Study of a possible genetic cause of

CHARGE association

A Thesis submitted in Accordance with the Requirements of The University of Glasgow for the Degree of Doctor of Medicine (MD)

By

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DECLARATION

I certify that the work presented in this thesis except where acknowledged has been performed by me, and that the results of this study have not been submitted for any other degree.

Diana S. Johnson

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LIST OF PUBLICATIONS AND SPOKEN PRESENTATIONS

Published Articles

Johnson D, Morrison N, Grant L, Turner T, Fantes J, Connor JM, Murday V. Confirmation of *CHD7* as a cause of CHARGE association identified by mapping a balanced chromosome translocation in affected monozygotic twins. J Med Genet. 2006 Mar;43(3):280-4. Epub 2005 Aug 23.

Gennery AR, Slatter MA, Rice J, Hoefsloot LH, Barge D, McLean-Tooke A, Montgomery T, Goodship JA, Burt AD, Flood TJ, Abinun M, Cant AJ, Johnson D. Mutations in *CHD7* in patients with CHARGE syndrome cause T-B + natural killer cell + severe combined immune deficiency and may cause Omenn-like syndrome. Clin Exp Immunol. 2008 Jul;153(1):75-80. Epub 2008 May 26.

Spoken Presentations

A Clinical and Molecular Study of 43 individuals with CHARGE syndrome. Johnson D, Rice J,Morrison N, Grant L, Turner T, Fantes J, Connor JM, Murday V. Presented by D.Johnson at the 18th European Dysmorphology meeting, Bishenberg, France, September 2007

CHARGE Syndrome – An Update. Presented by D.Johnson at the 1st Dysmorphology Study Day, Sheffield Children's Hospital, Sheffield, November 2007

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LIST OF ABBREVIATIONS

ABR	Auditory Brainstem Response
AVSD	Atrioventricular Septal Defect
ASD	Atrial Septal Defect
BAC	Bacterial Artificial Chromosome
BAHA	Bone Anchored Hearing Aid
CGH	Comparative Genomic Hybridization
CHD7	Chromodomain Helicase DNA-Binding 7
CNS	Central Nervous System
CPAP	Continuous Positive Airway Pressure
CT	Computerized Tomography
DER	Derivative
DNA	Deoxyribonucleic Acid
DORV	Double Outlet Right Ventricle
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FISH	Florescence In Situ Hybridization
HCG	Human Chorionic Gonadotrophin
IQ	Intelligence Quotient
MCA	Multiple Congenital Anomalies
MQH_20	Milli-Q Water
MLPA	Multiplex Ligation-Dependent Probe Amplification
MR	Mental Retardation
MRI	Magnetic Resonance Imaging
NG	Nasogastric
OA	Oesophageal Artesia
O.D.	Optical Density
OFC	Occipitofrontal Circumference
PAVD	Partial Anomalous Pulmonary Venous Drainage
PCR	Polymerase Chain Reaction
PDA	Patent Ductus Arteriosus
PEG	Percutaneous Enterogastrostomy
PFO	Patent Foramen Ovale
PS	Pulmonary Stenosis
PSI-BLAST	Position-Specific Iterative Basic Local Alignment Search Tool
RVOT	Right Ventricular Outflow Tract

SCBU	Special Care Baby Unit
SCC	Semicircular Canals
SCID	Severe Combined Immune Deficiency
SNHL	Sensorineural Hearing Loss
SNP	Single Nucleotide Polymorphism
SVC	Superior Vena Cava
SVD	Spontaneous Vaginal Delivery
TOF	Tracheoesophageal Fistula
UV	Ultra-Violet
VEP	Visual Evoked Potential
VSD	Ventricular-septal Defect

SUMMARY

CHARGE association, or syndrome as it is now known, is a condition where a number of congenital malformations are non-randomly associated in a recognizable pattern. There are two sets of diagnostic criteria for CHARGE syndrome which are in common usage at present (Blake *et al*., 1998; Verloes, 2005).

The etiology of CHARGE syndrome was unknown. We identified twin girls with CHARGE syndrome and a *de novo* apparently balanced chromosome translocation 46,XX,t(8;13)(q11.2;q22). By mapping the chromosome translocation breakpoints we found that the gene chromodomain-helicase-DNA-binding protein 7 (*CHD7*) located at 8q12 was disrupted in these girls. *CHD7* has a genomic length of 188kb with 9000 coding bases over 37 exons. It has a putative function as a transcription factor which makes it a good candidate gene for a condition which affects multiple body systems.

Concurrently with this study Vissers *et al* (2004) identified *CHD7* as a cause of CHARGE syndrome. They found two individuals with CHARGE syndrome with overlapping microdeletions detected by array CGH. By sequencing the 9 genes in this region in a cohort of 17 cases they identified a mutation in *CHD7* in 10 cases.

We ascertained a cohort of 45 patients with a diagnosis of CHARGE syndrome or possible CHARGE syndrome by scrutinizing the clinical genetics databases in Glasgow and Sheffield. Part of the cohort was accessed by receipt of samples from clinical genetics departments elsewhere in the U.K. and in Lisbon. Clinical information was acquired on this cohort either by examination and review of the clinical notes by the author or by completion of a proforma by the referring clinician.

Sequencing in this cohort of 45 patients was successful in 43 individuals. We identified 28 mutations; 16 nonsense, 10 frameshift and 2 splice site mutations. 20 of the mutations were

novel, 8 had been reported in other studies. The mutations were found throughout the gene with no particular hotspots. No genotype/phenotype correlations were found either in relationship to the position of the mutation within the gene or with regards to the type of mutation.

I have analyzed the phenotype in our cohort and compared it with the cases of CHARGE association reported prior to the availability of mutation analysis. I have also compared the phenotype in our mutation positive cases with those reported in other studies which were mutation-positive.

We report two individuals with rare findings in CHARGE syndrome; one with a palsy of the twelfth cranial nerve reported anecdotally only once before (Blake *et al*., 2008), and another child with a limb reduction defect which has been reported in five other cases (Aramaki *et al*., 2006; Asamoah *et al*.,2004; Van de Laar *et al*., 2007).

