.1 mM EDTA, pH 7.5). The tube was then left overnight at 37°C to ensure the DNA was properly dissolved.

10.2.4 X-gal staining for β-galactosidase activity

Staining of tissues, including ear punch, was done according to the method of Gossler and Zachgo (1993). Tissue samples to be stained were first of all fixed in fixative solution (0.2% glutaraldehyde in phosphate buffer containing 5mM EGTA and 2mM $MgCl_2$) for 30 to 120 minutes (depending on size) at room temperature. Very large tissue pieces were partially dissected to permit full penetration of reagent. The samples were then washed in three changes of washing solution (0.01% Na desoxycholate and 0.02% Nonidet P-40 in phosphate buffer containing 5mM EGTA and 2mM $MgCl_2$) for 30 minutes each at room temperature. Then the samples were stained in staining solution (0.5 mg/ml X-gal, 10mM $K_3[Fe(CN)_6]$ and 10mM $K_4[Fe(CN)_6]$ in washing solution) overnight in the dark at 37°C. After staining the samples were washed in washing solution.

The fixed and stained tissue samples were dehydrated, and embedded in paraffin wax. Sections about 10 μm thick were cut from the samples. Then the sections were dewaxed, mounted and visualised by microscopy.

Staining frozen sections is required when large tissues are to be analyzed. Frozen sections were cut by Iain McMillan in the Department of Pathology at Glasgow University Veterinary School. The procedure of staining was modified in the following ways: frozen sections about 10 μm thick were left to dry and firmly attach to glass slides that were gelatinized. Slides were fixed in fixative solution for 5 minutes at room temperature, washed three times in washing solution for 5 minutes each, and then stained in staining solution overnight (Histochemical staining trays are very convenient and allow easy handling of 20 slides at a time when two slides are put back to back). Then the slides were washed first for 5 minutes in washing solution, then 5 minutes in distilled water. After dehydrating sections in ethanol (70%, 96%, 100% EtOH, 5 minutes each), the slides were passed through xylene: EtOH 1:1 (1 minute) and xylene (1 minute), then mounted and visualised by microscopy.

10.2.5 Mutation frequency

By X-Gal staining, the cells having functional *LacZ* gene product, β-galactosidase, will be blue in colour. Any mutation affecting the enzymic activity of the β-galactosidase will turn the cells from blue to white. Thus, the mutation frequency in one tissue can be measured by screening sections of this tissue to count the fraction of colourless cells, i.e.

$$\text{Mutation frequency} = \frac{\text{Number of colourless cells}}{\text{Total cells}}$$

From the published data, for one organ, at least 1 million cells should be counted. Therefore, if there are 100 cells in one microscopic field, at least 10000 fields should be screened.

10.3 Results

10.3.1 Breeding protocol

LacZ transgenic mice (ROSA mice) with a homozygous wild-type p53 gene have been available to us. It has been shown that, in this strain, *LacZ* is constitutively expressed (Figure 10.1). By crossbreeding with p53 knock-out mice, according to classic mouse genetics, we have been able to obtain *LacZ* transgenic mice having heterozygous wild-type p53 or homozygous p53 null backgrounds. It is however important to avoid having two copies of the *LacZ* gene. The breeding procedure is designed as follows: ROSA mice were mated with p53 null mice to produce a generation F1, then *LacZ* positive F1 mice, which had one copy of the *LacZ* gene based on genetics, were backcrossed to p53 null mice or wild type mice (Figure 10.2). p53 genotype was determined by PCR (Figure 10.3 (A)), and *LacZ* genotype by staining of ear punch (Figure 10.3 (B)).

10.3.2 Staining

Staining of tissues is limited by the size of the specimen. Tissues tend to give staining problems due to impaired penetration of the substrate, despite attempts to cut them into very small pieces. Therefore, in these cases, staining of frozen sections is required.

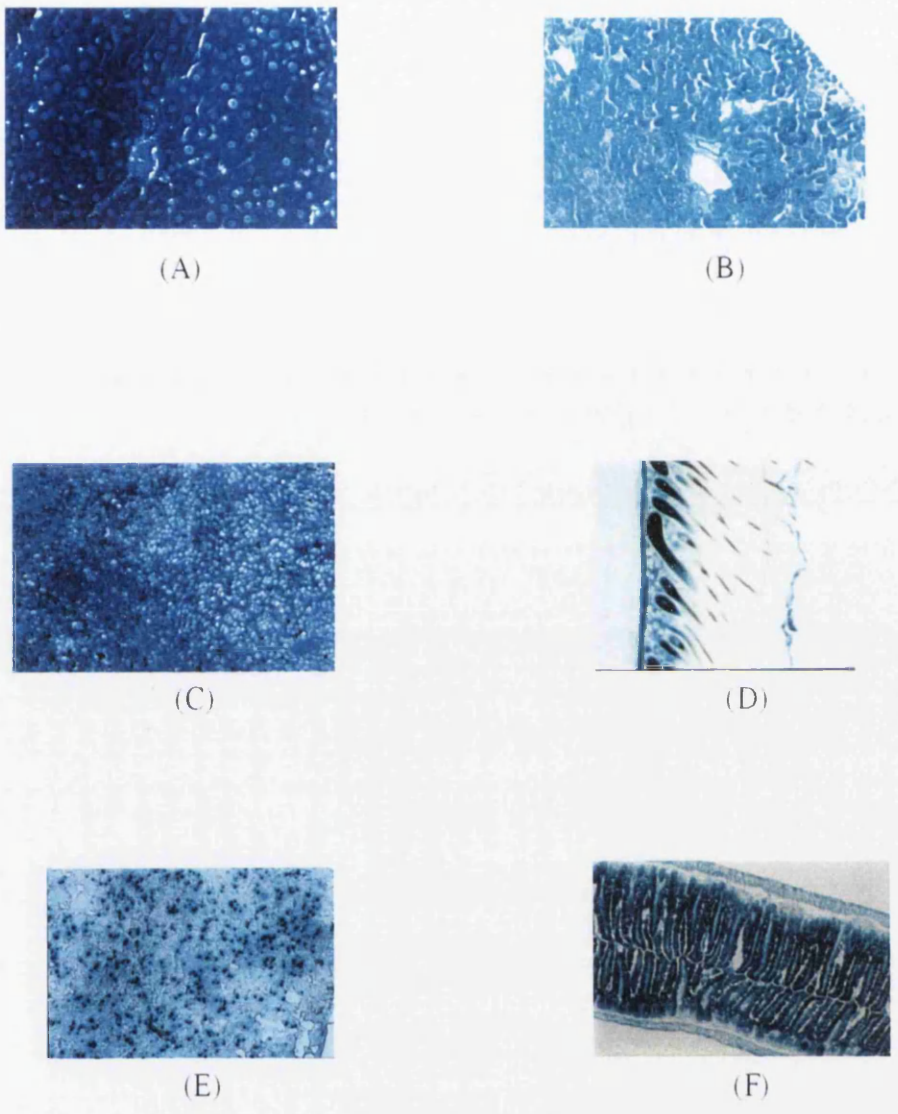


Figure 10.1 Expression of *LacZ* gene in different tissues of ROSA mice. (A) Liver, (B) Kidney, (C) Spleen, (D) Skin, (E) Brain and (F) Intestine.

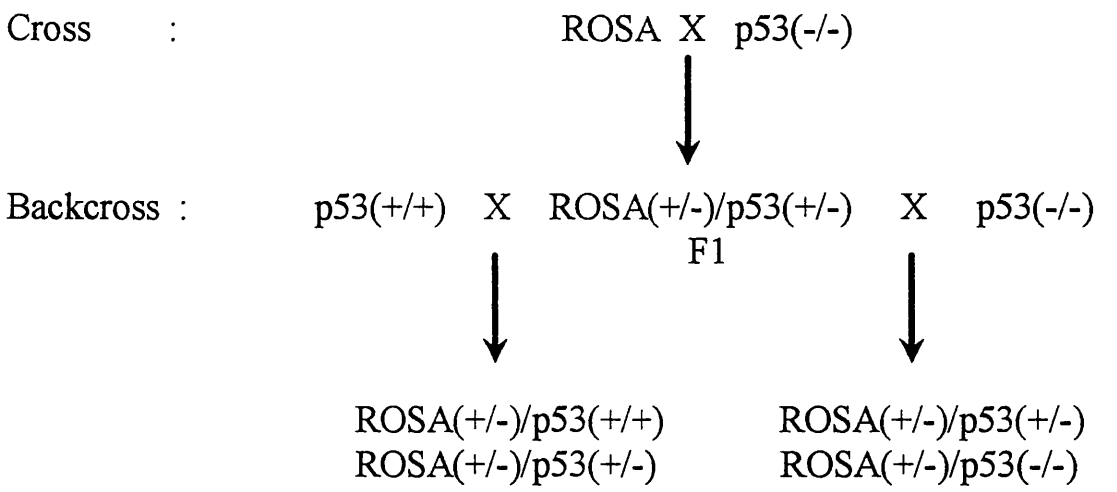
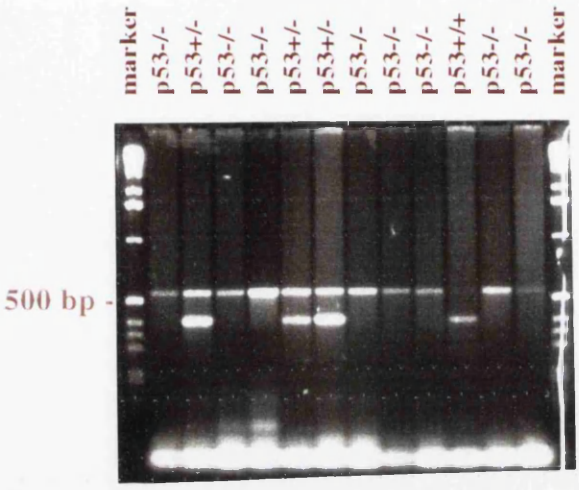


Figure 10.2 Experimental design of mouse breeding

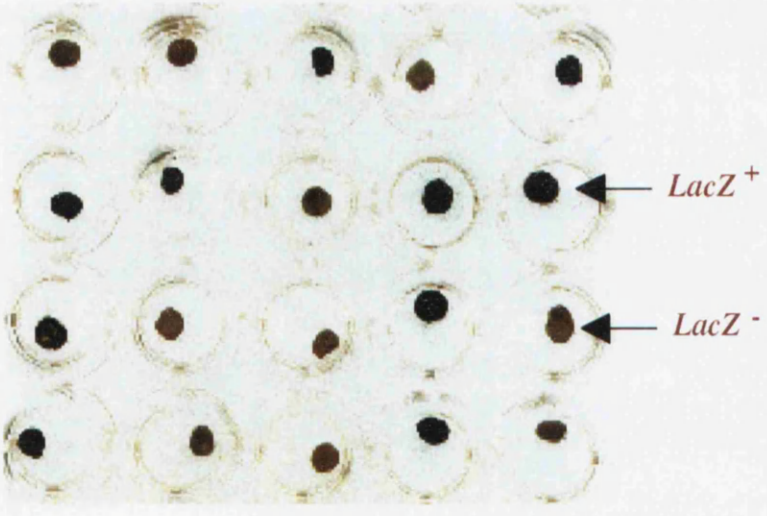
The frozen sections of six tissues (skin, intestine, liver, kidney, spleen and brain) were stained. The results shows that the expression of *LacZ* gene in the F1 backcross strain varied considerably in individual tissues and individual mice (Figure 10.4).

10.4 Discussion

The advantage of the new system (ROSA mice) to measure mutation *in vivo* is that almost all kinds of mutations (large or small deletions, point mutations etc.) should be detectable. The limitation of this new system is that it is only applicable to tissues where the expression of *LacZ* gene is uniform. Unfortunately, in our studies, after crossbreeding to p53 deficient mice, we found the expression of *lacZ* gene is highly non-uniform.



(A) p53 genotyping by PCR



(B) *LacZ* genotyping by x-gal staining ear punch

Figure 10.3 Genotyping of p53 and *LacZ* gene. (A) p53 genotyping by PCR; (B) *LacZ* genotyping by x-gal staining ear punch