Our notes review ascertained an incidence of CHARGE syndrome of 1/10,000.

INTRODUCTION

The recognition that an association between coloboma and a number of specific congenital malformations might constitute a new syndrome was first proposed in 1979 by Hall (Hall 1979) and independently by Hittner *et al* (1979). The acronym CHARGE was suggested by Pagon *et al* (1981), using the cardinal features which were thought to occur (**C**oloboma, **H**eart disease, **A**tresia of choanae, **R**etarded growth and development and/or central nervous system anomalies, **G**enital hypoplasia and **E**ar anomalies) (Pagon *et al*., 1981). These seven features which were found to be nonrandomly associated congenital abnormalities were used to devise the diagnostic criteria for CHARGE association. The consensus view was that to make a diagnosis of CHARGE association an individual should have either: a) Coloboma or choanal atresia + 3 of the other anomalies, or b) Coloboma + choanal atresia + 2 of the other anomalies (Pagon *et al*., 1981). The diagnostic criteria were updated in 1998 (Blake *et al*., 1998) when more importance was placed on the rarer malformations which appeared to be more specific to CHARGE association, including these as major criteria, whilst more nonspecific malformations were included in the minor criteria. These malformations occur commonly in CHARGE association but also occur in other malformation syndromes.

The major criteria are now considered to be coloboma, choanal atresia, characteristic ear anomaly and cranial nerve dysfunction. The minor criteria are genital hypoplasia, developmental delay, cardiovascular malformations, growth deficiency, orofacial cleft, tracheoesophageal fistula (TOF) and a distinctive face (Blake *et al*., 1998). Diagnosis now requires the presence of 4 major or 3 major + 3 minor criteria.

A further set of diagnostic criteria has subsequently been suggested by Verloes (Verloes 2005). This allows diagnoses of typical, atypical and partial CHARGE syndrome to be made. Using Verloes criteria the major criteria are coloboma, choanal atresia and hypoplastic semi-circular canals. The minor criteria are rhombencephalic dysfunction, hypothalamo-hypophyseal dysfunction, abnormal middle or external ear, malformation of mediastinal organs and mental retardation. Using these criteria a diagnosis of Typical CHARGE, Partial/ incomplete CHARGE or atypical CHARGE can be made as illustrated in table 1 below.

Table 1. Diagnostic Criteria for CHARGE syndrome (Verloes 2005)

Typical CHARGE	3 major criteria	2/3 major + 2/5 minor criteria
Partial/ incomplete CHARGE	2/3 major + 1/5 minor criteria	
Atypical CHARGE	2/3 major + 0/5 minor	1/3 major + 3/5 minor criteria

Using these criteria a diagnosis of Typical CHARGE can be made if there are 3 major criteria present or 2 major criteria in addition to at least 2 minor criteria. A diagnosis of Partial/incomplete CHARGE can be made if there are 2 major criteria and 1 of the minor criteria present. A diagnosis of Atypical CHARGE can be made if there are only 2 major criteria present or 1 major criterion and at least 3 minor criteria.

Both Blake's (Blake *et al*., 1998) and Verloes's (Verloes 2005) criteria are in current usage.

A review of over 100 reported cases in the literature is shown in Table 2. This illustrates the relative frequencies of the different malformations in reported cases. The anomalies which occur in CHARGE association involve disorder in the development of the optic vesicle, otic capsule, midline CNS structures and the upper pharynx. They are thought to be the result of abnormal differentiation of cephalic

mesoderm and ectoderm (otic placode and first branchial cleft). There is believed to be abnormal differentiation, migration and survival of neural crest cells. Abnormal interactions of neural crest cells (forming the first and second arch) occur with the cephalic mesoderm and the developing forebrain and there is concomitant disorder in the development of the rhombencephalon from which the neural crest cells have migrated. These events take place between the third and ninth weeks of gestation (Siebert *et al*., 1985; Kirby *et al*., 1990; Lin *et al*., 1990)

Table 2. Common malformations in CHARGE association and the percentage of individuals in which they occur. (Angelman 1961; Edwards *et al*., 1961; Stool *et al*., 1968; Buckfield *et al*., 1971; James *et al*., 1974; Ho *et al*., 1975; Sekhar *et al*., 1976; Sassani *et al*., 1977; Say *et al*., 1977; Hall 1979;Hittner *et al*., 1979; Lillquist *et al*., 1980;Davenport *et al*., 1986; Oley *et al*., 1988; and Dhooge *et al*., 1998)

Malformation	Cases	Percentage
Coloboma	83/105	79%
Heart disease	76/106	72%
Choanal atresia	53/100	53%
Retarded growth	74/89	83%
MR/CNS anomalies	88/100	88%
Genital hypoplasia	61/86	71%
Ear anomalies/deafness	94/104	90%
Micrognathia/cleft palate	37/66	56%
Facial palsy	46/94	49%
Swallowing difficulty	27/66	41%
Tracheoesophageal fistula	4/62	6.5%

Numerator is the number of individuals found with the malformation, denominator is the number examined. MR, mental retardation, CNS, central nervous system

Coloboma

Coloboma occurs due to failure of fusion of the choroidal fissure along the infero nasal aspect of the optic cup and stalk. The colobomata which occur in CHARGE patients

may range from the typical iris coloboma without visual impairment to coloboma involving the retina and optic disc. Some individuals have anophthalmos. In comparison with other individuals with isolated coloboma there are fewer than expected iris coloboma (Pagon *et al*., 1981). The prevalence of isolated coloboma is not known. One study found that coloboma and the related structural developmental eye defects of microphthalmia and anophthalmia have a combined birth prevalence of 2.4-3.5/10,000 (Campbell *et al*., 2002). Cataract and retinal detachment may occur as complications of coloboma (Davenport *et al*.,1986).

Heart Disease

Heart malformations in CHARGE are often severe. In the review by Lin *et al.* (1987) 42% had a conotruncal anomaly and 36% an aortic arch anomaly. Tetralogy of Fallot and double outlet right ventricle each occurred in 38% of cases. AVSD is also common. PDA is frequently found either alone or in combination with one of the other associated cardiac malformations. Aberrant subclavian arteries may also be present. Nonconcordance of heart defects may occur in affected sibs (Lin *et al*., 1987; Cyran *et al*., 1979; Wyse *et al*., 1993).

Choanal atresia

Choanal atresia is a malformation where there is persistence of the primitive membrane between the posterior nasal passages and the upper pharynx. The result is that air is unable to pass through the nose to the pharynx and then to the lungs. Neonates are obligate nasal breathers and thus when present, choanal atresia is frequently diagnosed soon after birth. The atresia may be bony or membranous, unilateral or bilateral (Pagon

et al., 1981). It is caused by failure of the primitive membrane to divide. Figures 1 and 2 illustrate the anatomical position of the obstruction (Bonafos *et al*., 2004).

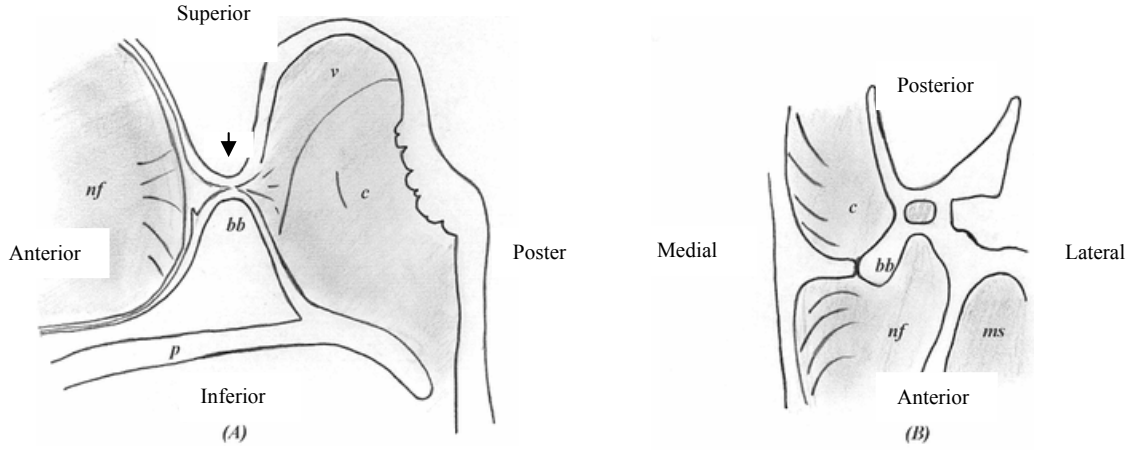

Figure 1 Schematic of bony choanal atresia. A. Lateral view. B. Axial view. c = cavum; nf = nasal fossae; ms = maxillary sinus; p = palate; v = vomer; bb = bone block. There is a voluminous bone block causing absence of communication between the cavum and the nasal fossae

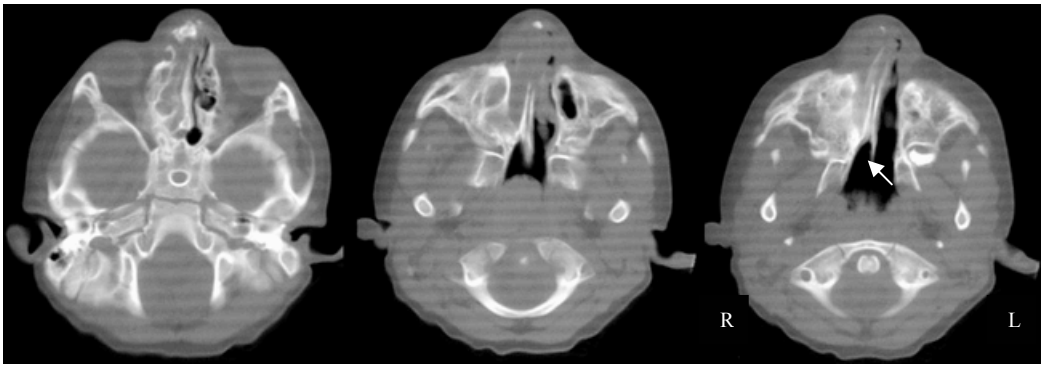

Figure 2 Sinusal computerized tomography scan. Axial section, bone window. Total right bone choanal atresia

The prevalence of choanal atresia is approximately 0.82/10,000. In 43% of individuals this occurs as an isolated anomaly (Harris *et al*., 1997). A study looking at airway obstruction in 30 CHARGE patients found 14 with pharyngomalacia/laryngomalacia or

both, 5 with tracheomalacia and 9 with multiple levels of airway obstruction. In total 67% of individuals had airway obstruction below the choanae (White *et al*., 2005).

Retarded growth

The majority of patients with CHARGE have normal birth weight but by six months old are below the third centile for weight and length (Pagon *et al*., 1981; Blake *et al*., 1993); many hospital admissions for treatment of congenital anomalies are undoubtedly responsible in part. However over 50% of children with CHARGE have gastroesophageal reflux, and pharyngeal in-coordination is also common (Blake *et al*., 1990, 1993). Both of these make feeding more difficult. Delayed bone age is also a frequent finding (Oley *et al*., 1988).

Retarded development/CNS anomalies

Initial studies stated that the majority of CHARGE patients have some learning disability which ranges from those with an IQ of 80 to profound retardation (Pagon *et al*., 1981). More recent studies show that up to 50% have a good intellectual outcome (Raqbi *et al*., 2003) and that poor outcome is best predicted by the presence of extensive bilateral coloboma, microcephaly and the presence of a brain malformation (Vervloed *et al*., 2006).