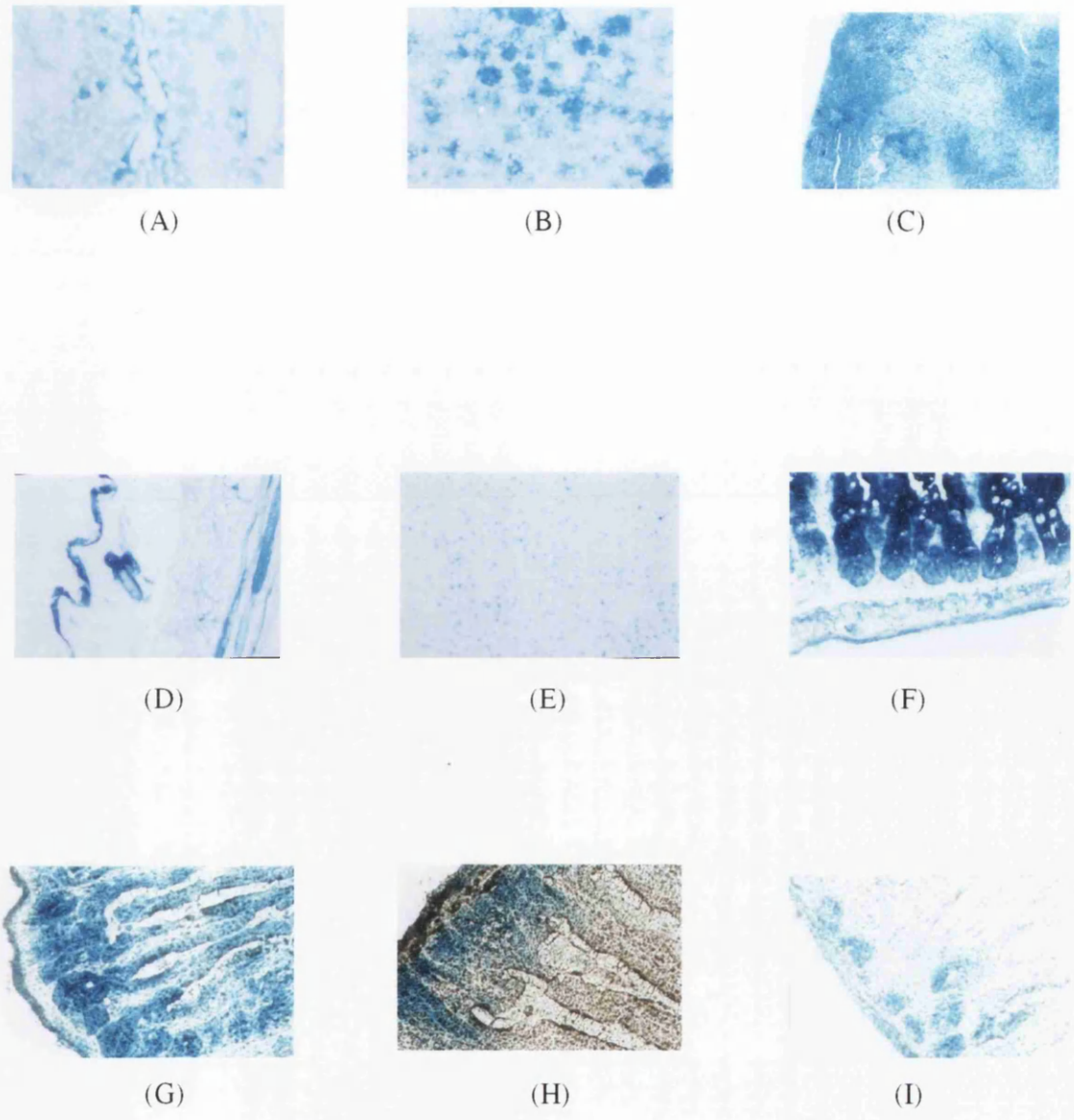


Figure 10.4 Expression of *LacZ* gene in different tissues of F1 backcrossing mice. (A) Liver, (B) Kidney, (C) Spleen, (D) Skin, (E) Brain, (F) Intestine, (G) Intestine, (H) Intestine and (I) Intestine. (A)-(F) obtained fromsame mice.

The expression of *LacZ* gene in F1 backcross strain varied in the tissues and mice possibly due to changing genetic background. In our studies, the genetic background of ROSA mice is C57BL/6J, whereas in p53 deficient mice it is 129/Sv. By crossbreeding two strains, F1 mice contain 50% C57BL/6J genetic background and 50% 129/Sv genetic background. By backcrossing to p53 deficient mice, the backcross strain contains 25% C57BL/6J genetic background and 75% 129/Sv genetic background. The promoter of *LacZ* may be influenced by the genetic background, which leads to different expression. We have concluded that the ROSA mouse system holds promise for the future studies, but future studies to control genetic background and homogeneity of *LacZ* expression in tissue will be necessary before system is usable for its intended purpose.

Chapter 11

Conclusions and Suggestions for Future Work

11.1 Multistage models of tumorigenesis

11.1.1 Spontaneous tumorigenesis

The modelling work described here began with multistage models which provide a convenient conceptual framework within which to view the process of tumorigenesis. The initial studies focused on exploration of the effect of changing number of stages, mutation rate (in its most general sense), and ultimate (i.e. steady state) stem cell number on the timing of appearance of tumours. As expected, tumours tended to occur earlier with lesser stage number, higher stem cell number and higher mutation rate (Figure 11.1). However, a striking observation was that each of these dependencies was more pronounced at lower than higher mutation rate. With increasing mutation rate, the rate of growth of the earliest transformed cell exerted an increasing influence on tumour latency independently of the other variables.

The multistage models have been used to predict tumour multiplicity. In general, the mean number of tumours per mouse and its variation increases with decrease in the number of stages, and with increase in the mutation rate, and with increase in the stem cell number (Figure 11.1). The time lag between the first-appearing tumour and consequent tumours increases with increase in stage number and with decrease in the mutation rate.

With competing tumour development in two tissues showing different growth patterns, these modelling studies have shown that tumours tend to occur earlier in the tissues which show a sudden burst of growth in early life. The effect of alternative growth patterns on tumour spectra is also dependent on stage number and the mutation rate. With fewer stages

and higher mutation rate, most of tumours will arise from the tissues which show a sudden burst of growth in early life. However, tumour spectra are also influenced by age. Tumours in the tissues which show a sudden burst of growth in early life are relatively over-presented amongst early-occurring tumours.

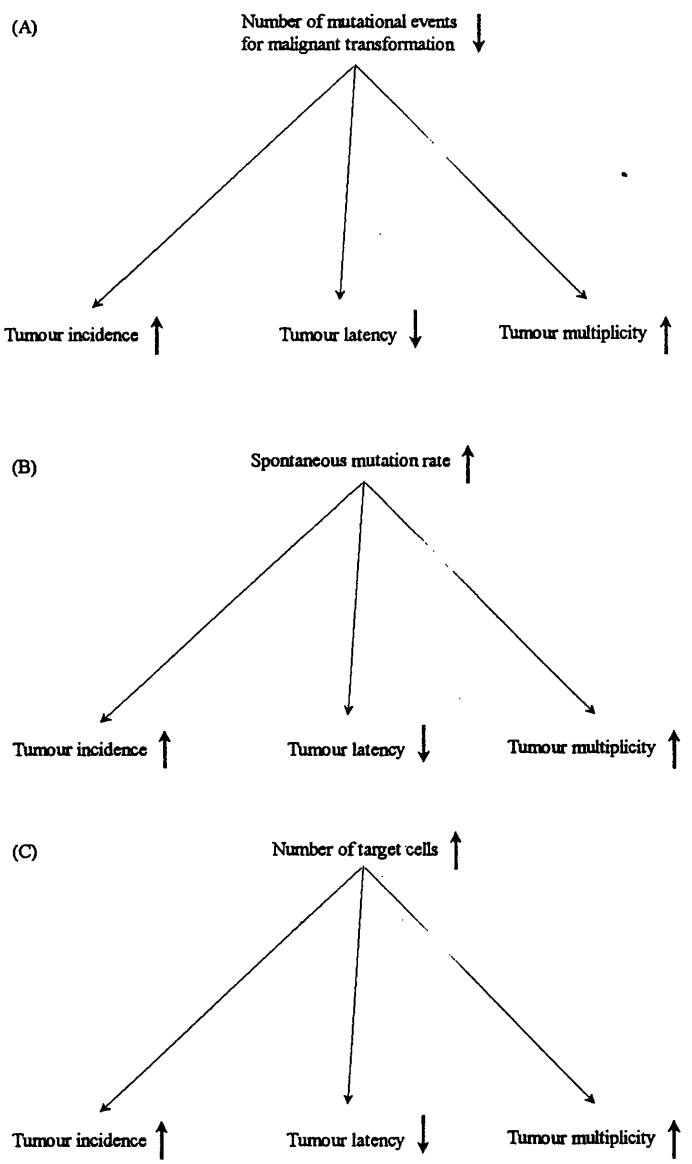


Figure 11.1 Influence of (A) the number of mutational events for malignant transformation, (B) the mutation rate, and (C) the number of target cells on tumour incidence, latency and multiplicity.

11.1.2 Radiation tumorigenesis

With the incorporation of radiation effects, i.e. cell killing and mutation induction, into the multistage model, the modelling studies have shown that a single dose of radiation usually (but not always) results in the earlier appearance of tumours (see Table 4.1 and Table 6.2) and increases in tumour number per mouse (Table 6.1). Dose-latency and dose-tumour-multiplicity relationships are determined by stage number, the spontaneous mutation rate, and age at exposure. Dose-latency findings may be grouped into three categories of relationship:

(1) The T_{50} (i.e. time until tumours are detected in 50% of the mice) decreases with increasing dose down to a minimum, with an increase following that minimum (most instances);

(2) T_{50} is flat with increasing dose;

(3) T_{50} increases with dose.

The dose-tumour-multiplicity relationship seems follows a constant pattern - the mean number of tumours per mouse increases with increasing dose up to maximum, followed by decrease. However, the shape of both the dose-latency and the dose-tumour relationship curves are determined by stage number, the spontaneous mutation rate at each stage, and age at exposure.

11.2 Multipath multistage models of tumorigenesis

The present studies have shown that tumour development from any route is dependent on the number of mutational events required for malignant transformation and mutation rate

in this route.

11.3 Multigate/multistage models of tumorigenesis

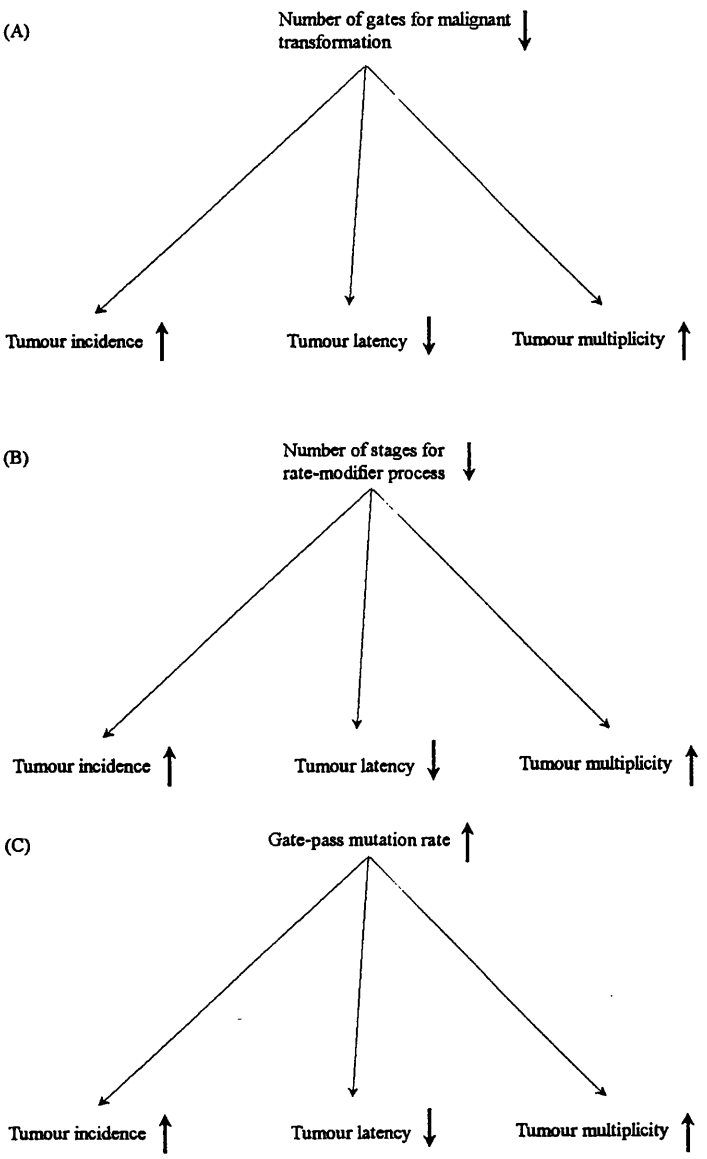
Much biological evidence exists indicating that tumours arise and progress through the accumulation of various genetic mutations. We have proposed that there are at least two types of mutations, i.e. directly tumorigenic mutations and indirectly tumorigenic mutations. We have also proposed a multigate/multistage model, which specifies that tumorigenesis requires a number of regulatory 'gates' to be passed, and a tumorigenic mutation of direct type alters a gateway gene and corresponds to a gate-pass event. However, indirect mutations are not enabling events in themselves but modifiers of the tumorigenic mutation rate. An important feature of these models is that mutation of the gateway genes alone (without modifier gene mutations) may lead to malignant transformation, whereas modifier mutations cannot achieve transformation without gate-pass events.

The present studies have shown that tumour incidence, latency and multiplicity are dependent on the number of gates required for malignant transformation and stages required for the rate-modifier process, the mutation rate of the gate-pass and modifier genes, and the rate-modifier factor (Figure 11.2).

The proportion of tumours arising by gate-pass mutations plus rate-modifier gene mutations increases with age, which implies that the tumours with modifier mutations will be relatively over-represented amongst late-occurring tumours. However, this relationship is

influenced by the number of gates and stages, the rate of the gate-pass and modifier mutations, and the rate-modifier factor.

It has been shown that tumours arising by gate-pass mutations only occur slightly earlier than those by the gate-pass plus modifier mutations, this difference is also dependent on the number of gates and stages, the rate of the gate-pass and modifier mutations, and the rate-modifier factor.



(Figure 11.2 to be continued)

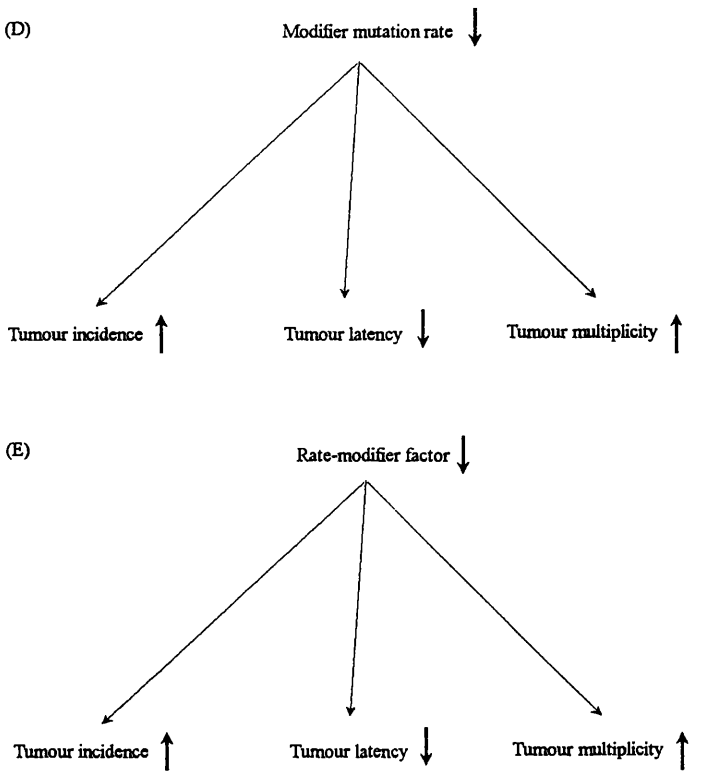


Figure 11.2 Influence of parameters in the multigate/multistage model on tumour incidence, latency and multiplicity.