CNS anomalies are quite common in CHARGE. A review of 47 patients by Lin *et al* (1990) found a CNS malformation in 55%. Of these 65% were forebrain malformations, 42% had arrhinencephaly, 12% holoprosencephaly and 12% other forebrain defects. The other 35% had a non-forebrain malformation. The presence of a CNS malformation has been significantly correlated with choanal atresia (Lin *et al*., 1990). A recent study by Chalouli *et al* (2005) of 14 patients with CHARGE showed

that all had olfactory deficiency and of these half were anosmic. The 9 individuals who had MRI studies all had anomalies of the olfactory bulb and/or tract, structures derived from the rhinencephalon (Chalouli *et al*., 2005). This is concordant with Pinto's study where in all 18 individuals in whom it was assessed there was defective smell and abnormality of the olfactory bulb. This was also associated with hypogonadotrophic hypogonadism (Pinto *et al*., 2005).

More than 75% of individuals are thought to have dysfunction of one or more cranial nerves (Byerly and Pauli, 1993). White *et al* (2005) studied 30 patients with CHARGE and found 60% had evidence of chronic aspiration with a further 20% with pharyngeal dysmotility. This is thought to be due to dysfunction of the 9^{th} and 10^{th} cranial nerves. (White *et al*., 2005)

Genital Hypoplasia

The microphallus and cryptorchidism that occurs in boys is easier to detect clinically than the labial hypoplasia, which may occur in girls. The cause of genital hypoplasia in some patients has been found in a number of studies to be due to pituitary or hypothalamic dysfunction (Pagon *et al*., 1981; Davenport *et al*., 1986; Blake *et al*., 1993; Pinto *et al*., 2005). In a study of 32 individuals with CHARGE Pinto *et al.* (2005) found 19/20 boys had micropenis/cryptorchidism. Of these 7 out of 9 tested were found to have low testosterone levels and 3 had growth hormone deficiency. The latter was associated with hypoplasia of the anterior pituitary. Treatment with testosterone restored normal penis length in 34% of those in whom it was small at birth. In addition to the genital anomalies delayed puberty is common (Pinto *et al*., 2005). Some girls may have absent vagina, uterus and ovaries (Ragan *et al*., 1999).

Ear anomalies/deafness

The ears are typically small and wide with reduced height, cup shaped or lop shaped. The ear lobes are small or absent. There is a prominent antihelix which may be discontinuous with the antitragus and a triangular concha.

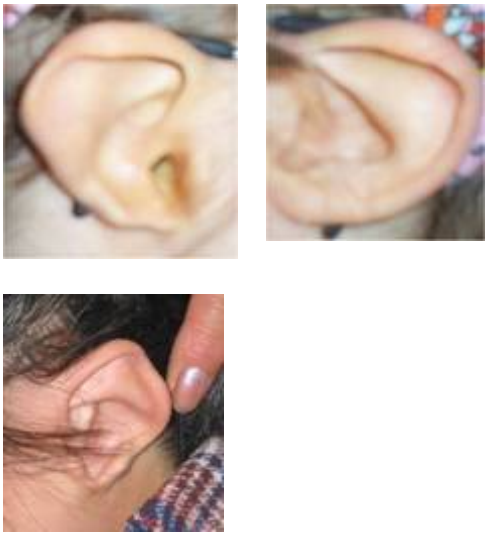

Figure 3. Typical CHARGE ears

(http://www.chargesyndrome.ca/IntroducingCHARGEbooklet.htm)

Deafness may be both conductive and sensorineural and can be slowly progressive. The audiogram has a typical 'wedge' shape (Davenport *et al*., 1986; Thelin *et al*., 1986). Conductive hearing loss occurs due to the persistence of middle ear effusions and ossicular malformations which may include misshapen footplate of the stapes, absent stapedius tendon/muscle and pyramidal process, and a short incus. The ossicular chain may be fixed. The oval window is often abnormal/absent and there may be osseous obliteration of the round window (Davenport *et al*., 1986; Dhooge *et al*., 1998; Verloes 2005). Sensorineural hearing loss may be due to semicircular canal or cochlea hypoplasia (Collins *et al*., 2002). Cochlea hypoplasia may affect only the upper turn of

the cochlea or be more severe resulting in the Mondini deformity. Anomalies of the semicircular canals have been found in more than 80% of those who have been scanned (Morgan *et al*., 1993). Deafness ranges from mild to profound and may be asymmetric. The presence of an external ear anomaly is usually associated with deafness in that ear (Pagon *et al*., 1981).

Limb anomalies

Limb anomalies are not common in CHARGE syndrome. Those, which have been reported, include a distal transverse crease which extends between the 2nd and 3rd finger web, 2-3 cutaneous syndactyly, tapered fingers, 5th finger clinodactyly and camptodactyly with absence of the distal phalanx of the 5th finger in one individual and nail hypoplasia, triphalangeal thumb and ectodactyly. There may be limited supination, talipes, sandal gap, tibial hemimelia and dimpling (Davenport *et al*., 1986; Oley *et al*., 1988; Meinecke *et al*., 1989; Prasad *et al*., 1997; Dhooge *et al*., 1998; Jongmans *et al*., 2006; Sanlaville *et al*., 2006).

Other anomalies

Amongst the other anomalies which may occur urological anomalies are quite common. Urethral atresia, ureteral reflux, urethrorectal and urethroperineal fistulae were reported by Davenport (Davenport *et al*., 1986). A review of genitourinary anomalies found urinary tract anomalies in 42%. These include solitary kidney, hydronephrosis, renal hypoplasia and duplex kidneys. Vesicoureteric reflux is also common occurring in 44% (Ragan *et al*., 1999).

Other rare anomalies include those involving the skeletal system, the endocrine system, the gastrointestinal system and the respiratory tract. Skeletal abnormalities include

hemivertibrae (Akisu *et al*., 1998), scoliosis (Oley *et al*., 1988), 11 or13 rib pairs (Pagon *et al*., 1981; Oley *et al*., 1988). Endocrine and immune system abnormalities include absent thymus, T cell dysfunction, absent parathyroid gland (Pagon *et al*., 1981; Oley *et al*., 1988), ectopic thyroid/hypothyroid (Oley *et al*., 1988; Marin *et al*., 1991). Absent thymus/thymic hypoplasia may be more common than previously thought. Sanlaville found this abnormality in 7/10 individuals at post mortem (Sanlaville *et al*., 2006). Gastrointestinal abnormalities include, omphalacele (Pagon *et al*., 1981; Oley *et al*., 1988), imperforate anus, accessory spleen (Davenport *et al*., 1986), duodenal atresia (Aramaki *et al*., 2006) and caecal volvulus (Lai and Feng, 2006). In the respiratory system in addition to choanal atresia, tracheo-, laryngo- and bronchomalacia may occur (Hsuch *et al*., 2004).

Individuals with CHARGE have a typical facial appearance. The face is square with bitemporal narrowing and a flattened nasal tip (Blake *et al*., 1998).

The estimated prevalence of CHARGE association is 1/8500-1/10,000 (Blake *et al*., 2003; Keller *et al*., 2000).

The cause for CHARGE association was unknown. The majority of cases have been sporadic and several different pathogenic mechanisms have been suggested. Evidence supporting a genetic cause includes the isolated reports of multiplex families with presumed autosomal dominant (Mitchell *et al*., 1985) and autosomal recessive inheritance (Awrich *et al*., 1982; Pagon *et al*., 1981). There are also several reports of concordant monozygotic twin pairs (Farquhar *et al*., 2002). There is phenotypic overlap with some chromosomal syndromes such as partial trisomy 13 and 4p-. A number of single cases with chromosome abnormalities have also been reported with features consistent with a diagnosis of CHARGE association to a greater or lesser degree. These include trisomy 18 (Lee *et al*., 1995), der (9)t(9;13), der(6)t(4;6)

(Sanlaville *et al*., 2002), ring chromosome 14 (Fledelius1996) and a case with a balanced translocation t(6;8)(6p8p;6q8q) (Hurst *et al*., 1991). The finding of many different chromosomal rearrangements suggests that the condition is heterogeneous with a number of different genetic causes.

Identifying the causative gene for a syndrome may be attempted in several ways. Linkage studies where there are sufficient numbers of affected individuals in families to achieve statistical power is one method, however since this requires multiplex families this is unsuitable for this condition. Candidate genes can be sequenced in affected individuals, although these are difficult to find when the biological basis of a condition is unknown. Genome scans can be undertaken with microsatellite markers to identify loss of heterozygosity. This is unlikely to be successful unless deletions are a significant cause. Comparative genomic hybridization (CGH) can also be used to identify small deletions. Other methods which identify genomic copy number changes include representational oligonucleotide microarray analysis (Lucito *et al*., 2003; Sebat *et al*., 2004) and single nucleotide polymorphism oligonucleotide arrays (SNP arrays) (Huang *et al*., 2004). Mapping chromosomal breakpoints in affected individuals with a balanced chromosome translocation would also be a strategy. The few families affected by CHARGE association are too small to do linkage studies. Three possible candidate genes, PAX2, TUPLE1 and SHH have been sequenced in twenty-seven individuals with CHARGE. They were thought to be good candidate genes but no mutations have been found (Tellier *et al*., 2000). Another candidate gene, PITX2 sequenced in twenty-nine patients with CHARGE also revealed no mutations (Martin *et al*., 2002). Genome scans of 10 patients looking for loss of heterozygosity did not detect any deletions (Lalani *et al*., 2003, 2005). CGH of twenty seven individuals identified two different chromosomal abnormalities but no regions which were

consistently deleted/duplicated (Sanlaville *et al*., 2002). Mapping the chromosome breakpoints in affected individuals with balanced translocations has been a successful strategy in identifying genes responsible for a variety of inherited disorders, from Duchenne muscular dystrophy (Worton *et al*., 1984; Ray *et al*., 1986) to rare sporadic genetic syndromes, which are difficult to identify through conventional linkage analysis.

A study mapping the translocation breakpoint in an individual with a balanced translocation involving chromosomes 2 and 7 identified the gene SEMA3E as a possible rare cause of CHARGE association (Lalani *et al*., 2004). Approximately 1/650 people have a balanced chromosome rearrangement and for the majority this has no phenotypic affect. A small percentage will have learning difficulty, congenital anomalies or a syndrome in association with their chromosomal rearrangement. If this is *de novo* it is presumed that the rearrangement involves a small deletion or that the chromosomal breakpoints disrupt a single gene which causes the phenotype in that individual. We identified a monozygotic twin pair with CHARGE association and a *de novo* chromosomal rearrangement 46,XX,t(8;13)(q11.2;q22). Both girls fully meet the diagnostic criteria for CHARGE association/syndrome (Blake *et al*., 1998). In addition, unlike many of the other chromosomal abnormalities described in association with CHARGE, which are unique, this rearrangement shared a common breakpoint with a previously reported case (Hurst *et al*., 1991).

The clinical diagnosis is paramount when using breakpoint mapping as a strategy to identify causative genes. The diagnosis was secure in the twins described and in the reported case with the common chromosome 8 breakpoint (Hurst *et al*., 1991).

HYPOTHESIS

The identical twins have CHARGE association due to a *de novo* chromosome translocation. One of the chromosome breakpoints in our probands disrupts a gene which causes CHARGE association.

AIMS

1. Map the translocation breakpoints in our probands.
2. Identify a candidate gene at the breakpoint.
3. Ascertain incidence and prevalence of individuals with CHARGE association born in Greater Glasgow.
4. Delineate the phenotype of individuals diagnosed with CHARGE.
5. If a causative gene is identified, to sequence the gene in other affected individuals to assess any genotype/phenotype correlations

EXPERIMENTAL APPROACH

Parental consent and local ethics approval was obtained for the study.

ASCERTAINMENT OF CHARGE PATIENTS

To ascertain individuals with CHARGE association lists of patients were obtained from the Genetics Department database for individuals with a diagnosis list of CHARGE and conditions which had overlapping features. Di George (tested negative for 22q11 deletion), congenital heart disease, optic nerve hypoplasia, developmental delay, and multiple congenital anomalies (MCA).

A list of patients was obtained from Yorkhill Hospital medical coding of all individuals presenting in the last 10 years with a code of coloboma, choanal atresia or MCA.