11.4 Interpretation of the role of p53 in tumorigenesis

These modelling studies have demonstrated a fundamental problem in the application of the classic multistage model to spontaneous and radiation-induced tumorigenesis in p53 deficient mice. The single pathway multistage models in which inactivation of each p53 allele represents a distinct stage predict excessively large numbers of tumours in both p53 heterozygotes and p53 null homozygotes. The analysis shows that this prediction applies for up to five stages being required for tumorigenesis in wild type mice and allow this category of models to be rejected.

Our analysis has identified two categories of explanation for Vogelstein's paradox, i.e. the unexpectedly small number of tumours developing in p53 deficient mice and in Li-Fraumeni patients inheriting defective p53 alleles (Vogelstein, 1990). The first of these, the multistage multipath model invokes a p53 independent pathway which exists in parallel with a p53 mediated route of tumorigenesis. Only the latter route is enhanced in p53 deficient genotypes. On this type of model, the mutation rates are independently fixed and these inherent rates are not changed by p53 inactivation. We have found that a p53 mediated 5-stage pathway which provides 20% of the tumours in wild type mice is consistent with the data.

The second mechanism, which we have called the multigate model postulates a single pathway (or gateway) with several gate-pass events (obligatory mutations) occurring at a rate which depends on p53 status. We have observed that the data can be accommodated by a 2- or 3-gate model in which the gate-pass mutation rate is amplified by a factor of about 10 when both p53 alleles are inactivated. From simulations to date 2-gate model seems better than the 3-gate model, but further evidence is required.

11.5 Future studies

11.5.1 Multigate/multistage model

The bulk of the work to date has been on spontaneous tumorigenesis and has involved exploration of the effect of the various parameters in the multigate/multistage model on tumour incidence, latency and multiplicity. We will extend this analysis to radiation-

induced tumorigenesis in p53 deficient mice, for which some experimental data has already been reported (Kemp et al., 1994). We expect that stochastic modelling of tumorigenesis in p53 deficient mice will contribute to an understanding of tumorigenesis in human Li-Fraumeni patients as well as to the role of p53 in human cancer more generally.

11.5.2 Analysis of tumorigenic data in other transgenic mice

The approach taken in our studies should also prove useful for analysis of tumorigenesis in other transgenic mouse models, such as Rb, Ras etc transgenic mice, especially where, as in the MSH2 deficient (mismatch repair deficient) transgenic model (Reitmar et al, 1995) the genetic defect would be expected to act as a modifier of putative gate-pass events. It is expected that these studies should make a substantive contribution to the understanding of the role of cancer genes in tumorigenesis.

11.5.3 Analysis of human tumorigenic data

The stochastic models of tumorigenesis which are developed in the present studies can be applicable to study tumorigenesis in human, especially inherited human cancers, such as Li-Fraumeni patients. We will undertake this analysis using available Li-Fraumeni data in future.

11.5.4 Experimental studies

We have observed that variability of *LacZ* gene expression makes the ROSA/p53 deficient mice unsuitable for in vivo evaluation of the altered mutation rate hypothesised to be caused by p53 inactivation. It is necessary to find a new mutation detection system and

compare the mutation frequency in mice of different p53 background. Until then, the modelling studies and in vitro studies (with somewhat conflicting data) provide the only available of the effect of p53 inactivation on mutation rate.

11.6 Conclusions

In conclusion, these studies have shown the complex relationships which exist between mutation rate, tumorigenic stage number and tumour latency and multiplicity in spontaneous tumorigenesis and radiation-induced tumorigenesis. Dose-response relationships are not necessarily simple and experimental or epidemiological observations may need to be interpreted using appropriate models. We have shown that the classical multistage model cannot explain tumorigenesis data in p53 deficient mice and have proposed alternative models for this. The multigate/multistage model has been developed mathematically for the first time. Some predictions of the model (eg magnitude of the modifying factor, age-incidence of p53-mediated and non-p53-mediated tumours) should be experimentally testable. Some other predictions, on the relationship between the number of target cells and tumour multiplicity, on the effect of prenatal and post-natal irradiation on tumour latency, should also be experimentally testable. It is hoped that some of the methods developed here will be applicable to tumour development in other mouse models, and ultimately to human cancer.

References

Adams J. and Cory S. (1991) Transgenic models of tumor development. Science 254: 1161-1167.

Akslen L.A. and Morkve O. (1992) Expression of p53 protein in cutaneous melanoma. Int. J. Cancer 52:13-16.

Albanes D. and Winick M. (1988) Are cell number and cell proliferation risk factors for cancer? J. Natl Cancer Inst. 80: 772-775.

Armitage P. and Doll R. (1954) The age distribution of cancer and a multistage theory of cancer. Br. J. Cancer 8: 1-12.

Armitage P. and Doll R. (1957) A two-stage theory of carcinogenesis in relation to the age distribution of human cancer. Br. J. Cancer 11: 161-169.

Armitage P. and Doll R. (1961) Stochastic models for carcinogenesis. In 'Fourth Berkeley Symposium on Mathematical Statistics and Probability', University of California, Berkeley.

Baker S.J., Markowitz S., Fearon E.R., Willson J.K. and Vogelstein B. (1990) Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249: 912-915.

Barrett J.C. (1987A) Genetic and epigenetic mechanism in carcinogenesis. In: Mechanisms of Environmental Carcinogenesis. Vol 1. Role of Genetic and Epigenetic Changes (Barret J.C. Ed.), CRC Press, Boca Raton, FL, pp 1-15.

Barrett J.C. (1987B) A multistep model for neoplastic development: role of genetic and epigenetic changes. In: Mechanisms of Environmental Carcinogenesis. Vol 2. Multistep Models of Carcinogenesis (Barret J.C. Ed.), CRC Press, Boca Raton, FL, pp 117-126.

Barrett J.C. (1993) Mechanism of multistep carcinogenesis and carcinogen risk assessment. Environmental Health Perspectives 100: 9-20.

Barrett J.C., Crawford B.D., Mixter L.O., Schechtman T.L., Ts'o P.O.P. and Pollack R. (1979) Correlation of *in vitro* growth properties and tumorigenicity of Syrian hamster cell lines. Cancer Res. 39: 1504.

Barrett J.C. and Fletcher W.F. (1987) Cellular and molecular mechanisms of multistep carcinogenesis in cell culture models. In: Mechanisms of Environmental Carcinogenesis. Vol 2. Multistep Models of Carcinogenesis (Barret J.C. Ed.), CRC Press, Boca Raton, FL, pp 73-116.

Barrett J.C. and Ts'o P.O.P. (1978) Relationship between somatic mutation and neoplastic transformation. Proc. Natl. Acad. Sci. U.S.A. 75: 3297.

Benedict W.F., Murphree A.L., Banerjee A., Spina C.A., Sparkes M.C. and Sparkes R.S. (1983) Patient with 13 chromosome deletion: evidence that the retinoblastoma gene is a recessive cancer gene. Science 219: 973-975.

Benedict W.F., Rucher N., Marker C. and Kouri R.E. (1975) Correlation between balance of specific chromosomes and expression of malignancy in hamster cells. J. Natl. Cancer Inst. 54: 157.

Bennett W.P., Hollstein M.C., He A., Zhu S.M., Resau J.H., Trump B.F., Metcalf R.A., Welsh J.A., Midgley C., Lane D.P., and Harris C.C. (1991) Archival analysis of p53 genetic and protein alterations in Chinese esophageal cancer. Oncogene 6: 1779-1784.

Berenblum I. (1941) The cocarcinogenic action of croton resin. Cancer Res. 1: 44-48.

Berns A. (1994) Is p53 the only real tumor suppressor gene? Current Biology 4:137-139.

Beroud C., Verdier F. and Soussi T. (1996) p53 gene mutation - software and database. Nucleic Acids Research 24: 147-150.

Berward Y. and Sachs L. (1963) *In vitro* cell transformation with chemical carcinogens. Nature 200: 1182.

Berward Y. and Sachs L. (1965) *In vitro* cell transformation of normal cells to tumor cells by carnogenic hydrocarbon. J. Natl. Cancer Inst. 35: 641.

Bishop J.M. (1985) Viral oncogenes. Cell 42: 23-38.

Bishop J.M. (1987) The molecular genetics of cancer. Science 235: 305-311.

Bishop J.M. (1991) Molecular themes of oncogenesis. Cell 64: 235-248.

Bishop J.M. (1995) Cancer: the rise of the genetic paradigm. Gene and Development 9: 1309-1315.

Bodmer W.F. and Thomlinson I. (1996) Population genetics of tumours. CIBA Foundation Symposia 197: 181-189.

Borek C. and Sachs L. (1966) *In vitro* cell transformation by X-irradiation. Nature 210: 276.

Bos J.L. (1988) The ras family and human carcinogenesis. Mutat. Res. 195: 255-271.

Bos J.L. (1989) Ras oncogenes in human cancer: a review. Cancer Res. 49: 4682-4689.

Bouffler S.D., Kemp C.J., Balmain A. and Cox R. (1995) Spontaneous and ionizing radiation-induced chromosomal-abnormalities in p53-deficient mice. Cancer Research 55: 3883-3889.

Boutwell R.K. (1964) Some biological aspects of skin carcinogenesis. Prog. Exp. Tumor Res. 4: 207-250.

Brodeur G.M., Seeger R.C., Schwab M., Varmus H.E. and Bishop J.M. (1984) Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224: 1121-1124.

Brown C.C. and Chu K.C. (1982) Approaches to epdemiologic analysis of prospective and retrospective studies: example of lung cancer and exposure to arsenic. In: Environmental Epidemiology: risk assessment (Prentice R.L. and Whittemore A.S. ed). SIAM, Philadephia.

Buckley I. (1988) Oncogenes and the nature of malignancy. Adv. in Cancer Res. 50:71-93.

Buick R.N. and Tannock I.F. (1992) Properties of malignant cells. In The Basic Science of Oncology, I. F. Tannock and R. P. Hill, ed., McGraw-Hill, Inc., New York, p139.

Burns F.J., Vanderlaan M., Sivak A. and Albert R.E. (1976) Regression kinetics of mouse skin papilloma. Cancer Res. 36: 1422-1425.

Cairns J. (1975) Mutation selection and the natural history of cancer. Nature 255: 197-200.

Caron de Fromentel C. and Soussi T. (1992) TP53 tumour suppressor gene: a model for investigating human mutagenesis. Genes, Chromosomes and Cancer 4: 1-15.

Chang F., Syrjänen S., Tervahaula A. and Syrjänen K. (1993) Tumorigenesis associated with the p53 tumour suppressor gene. Br. J. Cancer 68: 653-661.

Chen P.L., Chen Y., Bookstein R. and Lee W.H. (1991) Genetic mechanisms of tumor suppression by the human p53 gene. Science 250: 1576-1580.

Chu K.C., Brown C.C., Tarone R. and Tan W.Y. (1987) Differentiating between proposed mechanisms for tumor promotion in mouse skin using the multi-event model for cancer. J. Natl. Cancer Inst. 79: 789-796.

Clarke A.R., Purdie C.A., Harisson D.J., Morris R.G., Bird C.C., Hooper M.L. and Wyllie A.H. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 362: 849-852.

Cohen S.M. and Ellwein L.B. (1991) Cell proliferation in carcinogenesis. Science 249: 1007-1011.

Cohen S.M. and Ellwein L.B. (1991) Genetic errors, cell proliferation, and carcinogenesis. Cancer Res. 51: 6493-6505.

Cook P.J., Doll R. and Fellingham S.A. (1969) A mathematical model for the age distribution of cancer in man. Int. J. Cancer 4: 93-112.

Cooper G.M. (1982) Cellular transforming genes. Science 217: 801-806.

Cooper G.M. (1990) Oncogenes. Jones and Bartlett, Boston.

Cowell J.K. (1990) The nuclear oncoprotein: RB and p53. Semin. Cancer Biol. 1: 437-446.

Cox R. (1994) Molecular mechanisms of radiation oncogenesis. Int. J. Radiat. Biol. 65: 57-64.

Cross S.M., Sanchez C.A., Morgan C.A., Schimke M.K., Ramel S., Idzerda R.L., Raskind W.H. and Reid B.J. (1995) A p53-dependent mouse spindle checkpoint. Science 267: 1353-1356.

Crump K.S., Guess H.A. and Deal K.L. (1977) Confidence intervals and test of hypotheses concerning dose response relations inferred from animal carcinogenicity data. Biometrics 33: 437-451.

Crump K.S and Howe R.B. (1984) The multistage model with a time-dependent dose pattern: applications to carcinogenic risk assessment. Risk Anal. 4: 163-176.

Curtis S.B. (1986) Lethal and potentially lethal lesions induced by radiation - a unified repair model. Radiation Res. 106: 252-270.

Czosnek H.H., Bienz B., Givo D., Zakut-Houri R., Pravtcheva D.D., Ruddle F.H. and Oren M. (1984) The gene and the pseudogene of mouse p53 cellular tumor antigen are located on different chromosomes. Mol. Cell Bio. 4: 1638-1640.

Dalla-Favera R.S., Martinotti S., Gallo R.C., Erikson J. and Croce C.M. (1983) Translocation and rearragement of the c-myc oncogene locus in human undifferentiated B-cell lymphomas. Science 219: 963-997.

Day N.E. and Brown C.C. (1980) Multistage models and primary prevention of cancer. J. Natl. Cancer Inst. 64: 977-989.

Devroye L. (1986) Non-uniform random variate generation. Springer Verlag, New York.

Dewanji A., Moolgavkar S.H. and Luebeck E. (1991) Two-mutation model for carcinogenesis: joint analysis of premalignant and malignant lesions. Math. Biosciences 104: 97-109.