The hospital notes of all individuals identified above would be reviewed and a proforma developed to list clinical features completed.

All individuals with a diagnosis of CHARGE association/partial CHARGE association identified above would be invited for clinical review. They would be invited to take part in the study and blood samples would be obtained following written consent.

CASE REPORT OF IDENTICAL TWINS WITH A DE NOVO CHROMOSOME TRANSLOCATION

The twins were born at 31+6 weeks gestation by normal delivery to healthy unrelated parents. There was no family history of congenital malformations. Birth weights were 1390g (10^{th} centile) and 1450g (10^{th} centile) for twins 1 and 2 respectively. They were both intubated at birth for respiratory distress. Attempts to pass nasogastric tubes were unsuccessful. Choanal atresia was presumed and they were managed with oral airways

and gastric tubes. Once extubated CT scans confirmed bilateral bony and membranous occlusion. The choanae were successfully repaired at two months.

Both girls were found to have bilateral colobomata of the iris and fundi, with significant visual impairment due to retinal and macular involvement

On examination they both had external ear abnormalities and were subsequently found to have sensorineural deafness. Twin 2 was found to have profound sensorineural deafness on the left, with normal hearing on the right. High definition imaging of the ears was not performed but CT scans for the choanal atresia also demonstrated an under-aerated, presumably contracted, middle ear cavity and possible abnormities of the vestibular aqueducts. Twin 1 has bilateral profound sensorineural hearing loss with thresholds of 70-80db at all frequencies on both sides. She has the same abnormalities reported on CT.

Single umbilical arteries were noted but renal scans were normal. Echocardiography revealed patent ductus arteriosus requiring surgical ligation. Twin 2 also had a VSD, which required pulmonary banding pending closure.

They had normal cranial ultrasound scans.

Both girls had swallowing difficulties and had vomiting necessitating fundoplication.

Postnatal growth continued to be poor, below the 3rd centile, and both twins had significant developmental delay.

Twin 2 died suddenly at age 8 months. A post-mortem could not ascertain the cause of death.

Figure 4 shows the facial features and typical external ear abnormalities.

Karyotype revealed a translocation 46,XX,t(8;13)(q11.2;q22) in both girls but in neither of the parents.

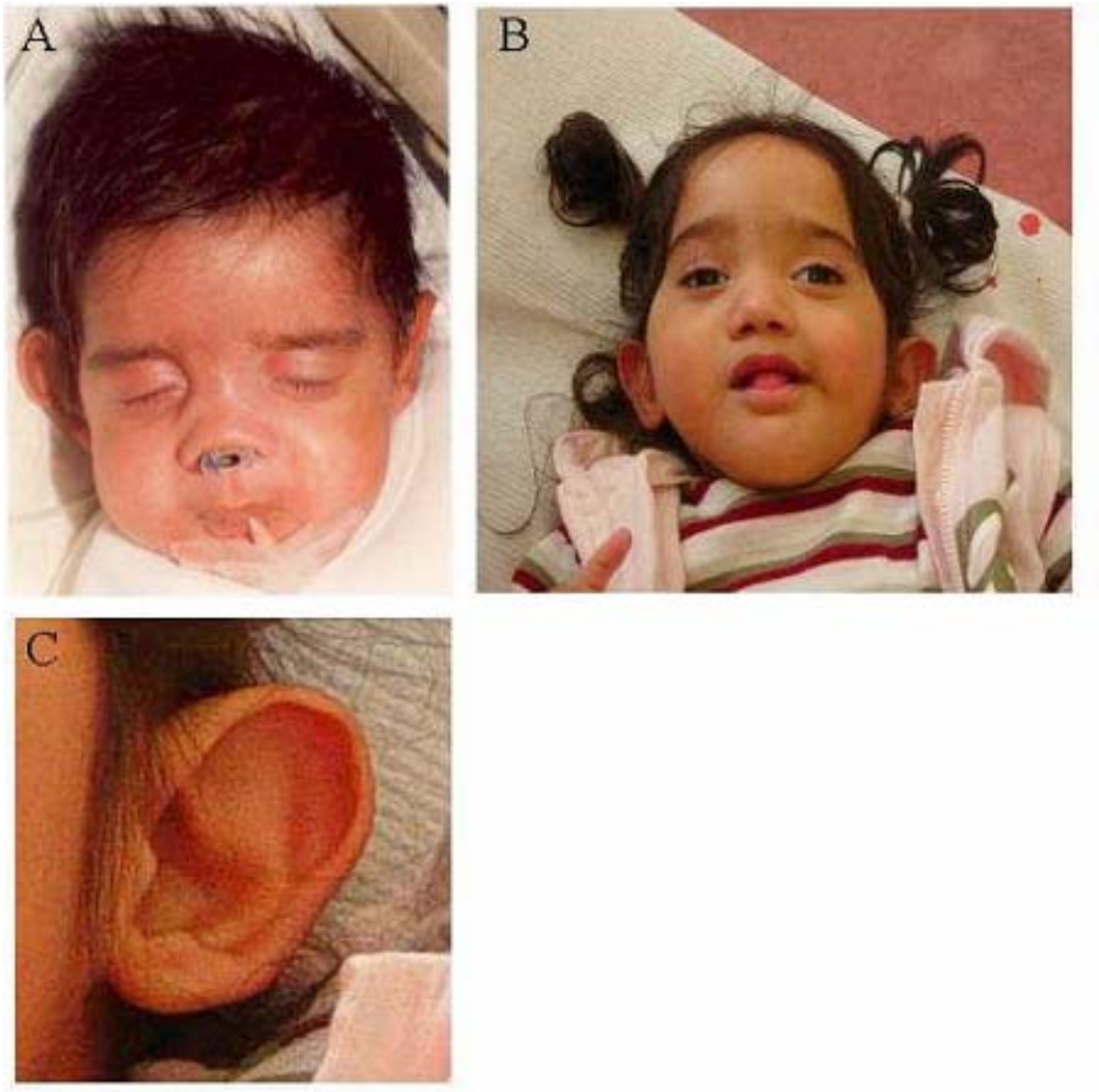

Figure 4 a) Twin 2 aged 2 months b) Twin 1 aged 2 years showing mildly dysmorphic features with laterally extended eyebrows with medial flare and c) A typical CHARGE ear, low set, protruding and featureless

Materials and Methods: Cytogenetics

Analysis of the probands chromosomes showed an apparently balanced translocation, 46,XX, t(8;13)(q11.2;q22) as shown in Figure 5.

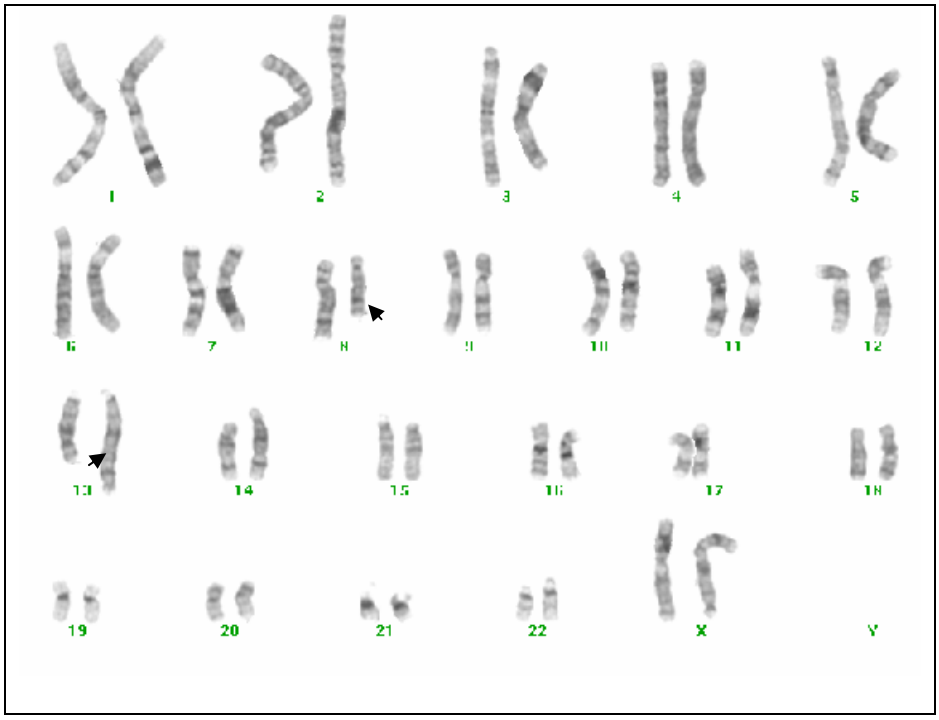

Figure 5. Photograph of karyotype of twin 1 showing translocation involving chromosomes 8 and 13 with breakpoints at q11.2 and q22 respectively.

Metaphase spreads were prepared from heparinised blood using standard cytogenetic techniques and fluorescence *in situ* hybridization (FISH) was performed with BAC, biotin labeled probes to characterize the translocation breakpoints on both chromosomes 8 and 13. The probes were selected using the UCSC and Ensembl Human Genome Browsers (http://genome.ucsc.edu/cgi-bin/hgGateway and http://www.ensembl.org/) at approximately 1MB intervals within the bands that were thought to be involved from the karyotype and extending into the bands on either side. The methods used for preparing the probes were as follows:

Preparation of BAC probes for FISH:

100ml of LB broth was prepared by adding two 1.1g tablets of Lennox L Broth (Sigma®) to 100ml of MQH_2O. 400ml of LB agar was prepared by adding eight 1.68g Lennox L agar tablets (Sigma®) to 400ml of MQH_2O. Both preparations were autoclaved and then left to cool. When the preparations were hand hot 100µl of 20mg/ml Chloramphenicol was added to the broth and 400µl of 20mg/ml Chloramphenicol was added to the agar. The plates were poured and allowed to cool for about 30 minutes. The BACs were then streaked on to the plates from the stabs using 10µl disposable loops. The plates were then incubated overnight at 37^0C. The LB broth was dispensed into sterile universal containers. Two isolated colonies from each plate were picked and placed in the broth. The broth was then incubated overnight at 37^0C, 200RPM. Two aliquots were taken from each culture and placed in sterile glycerol. 2µl of each glycerol stock was then used to inoculate 2.5ml of the sterile LB broth with Chloramphenicol. This preparation was then incubated overnight at 37^0C, 200RPM. BAC RP3 491L was cultured using the same method as the other BACs but using kanomycin instead of chloramphenicol in the cultures.

The DNA from the cultures was then isolated as per the CHORI BACPAC miniprep method with solutions supplied with the kit. (http://bacpac.chori.org/bacpacmini.htm)

Solutions used:

P1 (filter sterilized, 4^oC)
50mM Tris, pH 8
10 mM EDTA
100 ug/ml RNase A
P2 (filter sterilized, room temp)
0.2M NaOH
1% SDS
P3 (autoclaved, 4^oC)
3M KOAc, pH 5.5

TE Buffer (10mM Tris-HCL, pH8.0, 1mM EDTA pH8.0)

2ml of the BAC culture was pipetted into a 2ml eppendorf tube and spun at 13,000RPM for 3 minutes at room temperature. The supernatant was aspirated from the tube which was then inverted for approximately 30 seconds to allow further drying. The final remaining drops were then removed with a micropipette and Kim wipes. 0.3µl of P1 solution was then added to the tube and vortexed to resuspend the pellet. 0.3µl of P2 solution was then added to the tube whose contents were mixed by inverting it approximately 30 times. It was then left at room temperature for at least 5minutes. 0.3µl of P3 solution was then added to the tube which was inverted 5 times to mix the contents. The tube was then placed on ice for at least 5minutes. The tube was then spun at 13,000RPM for 10 minutes at 4^0C. After transferring the supernatant to a fresh tube 0.8µl of ice cold Isopropanol was added and the tube inverted 5 times to mix the contents. The tube was then placed on ice for at least 5minutes and then spun at 13,000RPM, 4^0C for 15minutes. After this spin the tube was kept in ice until it was ready for the next step. The supernatant was removed from the tube. 0.5µl of 70% ethanol which had been filtered at room temperature was added to the tube which was then inverted 5 times and spun at 4^0C for 5minutes. The ethanol was removed and a fresh 0.5µl of 70% filtered ethanol was added to the tube inverted 5 times and spun at 4^0C for 5minutes.The supernatant was then aspirated from the tube and the pellet was allowed to air dry at room temperature. When the pellet had become translucent 40µl of TE (pH 8) was added to the tube. This was then allowed to stand for one hour at room temperature to allow the pellet to resuspend. The BAC DNA was then kept stored at 4^0C.