Dewanji A., Venzon D.J. and Moolgavkar S.H. (1989) A stochastic two-stage model for cancer risk assessment. II. the number and size of premalignant clones. Risk Analysis 9: 179-187.

Dietrich W.F., Lander E.S., Smith J.S., Moser A.R., Gould K.A., Luongo C., Borenstein N., and Dove W. (1993) Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse. Cell 75: 631-639.

Diller L., Kassel J., Nelson C.E., Gryka M.A., Litwak G., Gebhardt M., Bressac B., Ozturk M., Baker S.J., Vogelstein B. and Friend S.H. (1990) p53 function as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol. 10: 5772-5781.

DiPaolo J.A. and Donovan P.J. (1967) Properties of Syrian hamster cells transformed in the presence of carcinogenic hydrocarbons. Exp. Cell Res. 48: 1361.

Donehower L.A. (1994) Tumor suppressor gene p53 and apoptosis. Cancer Bulletin 46: 161-166.

Donehower L.A. and Bradley A. (1993) The tumor suppressor p53. Biochim. Biophys. Acta 1155: 181-205.

Donehower L.A., Harvey M., Slagle B.L., McArthur M.J., Montgomery C.A., Butel J.S. and Bradley A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 356: 215-221.

Donehower L.A., Harvey M., Vogel H., McArthur M.J., Montgomery C.A., Park S.H., Thompson T., Ford R.J. and Bradley A. (1995) Effects of genetic background on tumorigenesis in p53-deficient mice. Molecular Carcinogenesis 14: 16-22.

Dongi R., Longoni A., Michieli P., Della Porta G. and Pierotti M.A. (1992) Analysis of p53 mutations in human thyroid carcinoma. In 'Mutant Oncogenes' edited by Lemoine, N. and Epenetos, A., Chapman and Hall: London, pp187-192.

Dowell S.P. and Hall P.A. (1994) The clinical relevance of the p53 tumour suppressor gene. Cytopathol. 5: 133-145.

Druker B.J., Mamon H.J. and Roberts T.M. (1989) Oncogenes, growth factors, and signal transduction. New England J. Med. 321: 1383-1391.

Eliyahu D., Michalovitz D., Eliyahu S., Pinhasi-Kimhi O. and Oren M. (1989) Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Natl Acad. Sci. USA 86: 8763-8767.

Elkind M.M (1984) Repair processes in radiation biology. Radiation Research 100: 425-449.

Enomoto T., Fujita M., Inoue M., Rice J.M., Nakajima R., Tanizawa O. and Nomura T. (1993) Alterations of the p53 tumor suppressor gene and its association with activation of the c-K-ras-2 protooncogene in premalignant and malignant lesions of the human uterine endometrium. Cancer Res. 53: 1883-1888.

Evans C.H. and DiPaolo J.A. (1975) Neoplastic transformation of guinea pig fetal cells in culture induced by chemical carcinogens. Cancer Res. 35: 1035.

Evans C.H. and DiPaolo J.A. (1982) Independent expression of chemical carcinogen-induced phenotypic properties frequently associated with the neoplastic state in a cultured guinea pig cell line. J. Natl. Cancer Inst. 69: 1175.

Farber E. (1984) The multistep nature of cancer development. Cancer Research 44: 4217-4223.

Farber E. (1987) Experimental induction of hepatocellular carcinoma as a paradigm for carcinogenesis. Clin. Physiol. Biochem. 5: 152-159.

Farber E. and Cameron R. (1980) The sequential analysis of cancer development. Advances in Cancer Research 31: 125-226.

Farber E. and Sarma D.S.R. (1987) Hepatocarcinogenesis: A dynamic cellular perspective. Laboratory Investigation 56: 4-22.

Farber E. Rotstein J.B. and Eriksson L.C. (1987) Cancer development as a multi-stage process'. In: Mechanisms of Environmental Carcinogenesis. Vol 2. Multistep Models of Carcinogenesis (Barret J.C. Ed.), CRC Press, Boca Raton, FL, pp 1-19.

Farmer G., Bargonetti J., Zhu H., Friedman P., Prywes R. and Prive C. (1992) Wild-type p53 activates transcription in vitro. Nature 358: 83-86.

Fearon E.R. and Vogelstein B. (1990) A genetic model for colorectal tumorigenesis. Cell 61: 759-767.

Finlay C.A., Hinds P.W. and Levine A.J. (1989) The p53 proto-oncogene can act as a suppressor of transformation. Cell 57: 1083-1093.

Fisher J.C. and Holloman J.H. (1951) A hypothesis for the origin of cancer foci. Cancer 4: 916-918.

Fowlis D.J. and Balmain A. (1993) Oncogenes and tumour suppressor genes in transgenic mouse models of neoplasia. Eur. J. Cancer 29A: 638-645.

Friedewald W.F. and Rous P. (1944) The initiating and promoting elements in tumor production. J. Exp. Med. 80:101-125.

Friedman P., Kern S., Vogelstein B. and Prives C. (1990) Wild-type, but not mutant human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. USA 87: 9275-9279.

Friedrich G. and Soriano P. (1991) Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes & Development 5: 1513-1523.

Fry R.J.M. (1981) Experimental radiation carcinogenesis: What have we learned? Radiation Research 87: 224-239.

Fry R.J.M. (1991) Radiation carcinogenesis in the whole-body system. Radiation Research 126: 157-161.

Fry R.J.M. and Storer J.B. (1987) External radiation carcinogenesis. Advances in Radiation Biology 13: 31-90.

Fukushima S., Imaida K., Sakata T., Okamura T., Shibata M. and Ito N. (1983) Promoting effects of sodium L-ascorbate of 2-stage urinary-bladder carcinogenesis in rats. Cancer Res. 43: 4454-4457.

Game J.C. (1993) DNA double-strand breaks and the RAD50-RAD57 gene in *Saccharomyces*. Seminars in Cancer Biology 4: 73-83.

Gonzalez F.J. (1996) Use of transgenic animals in carcinogenesis studies. Molecular Carcinogenesis 16: 63-67.

Goodhead D.T. (1994) Initial events in the cellular effects of ionizing radiations: clustered damage in DNA. Int. J. Radiat. Biol. 65: 7-17.

Gossler A. and Zachgo J. (1993) Gene and enhancer trap screens in ES cell chimeras. In: Gene Targeting: A Practical Approach (Joyner A.L. ed), Oxford University Press, Oxford, pp 181-214.

Greenblatt M.S., Bennett W.P., Hollstein M. and Harris C.C. (1994) Mutations in the p53 tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. Cancer Res.

54: 4855-4878.

Greenfield R.E., Ellwein L.B. and Cohen S.M. (1984) A general probabilistic model of carcinogenesis: analysis of experimental urinary bladder cancer. Carcinogenesis 5: 437-445.

Guerrero J., Villasonte A., Corces V. and Pellicer a. (1984) Activation of a c-K-*ras* oncogene by somatic mutation in mouse lymphomas induced by gamma radiation. Science 225: 1159-1162.

Gusterson B.A., Anbazhagan R., Warren W., Midgely C., Lane D.P., O'Hare M., Stamps A., Carter R. and Jayatilake H. (1991) Expression of p53 in premalignant and malignant squamous epithelium. Oncogene 6: 1785-1789.

Hall E.J. (1993) Review article: The gene as theme in the paradigm of cancer. Br. J. Radiol. 66: 1-11.

Hall E.J. (1994) Radiobiology for the radiologist, 4th edition. J.B. Lippincott Company: Philadelphia.

Harris C.C. (1996) p53 tumor suppressor gene: from the basic research laboratory to the clinic - an abridged historical perspective. Carcinogenesis 17:1187-1198.

Harris C.C. (1996) The 1995 Walter Hubert Lecture - molecular epidemiology of human cancer: insights from the mutational analysis of the p53 tumour-suppressor gene. Br. J. Cancer 73: 261-269.

Harris C.C. and Hollstein M. (1993) Clinical implications of the p53 tumor-suppressor gene. New England J. Med. 329: 1318-1327.

Harris H. (1971) Cell fusion and the analysis of malignancy. Proc. R. Soc. Lond. (Biol.) 179: 1-20.

Harris H. (1986) The genetic analysis of malignancy. Cell Science Suppl. 4: 431-444.

Hartwell L. and Weinert T. (1991) Genetic control mitotic fidelity in yeast and its relation to cancer, in Origins of Human Cancer: A Comprehensive Review (Brugge, J., Curran, T., Harlow, E., McCormick, F. eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 45-49.

Hartwell L. (1992) Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer. Cell 71: 543-546.

Havre P.A., Yuan J.L., Hedrick L., Cho K.R. and Glazer P.M. (1995) p53 inactivation by HPV16 E6 results in increased mutagenesis in human cells. Cancer Res. 55: 4420- 4424.

Havery M., McArthur M.J., Montgomery C.A., Bradley A. and Donehower L.A. (1993A) Genetic background alters the spectrum of tumors that develop in p53-deficient mice. FASEB J. 7: 938-943.

Harvey M., McArthur M.J., Montgomery C.A., Butel J.S., Bradley A. and Donehower L.A. (1993B) Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nature

Genet. 5: 225-229.

Harvey M., Sands A.T., Weiss R.S., Hegi M.E., Wiseman R.W., Pantazis P., Giovanella B.C., Tainsky M.A., Bradley A. and Donehower L.A. (1993C) *In vitro* growth characteristics of embryo fibroblasts isolated from p53-deficient mice. Oncogene 8: 2457-2467.

Henning H., Shores R., Wenk M.L., Spangler E.F., Tarone R. and Yuspa S.H. (1983) Malignant conversion of mouse skin tumors is increased by tumor initiators and unaffected by tumor promoters. Nature 304: 67-69.

Hesketh R. (1995) The oncogene factsbook. Academic Press: New York.

Hesketh R. (1994) The oncogene handbook. Academic Press: New York.

Hethcote H.W. and Knudson A.G. (1978) Model for the incidence of embryonal cancers: application to retinoblastoma. Proc Natl. Acad. Sci. USA 75: 2453-2457.

Hinds P.W. and Weinberg R.A. (1994) Tumor suppressor genes. Current Opinion in Genetics and Development 4: 135-141.

Holliday R. (1989) Chromosome error propagation and cancer. Trends Genet 5: 42-45.

Hollstein M., Shomer B., Greenblatt M., Soussi T., Hovig E., Montesano R. and Harris C.C. (1996) Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. Nucleic Acids Res. 24: 141-146.

Hollstein M., Sidransky D., Vogelstein B. and Harris C.C. (1991) p53 mutations in human cancers. Science 253: 49-53.

Horak E., Smith K., Bromley L., LeJeune S., Greenhall M., Lane D. and Harris A.L. (1991) Mutant p53, EGF receptor and c-erbB-2 expression in human breast cancer. Oncogene, 6: 2277-2284.

Hosono S., Chou M.J., Lee C.S. and Shih C. (1993) Infrequent mutation of p53 in hepatitis B virus positive primary hepatocellular carcinoma. Oncogene 8: 491-496.

Hu G.Y., Zhang W. and Deisseroth A.B. (1992) p53 gene-mutations in acute myelogenous leukemia. British J. of Haematology 81: 489-494.

Huberman E. and Sarchs L. (1966) Cell susceptibility to transformation and cytotoxicity by the carcinogenic hydrocarbon benzo(a)pyrene. Proc. Natl. Acad. Sci. U.S.A. 56: 1123.

Hupp T.R., Meek D.W., Midgley C.A. and Lane D.P. (1992) Regulation of the specific DNA binding function of p53. Cell 71: 875-886.

Hursting S.D., Perkins S.N. and Phang J.M. (1994) Calorie restriction delays spontaneous tumorigenesis in p53-knockout transgenic mice. Proc. Natl Acad. Sci. USA 91: 7036-7040.

Hutchinson F. (1985) Chemical changes induced in DNA by ionizing radiation. Prog. Nucl. Acid Res. Mol. Biol. 32: 115-154.

Hutchinson F. (1993) Molecular biology of mutagenesis of mammalian cells by ionizing radiation. Seminars in Cancer Biology 4: 85-92.

Iggo R., Gatter K., Bartek J., Lane D. and Harris A.L. (1990) Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet 335: 675-679.

Isobe M., Emanuel B.S., Givol D., Oren M. and Croce C.M. (1986) Localization of gene for human p53 tumor antigen to band 17p13. Nature 320: 84-85.

Jacks T. (1996) Lessons from the p53 mutant mouse. J. Cancer Res. Clin. Oncol. 122: 319-327.

Jacks T., Remington L., Williams B.O., Schmitt E.M., Halachmi S., Bronson R.T. and Weinberg R.A. (1994) Tumour spectrum analysis in p53-mutant mice. Current Biology 4: 1-7.

Jenkins J.R., Rudge K. and Currie G.A. (1984) Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature 312: 651-654.

Kalthoff H., Schmiegel W., Roeder C., et al (1993) p53 and K-ras alterations in pancreatic epithelial cell lesions. Oncogene 8:289-298.

Kastan M.B., Canman C.E. and Leonard C.J. (1995) p53, cell cycle control and apoptosis: implication for cancer. Cancer and Metastasis Reviews 14: 3-15.

Kastain M.B., Zhan Q.M., Eldeiry W.S., Carrier F., Jacks T., Walsh W.V., Plunkett B.S., Vogelstein B. and Fornace A.J. (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in Ataxia-Telangiectasia. Cell 71: 587-597.

Kemp C.J., Donehower L.A., Bradley A. and Balmain A. (1993) Reduction of p53 gene dosage does not increase initiationor promotion but enhances malignant progression of chemically induced skin tumors. Cell 74: 813-822.

Kemp C.J., Wheldon T. and Balmain A. (1994) p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis. Nature Genetics 8: 66-69.

Kern S.E., Pietenpol J.A., Thiagalingam S., Seymour A., Kinzler K.W. and Vogelstein B. (1992) Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 256: 827-830.

Kiaris H. and Spandidos D.A. (1995) Mutation of *ras* genes in human tumours (Review). Int. J. of Oncol. 7: 413-421.

Knudson A.G. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sciences USA 68: 820-823.

Knudson A.G. (1973) Mutation and human cancer. Adv. Cancer Res. 17: 317-352.

Knudson A.G. (1983) Hereditary cancer of man. Cancer Investigation 1: 187-193.

Knudson A.G. (1985) Hereditary cancer, oncogenes and antioncogenes. Cancer Res. 45:

1437-1443.

Knudson A.G. (1986) Genetics of human cancer. Annual Review of Genetics 20: 231-251.

Knudson A.G. (1988) The genetics of childhood-cancer. Bulletin Du Cancer 75: 135-138.

Knudson A.G. (1989A) Hereditary cancers: clues to mechanism of carcinogenesis. Br. J. Cancer 59: 661-666.

Knudson A.G. (1989B) Hereditary cancers: clues to mechanism of carcinogenesis. Br. J. Cancer 59: 817-818.

Knudson A.G. (1993) Antioncogenes and human cancer. Proc. Natl. Acad. Sci. U.S.A. 90: 10914-10921.

Knudson A.G. (1995) Foundations in cancer research - mutation and cancer - a personal odyssey. Advances in Cancer Research 67: 1-23.

Knudson A.G. (1996) Hereditary cancer - 2 hits revisited. J. Cancer Res. Clin. Oncol. 122: 135-140.

Knudson A.G., Hethcote H.W. and Brown B.W. (1975) Mutation and childhood cancer: a probabilistic model for the incidence of retinoblastoma. Proc. Natl. Acad. Sci. USA 72: 5116-5120.

Ko L.J. and Prives C. (1996) p53 - puzzle and paradigm. Gene and Development 10: 1054-1072.

Kobayashi I. and Takahashi N. (1988) Double-stranded gap repair of DNA by gene conversion in *Escherichia coli*. Genetics 119: 751-757.

Kohler S.W., Provost G.S., Fieck A., Kretz P.L., Bullock B., Sorge J.A., Putman D. and Short J.M. (1991) Spectra of spontaneous and induced mutations using a lambda ZAP^R *lacI* shuttle vector in transgenic mice. Proc. Natl. Acad. Sci. U.S.A. 88: 7958.

Krasin F. and Hutchinson F. (1977) Repair of DNA double-strand breaks in *Escherichia coli*, where requires recA function and the presence of a duplicate genome. J. Mol. Bio. 116: 81-98.

Krewski D., Crump K.S., Farmer J., Gaylor D.W., Howe R.,Portier C., Salsburg D., Sielken R.L. and Van Ryzin J. (1983) A comparison of statistical methods for low dose extrapolation ultilizing time-to-tumor data. Fund. Appl. Toxicol. 3: 140-160.

Kuroki T. and Sato H. (1968) Transformation and neoplastic development *in vitro* of hamster embryonic cells by 4-nitroquinoline-1-oxide and its derivatives. J. Natl. Cancer Inst. 41: 53.

Laird P.W., Zijderveld A., Linders K., Rudnicki M.A., Jaenisch R. and Berns A. (1991) Simplified mammalian DNA isolation procedure. Nucleic Acids Research 19: 15.

Lane D.P. (1992) p53, guardian of the genome. Nature 358:15-16.

Lane D.P. (1994A) p53 and human cancers. Brit. Med. Bull. 50: 582-599.

Lane D.P. (1994B) The regulation of p53 function - Steiner-Award Lecture. Int. J. Cancer 57: 623-627.

Lane D.P. and Benchimol S. (1990) p53: oncogene or anti-oncogene? Gene and Development 4: 1-8.

Lane D.P. and Crawford L.V. (1979) T antigen is bound to a host protein in SV40-transformed cells. Nature 278: 261-263.

Lee J.M., Abrahamson J.L.A., Bernstein A. (1994) DNA damage, oncogenesis and the p53 tumour-suppressor gene. Mutation Res. 307: 573-581.

Lee J.M., Abrahamson J.L.A., Kandel R., Donehower L.A. and Bernstein A. (1994) Susceptibility to radiation-carcinogenesis and accumulation of chromosomal breakage in p53 deficient mice. Oncogene 9: 3731-3736.

Lee E.Y.H.P., Chang C.Y., Hu N., Wang Y.C.J., Lai C.C., Herrup K., Lee W.H. and Bradley A. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 359: 295-300.

Leenhouts H.P. and Chadwick K.H. (1989) Recessive malignant genes, radiation induced cell transformation and animal carcinogenesis. In: Cell Transformation and Radiation-Induced cancer (Chadwick K.H., Seymour C. and Barnhart B., ed). Adam Hiler, Bristol and New York, pp 379-385.

Lehman T.A., Bennett W.P., Metcalf R.A., Reddel R., Welsh J.A., Ecker J., Modali R.V., Ullrich S., Romano J.W., Appella E., Testa J.R., Gerwin B.I. and Harris C.C. (1991) p53 mutations, ras mutations and p53-heat shock 70 protein complexes in human lung cell lines. Cancer Res. 51: 4090-4096.

Levine A.J. (1990) Tumor suppressor genes. BioEssays 12: 60-66.

Levine A.J. (1992) The p53 tumour suppressor gene and product. Cancer Surveys 12: 59-79.

Levine A.J. (1993) The tumor suppressor genes. Annu. Rev. Biochem. 62: 623-651.

Levine A.J., Momand J. and Finlay C.A. (1991) The p53 tumour suppressor gene. Nature 351: 453-456.

Levine A.J., Perry M.E., Chang A., Silver A., Dittmer D., Wu M. and Welsh D. (1994) The 1993 Walter Hubert Lecture: The role of the p53 tumour-suppressor gene in tumorigenesis. Br. J. Cancer 69: 409-416.

Linzer D.I. and Levine A.J. (1979) Characterization of a 54 K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell 17: 43-52.

Livingstone L.R., White A., Sprouse J., Livanos E., Jacks T. and Tlsty T. (1992) Altered

cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell 70: 923-935.

Little M.P. (1995) Are two mutations sufficient to cause cancer? Some generalization of the two-mutation model of carcinogenesis of Moolgavkar, Venzon, and Knudson, and of the multistage model of Armitage and Doll. Biometrics 51: 1278-1291.

Little M.P. (1996) Generalisations of the two-mutation and classical multi-stage models of carcinogenesis fitted to the Japanese atomic bomb survivor data. J. Radiol. Prot. 16: 7-24.

Little M.P., Hawkins M.M., Charles M.W. and Hildreth N.G. (1992) Fitting the Armitage-Doll model to radiation-exposed cohorts and implications for population cancer risks. Radiat. Res. 132: 207-221.

Little M.P., Muirhead C.R., Boice J.D. and Kleinerman R.A. (1995) Using multistage models to describe radiation-induced leukaemia. J. Radiol. Prot. 15: 315-334.

Little M.P., Muirhead C.R. and Stiller C.A. (1996) Modelling lymphocytic leukaemia incidence in England and Wales using generalizations of the two-mutation model of carcinogenesis of Moolgavkar, Venzon and Knudson. Statistics in Medicine 15: 1003-1022.

Loeb L.A. (1991) Mutator phenotype may be required for multistage carcinogenesis. Cancer Research 51: 3075-3079.

Lowe S.W., Schmitt E.M., Smith S.W., Osborne B.A. and Jacks T. (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362: 847-849.

Luebeck E.G., Curtis S.B., Cross F.T. and Moolgavkar S.H. (1996) Two-stage model of radon-induced malignant lung tumors in rats: effect of cell killing. Radiation Res. 145: 163-173.

Luebeck E.G. and Moolgavkar S.H. (1991) Stochastic analysis of intermediate lesions in carcinogenesis experiments. Risk Analysis 11: 149-157.

Maestro R., Dolcetti R., Gasparotto D., Doglioni C., Pelucchi S., Barzan L., Gradi E. and Boiocchi M. (1992) High frequency of p53 alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. Oncogene 7: 1159-1166.

Maisel H. and Gnugnoli G. (1972) Simulation of discrete stochastic systems. Science Research Associate, Int..

Malkin D. (1994A) Germline p53 mutations and heritable cancer. Annu. Rev. Genet. 28: 443-465.

Malkin D. (1994B) p53 and the Li-Fraumeni syndrome. Biochimica et Biophysica Acta 1198: 197-213.

Malkin D., Li F.P., Strong L.C., Fraumeni J.F., Nelson C.E., Kim D.H., Kassel J., Gryka M.A., Bischoff F.Z., Tainsky M.A. and Friend S.H. (1990) Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 250: 1233-1238.

Martin H.M., Filipe M.I., Morris R.W., Lane D.P. and Silvestre F. (1992) p53 expression and prognosis in gastric carcinoma. Int. J. Cancer 50: 859-862.

Mashiyama S., Murakami Y., Yoshimoto T. and Hayashi K. (1991) Detection of p53 gene mutation in human brain tumors by single-strand conformation polymorphism analysis of polymerase chain reaction products. Oncogene 6: 1313-1318.

Mercer W.E., Shields M.T., Amin M., Sauve G.J., Apella E., Romano J.W. and Ullrich S.J. (1990) Negative growth requlation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl Acad. Sci. USA 87: 6166-6170.

Merregaert J., Michiels L., Vanderrauwelaert E., Lommel M., Golwinkler R. and Janowski M. (1986) Oncogene involvement in radiation-induced and virus-induced mouse osteosarcomas. Leukemia Res. 10: 915-921.

Midgley C.A., Owens B., Briscoe C.V., Thomas D.B., Lane D.P. and Hall P.A. (1995) Coupling between gamma-irradiation, p53 induction and the apoptotic response depends upon cell-type in-vivo. J. Cell Science 108: 1843-1848.

Miller C., Mohandas T., Wolf D., Prokocimer M., Rotter V. and Koeffler H.P. (1986) Human p53 gene localiztied to short arm of chromosome 17. Nature 319: 783-784.

Miller W.H. and Dmitrovsky E. (1991) Oncogenes and clinical oncology. Current Opinion in Oncology 3: 65-69.

Milner B.J., Allan L.A., Eccles D.M., Kitchener H.C., Leonard R.C.F., Kelly K.F., Parkin D.E. and Haites N.E. (1993) p53 mutation is a common genetic event in ovarian carcinoma. Cancer Res. 53: 2128-2132.

Mitsudomi T., Steinberg S.M., Nau M.M. et al. (1992) p53 gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of ras mutations and clinical features. Oncogene 7: 171-180.

Moles J.P., Moyret C., Guilot B., Jeanteur P., Guilhou J.J., Theilet C. and Basset-Seguin N. (1993) p53 gene mutations in human epithelial skin cancers. Oncogene 8: 583-588.

Moolgavkar S.H. (1983) Model for human carcinogenesis: action of environmental agents. Environ. Health Perspect. 50: 285-291.

Moolgavkar S.H. (1986A) Carcinogenesis modeling: from molecular biology to epidemiology. Ann. Rev. Public Health 7: 151-169.

Moolgavkar S.H. (1986B) Hormones and multistage carcinogenesis. Cancer Survey 3: 183-214.

Moolgavkar S.H. (1990) Scientific issues in quantitative cancer risk assessment. Birkhäuser: Boston.

Moolgavkar S.H., Cross F.T., Luebeck G. and Dagle G.E. (1990A) A two-mutation model for radon-induced lung tumors in rats. Radiat. Res. 121: 28-37.

Moolgavkar S.H., Dewanji A. and Venzon (1988) A stochastic two-stage model for cancer risk assessment. I. The hazard function and the probability of tumor. Risk Anal. 8: 383-392.

Moolgavkar S.H. and Knudson A. (1981) Mutation and cancer: a model for human carcinogenesis. J. Natl. Cancer Inst. 66: 1037-1052.

Moolgavkar S.H. and Luebeck E.G. (1990) Two-event model for carcinogenesis: biological, mathematical, and statistical considerations. Risk Anal. 10: 323-341.

Moolgavkar S.H. and Luebeck E.G. (1992) Multistage carcinogenesis: population-based model for colon cancer. J. Natl. Cancer Inst. 66:1037-1052.

Moolgavkar S.H., Luebeck E.G., De Gunst M., Port R.E. and Schwarz M. (1990B) Quantitative analysis of enxyme-altered foci in rat hepatocarcinogenesis experiments - I. single agent regimen. Carcinogenesis 11: 1271-1278.

Moolgavkar S.H. and Venzon, D. (1979) Two-event models for carcinogenesis: incidence curves for childhood and adult tumours. Math. Biosc. 47: 55-77.

Mowat M.A., Cheng A., Kimura N., Bernstein A. and Benchimol S. (1985) Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus. Nature 313: 633-636.

Mulligan L.M., Matlashewski G.J., Scrable H.J. and Cavenee W.K. (1990) Mechanisms of p53 loss in human sarcomas. Proc. Natl Acad. Sci. USA 87: 5863-5867.

Murdoch D.J. and Krewski D. (1988) Carcinogenic risk assessment with time-dependent exposure pattern. Risk Anal. 8: 521-530.

Myhr B.C. (1991) Validation studies with Muta™ mouse: a transgenic mouse model for detecting mutations *in vivo*. Eviron. Mol. Mutage. 18: 308.

Nettesheim P., Fitzgerald D.J., Kitamura K., Walker C.L., Gilmer T.M., Barrett J.C. and Gray T.E. (1987) In vitro analysis of multistage carcinogenesis. Environ. Health Perspect. 75: 71-79.

Newbold R.F., Overell R.W., and Connell J.R. (1982) Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens. Nature 299: 633.

Nikolaidou A., Liloglou T., Malliri A., Ergazaki M., Tiniakos D. and Spandidos D.A. (1993) Detection of hepatitis B virus DNA and mutations in K-ras and p53 genes in human hepatocellular carcinomas. Int. J. Oncol. 3: 593-601.

Nishino H., Knoll A., Buettner V.L., Frisk C.S., Maruta Y., Haavik J. and Sommer S.S. (1995) p53 wild-type and p53 nullizygous Big Blue transgenic mice have similar frequencies and patterns of observed mutation in liver, spleen and brain. Oncogene 11: 263-270.

Nording C.O. (1953) A new theory on the cancer inducing mechanism. Br. J. Cancer 7: 68-72.

Nowell P.C. (1976) The clonal evolation of tumor cell populations. Science 194: 23-28.

Oren M. (1994) Relationship of p53 to the control of apoptotic cell death. Semimars in Cancer Biology 5: 221-227.

Park D.J., Nakamura H., Chumakov A.M., Said J.W., Miller C.W., Chen D.L. and Koeffler H.P. (1994) Transactivational and DNA binding abilities of endogenous p53 in p53 mutant cell lines. Oncogene 9: 1899-1906.

Peraino C., Fry R.J.M, and Staffeldt E. (1971) Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene. Cancer Res. 31: 1506.

Peto R. (1977) Epidemiology, multistage models, and short-term mutagenecity tests. In: Origins of Human Cancer, Book C: Human Risk Assessment (Hiatt H.H., Watson J.A. and Winsten J.A. ed.). Cold Spring Harbor, NY: Cold Spring Harbor Lab, pp1403-1428.

Perry M.E. and Levine A.J. (1993) Tumor-suppressor p53 and the cell cycle. Current Opinion in Genetics and Development 3: 50-54.

Phillips E.N., Xia F., Kelsey K.T. and Liber H.L. (1995) Spectra of spontaneous and X-ray-induced mutations at the HPRT locus in related human lymphoblast cell lines that express wild-type or mutant p53. Radiation Res. 143: 255-262.

Pierotti M.A. and Dragani T.A. (1992) Genetics and cancer. Current Opinion in Oncology 4: 127-133.

Pietenpol J.M., Tokino T., Thiagalingam S., El-Deiry W.S., Kinzler K.W. and Vogelstein B.S. (1994) Sequence-specific transcriptional activation is essential for growth suppression by p53. Proc. Natl. Acad. Sci. USA 91: 1998-2002.

Ponder B.A.J. (1990) Inherited predisposition to cancer. Trends in Genetics 6: 213-218.

Portier C.J. (1987) Statistical properties of a two-stage model of carcinogenesis. Environ. Health Perspect. 76: 125-131.

Portier C.P., Kopp-Schneider A. and Sherman C.D. (1993) Using cell replication data in mathematical modelling in carcinogenesis. Environ. Health Perspect. 101 (Supp 5): 79-86.

Portier M., Moles J.P., Mazars G.R., Jeanteur P., Bataille R., Klein B. and Theillet C. (1992) p53 and *RAS* gene mutations in multiple myeloma. Oncogene 7: 2539-2543.

Price A. (1993) The repair of ionising radiation-induced damage to DNA. Seminars in Cancer Biology 4: 61-71.

Purdie C.A., Harrison D.J., Peter A., Dobbie L., White S., Howie S.E.M., Salter D.M., Bird C.C., Wyllie A.H., Hooper M.L. and Clarke A.R. (1994) Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. Oncogene, 9, 603-609.

Purdie C.A., O'Grady J., Piris J., Wyllie A.H. and Bird C.C. (1991) p53 expression in colorectal tumors. Am. J. Pathol. 138: 807-813.

Reitmar A.H., Schmits R., Ewel A., Bapat B., Redston M., Mitri M., Waterhouse P., Mittrucker H.W., Wakeham A., Liu B., Thomason A., Griesser H., Gallinger S., Ballhausen W.G., Fishel R. and Mak T.W. (1995) MSH2 mice are viable and susceptible to lymphoid tumours. Nature Genetics 11: 64-70.

Renan M.J. (1992) Point mutation, deletions, and radiation carcinogenesis. Radiation Research 131: 227-228.

Renan M.J.(1993) How many mutations are required for tumorigenesis? Implications from human cancer data. Molecular Carcinogenesis 7: 139-146.

Rodenhuis S. and Slebos R.J. (1992) Clinical significance of ras oncogenes in human lung cancer. Cancer Res. 52: 2665s-2669s.

Rodrigues N.R., Rowan A., Smith M.E.F., Kerr I.B., Bodmer W.F., Gannon J.V. and Lane D.P. (1990) p53 mutations in colorectal cancer. Proc. Natl Acad. Sci. USA 87: 7555-7559.

Rotter V., Wolf D., Pravtcheva D. and Ruddle F.H. (1984) Chromosomal assignment of the murine gene encoding the transformation-related protein p53. Mol. Cell Biol. 4: 383-385.

Rous P., and Kidd J.G. (1941) Conditional neoplasms and subthreshold neoplastic states. A study of tar tumors in rabbits. J. Exp. Med. 73: 365-384.

Rovinski B. and Benchimol S. (1988) Immortalization of rat embryo fibroblasts by the cellular p53 oncogene. Oncogene 2: 445-452.

Ruggeri B., Zhang S.Y., Caamano J., DiRado M., Flynn S.D. and Klein-Szanto A.J.P. (1992) Human pancreatic carcinomas and cell lines reveal frequent and multiple alterations in the p53 and Rb-1 tumor suppressor genes. Oncogene 7: 1503-1511.

Sager R. (1989) Tumor supressor genes: The puzzle and promise. Science 246: 1406-1412.

Sands A.T., Suraokar M.B., Sanchez A., Marth J.E., Donehower L.A. and Bradley A. (1995) p53 deficiency does not affect the accumulation of point mutations in a transgene target. Proc. Natl Acad. Sci. USA 92: 8517-8521.

Sankaranarayanan S. (1993) Ionizing radiation, genetic risk estimation and molecular biology: impact and inferences. Trends in Genetics 9: 79-84.

Sasaki S. (1991) Influence of the age of mice at exposure to radiation on life-shortening and carcinogenesis. J. Radiat. Res., Supplement 2: 73-85.

Schwarz M., Buchman A., Schulte M., Pearson D. and Kunz W. (1989) Heterogeneity of enzyme-altered foci in rat liver. Toxicology Letters 49: 297-317.

Selivanova G. and Wiman K. (1995) p53: a cell cycle regulator activated by DNA damage. Advances in Cancer Research 66: 143-180.

Shaw P., Tardy S., Benito E., Obrador A. and Costa J. (1991) Occurrence of Ki-ras and p53 mutations in primary colorectal tumors. Oncogene 6: 2121-2128.

Sherman C.D. and Portier C.J. (1995) Quantitative analysis of multiple phenotype enzyme-altered foci in rat hepatocarcinogenesis experiments: the multipath/multistage model. Carcinogenesis 16: 2499-2506.

Sherman C.D. and Portier C.J. (1994) The multipath/multistage model of carcinogenesis. Informatik, Biometrie und Epidemiologie in Medizin und Biologie 25: 250-254.

Sherman C.D., Portier C.J. and Kopp-Schneider A. (1994) Multistage models of carcinogenesis: an approximation method for the size and number distribution of late-stage clones. Risk Analysis 14: 1039-1048.

Silini G. (1986) Experimental radiation carcinogenesis. In: Radiation Carcinogenesis and DNA Alterations (Burns F.J. and Silini G., ed). Plenum Press, New York.

Sinn E., Muller W., Pattengale P., Tepler I., Wallace R. and Leder P. (1987) Coexpression of MMTV/V-HA-Ras and MMTV/C-Myc genes in transgenic mice - synergistic action of oncogenes in vivo. Cell 49: 465-475.

Smith M.L. and Fornace A.J. (1995) Genomic instability and the role of p53 mutations in cancer cells. Current Opinion in Oncology 7: 69-75.

Soussi T., Caron de Fromentel C. and May P. (1990) Structural aspects of the p53 protein in relation to gene evolution. Oncogene 5: 945-952.

Srivastava S., Zou Z., Pirollo K., Blattner W. and Chang E.H. (1990) Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature 348: 747-749.

Stanbridge E.J. (1976) Suppression of malignancy in human cells. Nature 260: 17-20.

Stoler A.B. (1991) Genes and cancer. Bri. Med. Bull. 47: 64-75.

Takahashi T., Suzuki H., Hida T., Sekido Y., Ariyoshi Y. and Ueda R. (1991) The p53 gene is very frequently mutated in small-cell lung cancer with a distinct nucleotide substitution pattern. Oncogene 6:1775-1778.

Tan W.Y. (1991) Stochastic models of carcinogenesis. Marcel Dekker, Inc., New York.

Tan W.Y. and Chen C. (1990) A stochastic model of carcinogenesis - multiple pathways. In: Mathematical Population Dynamics (Arino O., Axelrod D.E. and Kimmel M. ed.). Marcel Dekker Inc., New York, Chapter 31.

Tan W.Y. and Gastardo M.T. (1985) On the assessment of effects of environmental agents on cancer tumor development by a two-stage model of carcinogenesis. Math. Biosciences 74: 143-155.

Tan W.Y. and Singh K. (1990) A mixed model of carcinogenesis - with application to retinoblastoma. Math. Biosciences 98: 201-211.

Thacker J. (1992) Radiation-induced mutation in mammalian cells at low doses and dose-rates. Advances in Radiation Biology 16: 77-117.

Thacker J. and Cox R. (1975) Mutation induction and inactivation in mammalian cells exposed to ionizing radiation. Nature 258: 429-431.

Thomas D.C. (1982) Temporal effects and interactions in cancer: implications of carcinogenic modles. In: Environmental Epidemiology: risk assessment (Prentice R.L. and Whittemore A.S. ed). SIAM, Philadephia.

Tobias C.A. (1985) The repair-misrepair model in radiobiology: comparison to other models. Radiation Research 104 (suppl. 8): s-77 -s-95.

Tsuda H., Fukushima S., Imaida K., Kurata Y. and Ito N. (1983) Organ-specific promoting effect of phenobarbital and saccharin in induction of thyroid, liver, and urinary-bladder tumors in rats after initiation with N-nitrosomethylurea. Cancer Res. 43: 3292-3296.

United Nations Scientific Committee on the Effects of Atomic Radiation (1986) Genetic and somatic effects of ionizing radiation. UNSCEAR: New York.

United Nations Scientific Committee on the Effects of Atomic Radiation (1988) Sources and effects of ionizing radiation. UNSCEAR: New York.

Upton A.C. (1986) Historical perspectives on radiation carcinogenesis. In 'Radiation Carcinogenesis' edited by Upton, A.C., Albert, R.E., Burns, F.J., and Shore, R.E.. Elsevier Science Publishing Co., New York.

Urlaub G., Mitchell P.J., Kas E., Chasin L.A., Funge V.L., Myoda T.T. and Hamlin J. (1986) Effect of gamma rays at the dihydrofolate reductase locus: deletion and inversions. Som. Cell Mol. Genet. 12: 555-566.

Vahakangas K.H., Samet J.M., Metcalf R.A., Welsh J.A., Bennett W.P., Lane D.P. and Harris C.C. (1992) Mutation of p53 and ras genes in radon associated lung cancer from uranium miners. Lancet 339: 576-580.

Van Ryzin J. (1980) Quantitative risk assessment. J. Occup. Medicine 22: 321-326.

Varley J.M., Brammar W.J., Lane D.P., Swallow J.E., Dolan C. and Walker R.A. (1991) Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinoma. Oncogene 6: 413-421

Varmus H.E. (1984) The molecular genetics of cellular oncogenes. Annu. Rev. Genet. 18: 553-612.

Varmus H.E. (1988) Retroviruses. Science 240: 1427-1435.

Viney J.L. (1995) Transgenic and gene knockout mice in cancer research. Cancer and Metastasis Reviews 14: 77-90.

Vogelstein B. (1990) A deadly inheritance. Nature 348: 681-682.

Vogelstein B. and Kinzler K.W. (1992) p53 function and dysfunction. Cell 70: 523-526.

Vogelstein B. and Kinzler K.W. (1993) The multistep nature of cancer. Trends in Genetics 9: 138-141.

Wallace S.S. (1983) Detection and repair of DNA-base damages produced by ionizing-radiation. Environmental Mutagenesis 5: 769-788.

Ward J.F. (1988) DNA damage proudced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and repairability. Prog. Nucl. Acid Res. Mol. Biol. 35: 95-125.

Ward J.F. (1995) Radiation mutagenesis: the initial DNA lesions responsible. Radiation Reserach 142:362-268.

Weinberg R.A. (1988) The genetic origin of human cancer. Cancer 61: 1963-1968.

Weinberg R.A. (1989) Oncogenes, antioncogenes and the molecular bases of multistep carcinogenesis. Cancer Research 49: 3713-3721.

Weinberg R.A. (1991) Tumor suppressor genes. Science 254: 1138-1146.

Weichselbaum R.R., Bechett M.A. and Diamond A.A. (1989) An important step in radiation carcinogenesis may be inactivation of cellular genes. Int. J. Radiation Oncology Bio. Phys. 16: 277-282.

Weinert T. and Lydall D. (1993) Cell cycle checkpoints, genetic instability and cancer. Seminars in Cancer Biology 4: 129-140.

Wheldon T.E. (1988) Mathematical models in cancer research. Adam Hilger, Bristol and Philadelphia.

White E. (1996) Life, death, and the pursuit of apoptosis. Gene and Development 10: 1-15.

Whittermore A. and Keller J.B. (1978) Quantitative theory of carcinogenesis. SIAM Review 20: 1-30.

Wichmann B.A. and Hill I.D. (1982) Algorithm AS 183. An efficient and portable pseudo-random number generator. Appl. Statist. 31: 188-190.

Xia F., Wang X., Wang Y.H., Tsang N.M., Yandell D.W., Kelsey K.T. and Liber H.L. (1995) Altered p53 status correlates with differences in sensitivity to radiation-induced mutation and apoptosis in two closely related human lymphoblast lines. Cancer Research 55: 12-15.

Yin Y., Tainsky M.A., Bischoff F.Z., Strong L.C. and Hall G.M. (1992) Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell 70: 937-948.

Yokota J. and Sugimura T. (1993) Multiple steps in carcinogenesis involving alterations of multiple tumour suppressor genes. FASEB J. 7: 920-925.

Yonish-Rouach E., Resnitzky D., Lotem J., Sachs L., Kimchi A. and Oren M. (1991) Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin 6. Nature 352: 345-347.

Yuan J.L., Yeasky T., M., Havre P.A. and Glazer P.M. (1995) Induction of p53 in mouse cells decreases mutagenesis by UV radiation. Carcinogenesis 16: 2295-2300.

Zhang W., Guo X.Y., Hu G.Y., Liu W.B., Shay J.W. and Deisseroth A.B. (1994) A temperature-sensitive mutant of human p53. EMBO J. 13: 2535-2544.

Appendices

Appendix 1

Mathematical Development of the Multistage Model of Tumorigenesis

The model of tumorigenesis, as illustrated in Chapter 5, is a k-stage model of tumorigenesis with growth and differentiation of all cell types. Let $X_0(t)$, $X_1(t)$, ..., $X_{k-1}(t)$, $X_k(t)$ and $T(t)$ represent the number of stem cells, 1-stage mutants, ..., k-1-stage mutants, tumour cells arising from stem cells and tumours by time t, respectively. At time $t=0$, $X_0(0)=x_0$, $X_j(t)=0$, (j=1, 2, ..., k), and $T(0)=0$. In a small time interval $[t, t+\Delta t]$, a j-stage mutant may

(1) divide into two j-stage mutants at rate $b_j(t)\Delta t+o(\Delta t)$;

(2) die (or differentiate) at rate $d_j(t)\Delta t+o(\Delta t)$;

(3) divide into one j-stage mutant and one j+1-stage mutant at rate $\mu_j\Delta t+o(\Delta t)$. j=0,1, ...,k-1 where a 0-stage mutant is a stem cell and a k-stage mutant is a tumour cell; or

(4) stay unchanged.

All cells go through the above processes independently of other cells.

1.1 The expected number of cells of each type

Let $\Psi(s_0, s_1, ..., s_k; t)$ be the probability generating function (PGF) of $X_j(t)$, j=0, 1, ..., k. The Kolmogorov forward equation for $\Psi(s_0, s_1, ..., s_k; t)$ is (Tan, 1991, chapter 6)

$$\begin{aligned}\frac{\partial\Psi(s_0,s_1,\ldots,s_k;t)}{\partial t} = \sum_{j=0}^{k-1} \{ & b_j(t)s_j^2 + \mu_{j+1}s_j s_{j+1} \\ & - [b_j(t) + d_j(t) + \mu_{j+1}]s_j \\ & + d_j(t)\}\frac{\partial\Psi(s_0,s_1,\ldots,s_k;t)}{\partial s_j}\end{aligned} \qquad \text{(A1.1)}$$

according to the definition of PGF, obtaining

$$EX_j(t) = \frac{\partial \Psi(1,1,\ldots,1;t)}{\partial s_j} \quad j = 0,1,\ldots,k \tag{A1.2}$$

From (A1.1) and (A1.2), we obtain

$$\begin{cases} \dfrac{dEX_0(t)}{dt} = [b_0(t) - d_0(t)]EX_0(t) \\ \dfrac{dEX_j(t)}{dt} = \mu_j EX_{j-1}(t) + [b_j(t) - d_j(t)]EX_j(t) \quad (j = 1,2,\ldots,k-1) \\ \dfrac{dEX_k(t)}{dt} = \mu_k EX_{k-1}(t) \end{cases} \tag{A1.3}$$

so that,

$$\begin{cases} EX_0(t) = e^{\int_0^t [b_0(s) - d_0(s)]ds} \\ EX_j(t) = \int_0^t \mu_j \, EX_{j-1}(\tau) \, e^{\int_\tau^t [b_j(s) - d_j(s)]ds} \, d\tau \quad (j = 1,2,\ldots,k-1) \\ EX_k(t) = \int_0^t \mu_k EX_{k-1}(\tau) d\tau \end{cases} \tag{A1.4}$$

1.2 The expected number of tumours

The expected number of tumours by time t, ET(t), is

$$ET(t) = \int_0^t F_D(u, t) \, \mu_k EX_{k-1}(u) du \tag{A1.6}$$

where $F_D(u,t)$ is the probability that a tumour cell at time u is detectable as a tumour by time t (see Tan, 1991, p60-61).

Proof. To prove the above equation (A1.6), partition the time interval [0, t] by $I_j=[t_{j-1}, t_j)$, j=1, .., n-1, and $I_n=[t_{n-1}, t_n]$, where $t_j = j\ \Delta t$ and $n\ \Delta t=t$ with $t_n = t$. Let M_j be the number of tumour cells generated from normal stem cells during the time interval I_j, $M_j = X_k(t_j)-X_k(t_{j-1})$, j=1, ..., n. Then the expectation number of the tumours formed from these M_j tumour cells is $M_jF(t_j,t)$. Taking the expectation over M_j, we obtain $[EX_k(t_j)-EX_k(t_{j-1})]F(t_j,t)$, so that

$$\begin{aligned} ET(t) &= \lim_{\Delta t\to 0}\sum_{j=1}^{n}[EX_k(t_j)-EX_k(t_{j-1})]F_D(t_j,t) \\ &= \lim_{\Delta t\to 0}\sum_{j=1}^{n}\frac{[EX_k(t_j)-EX_k(t_{j-1})]}{\Delta t}F_D(t_j,t)\,\Delta t \\ &= \int_0^t \frac{dEX_k(u)}{du}F_D(u,t)du \\ &= \int_0^t F_D(u,t)dEX_k(u) \end{aligned} \qquad \text{(A1.7)}$$

From (A1.3), we can obtain

$$ET(t) = \int_0^t \mu_k EX_{k-1}(u)F_D(u,t)du$$

In practice, it is very difficult or impossible to compute exactly the incidence rate of tumours, the expected number of cells of each type and the expected number of tumours, especially when the number of stages is greater than 2. However, computer simulation provides a very powerful tool. Thus, in our studies, computer simulations have been used to explore the properties of the various models that we considered (see appendix 3).

Appendix 2

Mathematical Development of the 3-gate/2-stage Model of Tumorigenesis

The model of tumorigenesis, described in Chapter 8 (Figure 8.2), is a 3-gate/2-stage model. Let $X_{01}(t)$, $X_{02}(t)$, $X_{03}(t)$, $X_{11}(t)$, $X_{12}(t)$, $X_{13}(t)$, $X_{21}(t)$, $X_{22}(t)$, $X_{23}(t)$ and $Y(t)$ represent the numbers of stem cells with $p53^{+/+}$ ($S/p53^{+/+}$), stem cells with $p53^{+/-}$ ($S/p53^{+/-}$), stem cells with $p53^{-/-}$ ($S/p53^{-/-}$), gate-1 mutants with $p53^{+/+}$ ($G1/p53^{+/+}$), gate-1 mutants with $p53^{+/-}$ ($G1/p53^{+/-}$), gate-1 mutants with $p53^{-/-}$ ($G1/p53^{-/-}$), gate-2 mutants with $p53^{+/+}$ ($G2/p53^{+/+}$), gate-2 mutants with $p53^{+/-}$ ($G2/p53^{+/-}$), gate-2 mutants with $p53^{-/-}$ ($G2/p53^{-/-}$), and malignant cells generated from stem cells by time t, respectively. At time $t=0$, $X_{01}(t)=1$, $X_{02}(t)=0$, $X_{03}(t)=0$, $X_{11}(t)=0$, $X_{12}(t)=0$, $X_{13}(t)=0$, $X_{21}(t)=0$, $X_{22}(t)=0$, $X_{23}(t)=0$ and $Y(t)=0$. In a small time interval $[t, t+\Delta t)$, the probability of each event happening is shown in Table A2.1. The probability of more than one event is $o(\Delta t)$, defined such that: $\lim[o(\Delta t)/\Delta t] = 0$ as Δt approaches 0.

2.1 The expected number of cells of each type

One focus of interest in tumorigenesis lies in investigating the expected number of cells in each stage. The Kolmogorov forward equations provide a means for calculating these. Let $P_t(i_1, i_2, i_3, j_1, j_2, j_3, l_1, l_2, l_3, k) = Pr\{ X_{01}(t)=i_1, X_{02}(t)=i_2, X_{03}(t)=i_3, X_{11}(t)=j_1, X_{12}(t)=j_2, X_{13}(t)=j_3, X_{21}(t)=l_1, X_{22}(t)=l_2, X_{23}(t)=l_3, Y(t)=k\}$. The Kolmogorov forward equations yield the partial differential equation:

Table A2.1 Transitions and transition probabilities of the multigate process for tumorigenesis

Parent at t	Progenies at t+Δt	Probabilities
1 S/p53$^{+/+}$ cell	2 S/p53$^{+/+}$ cells	$b_0(t)\Delta t + o(\Delta t)$
	1 S/p53$^{+/+}$ and 1 G1/p53$^{+/+}$ cell	$\mu_1(t)\Delta t + o(\Delta t)$
	1 S/p53$^{+/+}$ and 1 S/p53$^{+/-}$ cell	$\lambda_1(t)\Delta t + o(\Delta t)$
	differentiation/death cell	$d_0(t)\Delta t + o(\Delta t)$
1 S/p53$^{+/-}$ cell	2 S/p53$^{+/-}$ cells	$b_0(t)\Delta t + o(\Delta t)$
	1 S/p53$^{+/-}$ and 1 G1/p53$^{+/-}$ cell	$\mu_1(t)\Delta t + o(\Delta t)$
	1 S/p53$^{+/-}$ and 1 S/p53$^{-/-}$ cell	$\lambda_2(t)\Delta t + o(\Delta t)$
	differentiation/death cell	$d_0(t)\Delta t + o(\Delta t)$
1 S/p53$^{-/-}$ cell	2 S/p53$^{-/-}$ cells	$b_0(t)\Delta t + o(\Delta t)$
	1 S/p53$^{-/-}$ and 1 G1/p53$^{-/-}$ cell	$k\mu_1(t)\Delta t + o(\Delta t)$
	differentiation/death cell	$d_0(t)\Delta t + o(\Delta t)$
1 G1/p53$^{+/+}$ cell	2 G1/p53$^{+/+}$ cells	$b_1(t)\Delta t + o(\Delta t)$
	1 G1/p53$^{+/+}$ and 1 G2/p53$^{+/+}$ cell	$\mu_2(t)\Delta t + o(\Delta t)$
	1 G1/p53$^{+/+}$ and 1 G1/p53$^{+/-}$ cell	$\lambda_1(t)\Delta t + o(\Delta t)$
	1differentiation/death cell	$d_1(t)\Delta t + o(\Delta t)$
1 G1/p53$^{+/-}$ cell	2 G1/p53$^{+/-}$ cells	$b_1(t)\Delta t + o(\Delta t)$
	1 G1/p53$^{+/-}$ and 1 G2/p53$^{+/-}$ cell	$\mu_2(t)\Delta t + o(\Delta t)$
	1 G1/p53$^{+/-}$ and 1 G1/p53$^{-/-}$ cell	$\lambda_2(t)\Delta t + o(\Delta t)$
	differentiation/death cell	$d_1(t)\Delta t + o(\Delta t)$
1 G1/p53$^{-/-}$ cell	2 G1/p53$^{-/-}$ cells	$b_1(t)\Delta t + o(\Delta t)$
	1 G1/p53$^{-/-}$ and 1 G2/p53$^{-/-}$ cell	$k\mu_2(t)\Delta t + o(\Delta t)$
	differentiation/death cell	$d_1(t)\Delta t + o(\Delta t)$
1 G2/p53$^{+/+}$ cell	2 G2/p53$^{+/+}$ cells	$b_2(t)\Delta t + o(\Delta t)$
	1 G2/p53$^{+/+}$ and 1 malignant cell	$\mu_3(t)\Delta t + o(\Delta t)$
	1 G2/p53$^{+/+}$ and 1 G2/p53$^{+/-}$ cell	$\lambda_1(t)\Delta t + o(\Delta t)$
	differentiation/death cell	$d_2(t)\Delta t + o(\Delta t)$
1 G2/p53$^{+/-}$ cell	2 G2/p53$^{+/-}$ cells	$b_2(t)\Delta t + o(\Delta t)$
	1 G2/p53$^{+/-}$ and 1 malignant cell	$\mu_3(t)\Delta t + o(\Delta t)$
	1 G2/p53$^{+/-}$ and 1 G2/p53$^{-/-}$ cell	$\lambda_2(t)\Delta t + o(\Delta t)$
	differentiation/death cell	$d_2(t)\Delta t + o(\Delta t)$
1 G2/p53$^{-/-}$ cell	2 G2/p53$^{-/-}$ cells	$b_2(t)\Delta t + o(\Delta t)$
	1 G2/p53$^{-/-}$ and 1 malignant cell	$k\mu_3(t)\Delta t + o(\Delta t)$
	differentiation/death cell	$d_2(t)\Delta t + o(\Delta t)$

$$
\begin{aligned}
\frac{dP_t(i_1,i_2,i_3,j_1,j_2,j_3,l_1,l_2,l_3,k)}{dt} = &-\{i_1[\mu_1+\lambda_1+b_0(t)+d_0(t)]+i_2[\mu_1+\lambda_2+b_0(t)+d_0(t)]+i_3[k\mu_1+b_0(t)+d_0(t)] \\
&+j_1[\mu_2+\lambda_1+b_1(t)+d_1(t)]+j_2[\mu_2+\lambda_2+b_1(t)+d_1(t)]+j_3[k\mu_2+b_1(t)+d_1(t)] \\
&+l_1[\mu_3+\lambda_1+b_2(t)+d_2(t)]+l_2[\mu_3+\lambda_2+b_2(t)+d_2(t)] \\
&+l_3[k\mu_3+b_2(t)+d_2(t)]\}P_t(i_1,i_2,i_3,j_1,j_2,j_3,l_1,l_2,l_3,k) \\
&+(i_1-1)b_0(t)P_t(i_1-1,i_2,i_3,j_1,j_2,j_3,l_1,l_2,l_3,k) \\
&+(i_1+1)d_0(t)P_t(i_1+1,i_2,i_3,j_1,j_2,j_3,l_1,l_2,l_3,k) \\
&+[(i_2-1)b_0(t)+i_1\lambda_1]P_t(i_1,i_2-1,i_3,j_1,j_2,j_3,l_1,l_2,l_3,k) \\
&+(i_2+1)d_0(t)P_t(i_1,i_2+1,i_3,j_1,j_2,j_3,l_1,l_2,l_3,k) \\
&+[(i_3-1)b_0(t)+i_2\lambda_2]P_t(i_1,i_2,i_3-1,j_1,j_2,j_3,l_1,l_2,l_3,k) \\
&+(i_3+1)d_0(t)P_t(i_1,i_2,i_3+1,j_1,j_2,j_3,l_1,l_2,l_3,k) \\
&+[(j_1-1)b_1(t)+i_1\mu_1]P_t(i_1,i_2,i_3,j_1-1,j_2,j_3,l_1,l_2,l_3,k) \\
&+(j_1+1)d_1(t)P_t(i_1,i_2,i_3,j_1+1,j_2,j_3,l_1,l_2,l_3,k) \\
&+[(j_2-1)b_1(t)+i_2\mu_1+j_1\lambda_1]P_t(i_1,i_2,i_3,j_1,j_2-1,j_3,l_1,l_2,l_3,k) \\
&+(j_2+1)d_1(t)P_t(i_1,i_2,i_3,j_1,j_2+1,j_3,l_1,l_2,l_3,k) \\
&+[(j_3-1)b_1(t)+i_3k\mu_1+j_2\lambda_2]P_t(i_1,i_2,i_3,j_1,j_2,j_3-1,l_1,l_2,l_3,k) \\
&+(j_3+1)d_1(t)P_t(i_1,i_2,i_3,j_1,j_2,j_3+1,l_1,l_2,l_3,k) \qquad \text{(A2.1)} \\
&+[(l_1-1)b_2(t)+j_1\mu_2]P_t(i_1,i_2,i_3,j_1,j_2,j_3,l_1-1,l_2,l_3,k) \\
&+(l_1+1)d_2(t)P_t(i_1,i_2,i_3,j_1,j_2,j_3,l_1+1,l_2,l_3,k) \\
&+[(l_2-1)b_2(t)+j_2\mu_2+l_1\lambda_1]P_t(i_1,i_2,i_3,j_1,j_2,j_3,l_1,l_2-1,l_3,k) \\
&+(l_2+1)d_2(t)P_t(i_1,i_2,i_3,j_1,j_2,j_3,l_1,l_2+1,l_3,k) \\
&+[(l_3-1)b_2(t)+j_3k\mu_2+l_2\lambda_2]P_t(i_1,i_2,i_3,j_1,j_2-1,j_3,l_1,l_2,l_3-1,k) \\
&+(l_3+1)d_2(t)P_t(i_1,i_2,i_3,j_1,j_2,j_3,l_1,l_2,l_3+1,k) \\
&+[l_1\mu_3+l_2\mu_3+l_3k\mu_3]P_t(i_1,i_2,i_3,j_1,j_2,j_3,l_1,l_2,l_3,k-1)
\end{aligned}
$$

Let $G(s_1, s_2, s_3, z_1, z_2, z_3, r_1, r_2, r_3, y; t)$ be the joint probability generating function of $X_{01}(t)$, $X_{02}(t)$, $X_{03}(t)$, $X_{11}(t)$, $X_{12}(t)$, $X_{13}(t)$, $X_{21}(t)$, $X_{22}(t)$, $X_{23}(t)$ and $Y(t)$ given the initial conditions. From (A2.1),

$$\frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial t} = \{b_0(t)s_1^2 + \lambda_1 s_1 s_2 + \mu_1 s_1 z_1 - s_1[\mu_1 + \lambda_1 + b_0(t) + d_0(t)] + d_0(t)\} \frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial s_1}$$

$$+ \{b_0(t)s_2^2 + \lambda_2 s_2 s_3 + \mu_1 s_2 z_2 - s_2[\mu_1 + \lambda_2 + b_0(t) + d_0(t)] + d_0(t)\} \frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial s_2}$$

$$+ \{b_0(t)s_3^2 + k\mu_1 s_3 z_3 - s_3[k\mu_1 + b_0(t) + d_0(t)] + d_0(t)\} \frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial s_3}$$

$$+ \{b_1(t)z_1^2 + \lambda_1 z_1 z_2 + \mu_2 z_1 r_1 - z_1[\mu_2 + \lambda_1 + b_1(t) + d_1(t)] + d_1(t)\} \frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial z_1}$$

$$+ \{b_1(t)z_2^2 + \lambda_2 z_2 z_3 + \mu_2 z_2 r_2 - z_2[\mu_2 + \lambda_2 + b_1(t) + d_1(t)] + d_1(t)\} \frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial z_2}$$

$$+ \{b_1(t)z_3^2 + k\mu_2 z_3 r_3 - z_3[k\mu_2 + b_1(t) + d_1(t)] + d_1(t)\} \frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial z_3}$$

$$+ \{b_2(t)r_1^2 + \lambda_1 r_1 r_2 + \mu_3 r_1 y - r_1[\mu_3 + \lambda_1 + b_2(t) + d_2(t)] + d_2(t)\} \frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial r_1}$$

$$+ \{b_2(t)r_2^2 + \lambda_2 r_2 r_3 + \mu_3 r_2 y - r_2[\mu_3 + \lambda_2 + b_2(t) + d_2(t)] + d_2(t)\} \frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial r_2}$$

$$+ \{b_2(t)r_3^2 + k\mu_3 r_3 y - r_3[k\mu_3 + b_2(t) + d_2(t)] + d_2(t)\} \frac{\partial G(s_1,s_2,s_3,z_1,z_2,z_3,r_1,r_2,r_3,y;t)}{\partial r_3} \qquad \text{(A2.2)}$$

From (A2.2), we can obtain the expected number of cells of each type.

$$\begin{cases} \dfrac{dEX_{01}(t)}{dt} = [b_0(t) - d_0(t)]EX_{01}(t) \\ \dfrac{dEX_{02}(t)}{dt} = [b_0(t) - d_0(t)]EX_{02}(t) + \lambda_1 EX_{01}(t) \\ \dfrac{dEX_{03}(t)}{dt} = [b_0(t) - d_0(t)]EX_{03}(t) + \lambda_2 EX_{02}(t) \\ \dfrac{dEX_{j1}(t)}{dt} = [b_j(t) - d_j(t)]EX_{j1}(t) + \mu_j EX_{j-1\,1}(t) \quad (j=1,2) \\ \dfrac{dEX_{j2}(t)}{dt} = [b_j(t) - d_j(t)]EX_{j2}(t) + \mu_j EX_{j-1\,2}(t) + \lambda_1 EX_{j1}(t) \quad (j=1,2) \\ \dfrac{dEX_{j3}(t)}{dt} = [b_j(t) - d_j(t)]EX_{j3}(t) + k\mu_j EX_{j-1\,3}(t) + \lambda_2 EX_{j2}(t) \quad (j=1,2) \\ \dfrac{dEY(t)}{dt} = \mu_3 EX_{21}(t) + \mu_3 EX_{22}(t) + k\mu_3 EX_{23}(t) \end{cases} \tag{A2.3}$$

2.2 The expected number of tumours

Let T(t) represent the number of tumours occurring by time t. Thus, the expected number of tumours, ET(t), is

$$ET(t) = \int_0^t F_D(u, t)\ \{\mu_3 EX_{21}(u) + \mu_3 EX_{22}(u) + k\mu_3 EX_{23}\}du \tag{A2.4}$$

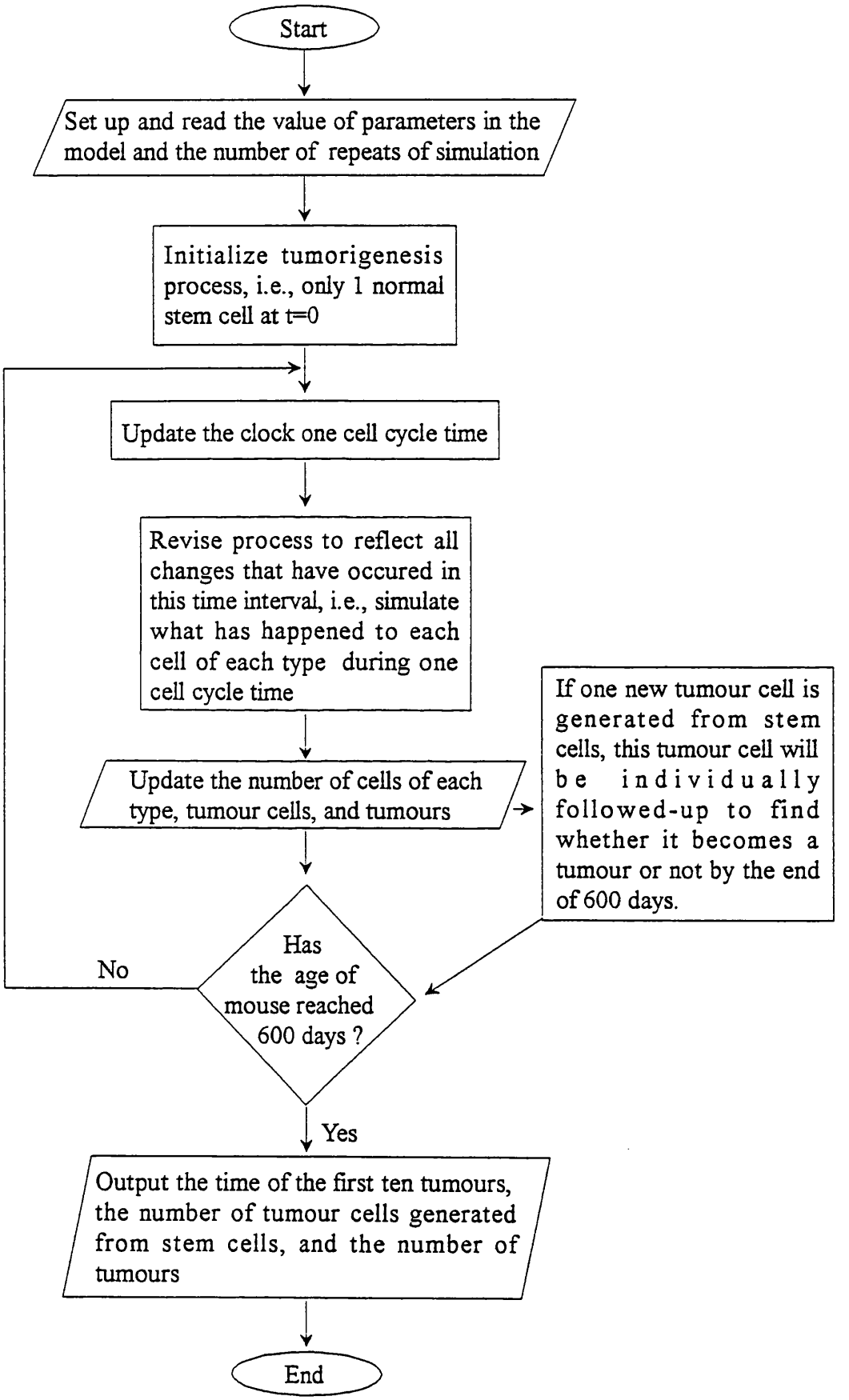
where $F_D(u,t)$ is the probability that a tumour cell at time u is detectable as a tumour by time t. The proof is similar to that shown in appendix 1.

In practice, it is also very difficult to compute the incidence rate of tumours, the expected

number of cells of each type and the expected number of tumours, specially when the number of gates is greater than 2. Here too, the computer simulation provides a very powerful tool, and has been used to explore the properties of these models.

Appendix 3

A General Flow Chart of the Computer Simulation for Tumorigenesis Models



Stochastic simulation process shown above can be described as the time-slice approach, in which the tumorigenic process is viewed as changing in all of its aspects over time. Its status is updated, in units of one cell cycle time, until a prescribed amount of time has elapsed. In our studies, we suppose the cell cycle time is not influenced by the mutional events.

In each cell division, cellular behaviour is as described in Chapter 3. For normal stem cells and mutants, the distribution of the number of cells was computed without following the fate of every cell: homogeneous patches of normal stem cells or mutants were treated as groups. For such groups only the number of cells were updated, using a multinomial random number generator, but using Poisson and normal random number generators to provide approximation when the number of cells is very large. Malignant cells arisen from normal stem cell are followed individually. All computer programs were coded in FORTRAN which can be excuted in DOS and in Unix systems.

The method for estimating the parameters in the models, which involves matching the tumour-free survival distribution of the experimental data with simulated data by a Monte Carlo method, is as follows: Partion the time interval [0, t] by $I_j=[t_{j-1}, t_j)$, j=1, ..., m-1, and $I_m=[t_{m-1}, t_m]$, where $t_j = j*\Delta t$ and $m*\Delta t=t$ with $t=t_m$. Let N_j and n_j be the number of mice that acquired tumour during I_j for the simulated and experimental data respectively. The parameters are estimated by minimizing the chi-square statistic:

$$\chi^2 = \sum_{j=1}^{m} \frac{(N_j - n_j)^2}{N_j + n_j} \qquad \text{(A3)}$$

In our studies, Δt=7 days, m=86.

Appendix 4

Papers published

1. Mao J.H. and Wheldon T.E. (1995) A Stochastic model for multistage tumorigenesis in developing and adult mice. Mathematical Biosciences 129: 95-110.

2. Mao J.H. and Wheldon T.E. Multistage models for radiation tumorigenesis in p53 deficient transgenic mice: computer simulation of effect of single doses of radiation. In: 'Advances in Mathematical Population Dynamics: Molecules, Cells and Man', edited by Arino O., Axelrod D. and Kimmel M., London: World Scientific (in press).

3. Mao J.H., Lindsay K.A., Balmain A. and Wheldon T.E. Stochastic modelling of tumorigenesis in p53 deficient mice supports a role for wild-type p53 in the maintenance of genetic uniformity in cell populations. British J. of Cancer (in press).

Appendix 5

Presentations at international conferences

1. A Stochastic model for multistage tumorigenesis in developing and adult mice. Oral presentation on ICMS Workshop: tumour growth and development, Edinburgh, UK, February 1995.

2. Multistage models for radiation tumorigenesis in transgenic mice: effect of single doses of radiation. Oral presentation on 4th International Conference on Mathematical Population Dynamics, Houston, USA, May 1995.