Nick translation with biotin:

Nick translation of the BAC DNA was carried out as follows;

Solutions used:

10x dNTP mix	0.2mM each dCTP, dGTP, dTTP 0.1mM dATP 0.1mM Biotin-14-ATP 500mM Tris HCL (pH7.8) 50mM Magnesium chloride 100mM 2-mercaptoethanol 100ug/ml bovine serum albumin)
10x enzyme mix	0.5U/ul DNA Polymerase 1 0.007U/ul DNase 1 50 mM Tris-HCL (pH7.5) 5mM Magnesium chloride 0.1mM phenylmethylsulphonyl fluoride 50% glycerol 100ug/ml bovine serum albumin)
Stop buffer	0.5M EDTA (pH 8.0)

5µl of the 10x dNTP mix, 5µl of the BAC DNA, 35µl of MQH_2O, and 5µl of the 10x enzyme mix were added together whilst keeping cool on ice. The mixture was then vortexed and spun at 15,000RPM for 5 seconds. The mix was then incubated at 15^0C for 90minutes. 5µl of stop buffer was then added followed by 4.6µl of sodium acetate pH5 (Sigma S-7899), 1µl of 20mg/ml glycogen (Boehringer Mannheim 901393) and 122µl of iced ethanol. This mixture was then vortexed, frozen at -72^0C for 15 minutes and then spun at 14,000RPM for 30 minutes at room temperature. The supernatant was then aspirated from the preparation, and the pellet was vacuum desiccated for 20 minutes. 10µl of TE (pH8) was then added to the pellet. This was vortexed and spun briefly and then left at room temperature for 2 hours until the pellet had completely dissolved.

Preparing probe;

Solutions:

Hyb Buffer	5ml formamide (Fluka 47670) 2ml 50% dextran sulphate 0.5ml 20xSSC 2.5ml sterile water	50% 10% 1xSSC
20xSSC	3M Sodium chloride 0.3M Trisodium citrate made up to 1L with purified water	
2x SSC	50ml 20xSSC 450ml purified water	
70% formamide	35ml formamide (Fluka47670) 5ml 20xSSC 10ml purified water	
50% formamide	25ml formamide (Fluka47670) 5ml 20xSSC 20ml purified water	
Milk wash	25g Marvel 400ml distilled water 100ml 20xSSC 250µl Tween	
FITC avidin	2.5µl fluoroscein avidin DCS 1ml milk wash	
Biotinylated anti-avidin	2.5µl Biotinylated anti-avidin D 1ml milk wash	
x4SSC with T	250µl Tween 20 500ml 4xSSC	
Counterstain	30µl of 40µg/ml DAPI 150µl citifluor AF1 mountant	

0.5µl of probe DNA, 1µl of total human DNA and 8.5µl of Hyb buffer were added to an eppendorf tube. The preparation was vortexed and spun, and then incubated first at 72^0C for 5-10minutes and then at 37^0C for one hour.

Metaphase spreads were prepared from heparinised blood using standard cytogenetic techniques. The slides were then washed by placing in a series of coplin jars first with x2SSC for 2 minutes, then 70% ethanol for 2 minutes, 85% ethanol for 2 minutes and 100% ethanol for 2 minutes. The slides were allowed to air dry. The chromosome spreads were then denatured in 70% formamide at 72^0C for 2 minutes then put in 70% ethanol at -20^0C for 2 minutes. The ethanol series was repeated and the slides allowed to air dry. 5µl of the probe was pipetted onto the slide and covered with a cover slip. The cover slip was sealed in place by pipetting cow gum around the edge of the slip. The slides were then incubated overnight in a water bath at 37^0C. The cover slip was removed and the slides were then washed twice in 50% formamide at 45^0C for 5 minutes. The slides were then washed twice in X2SSC at 45^0C for 5 minutes. The slides were then blocked with milk wash, covered with parafilm and incubated at 37^0C for10 minutes. The parafilm was then removed and 150µl FITC avidin added to the slide. This was covered with parafilm and incubated at 37^0C for 15 minutes. After removing the parafilm the slides were then washed twice in x4SSC with T for 5 minutes at room temperature. 150µl of biotinylated antiavidin was then placed on the slide, covered with parafilm and incubate at 37^0C for 15minutes. The parafilm was then removed and the slides were washed twice in x4SSC with T for 5 minutes. 150µl FITC avidin was then added to the slide. This was covered with parafilm and incubated at 37^0C for 15 minutes. After removing the parafilm the slides were then washed twice in x4SSC with T for 5 minutes at room temperature. 150µl Citifluar and 30µl dapi counter stain were then placed on the slide and covered with a cover slip. All probes were first applied to control chromosomes to check their approximate locations. 'Walking' along the chromosome in this way it was hoped that a single clone would be found to span each breakpoint. The sequence of this clone could then be used to

make sequential 10kb probes to further refine the breakpoints. The Ensembl Human Genome Browsers would be consulted to see if the breakpoints occurred in or near any genes which might be good candidates for CHARGE association. The Clones were supplied by the MRC Human Genetics Unit, Edinburgh and the Sanger Institute Mapping Core group. DNA was extracted according to the CHORI BACPAC Resources miniprep method (http://www.chori.org/bacpac/). FISH signals were visualized using the Cytovision image analysis system (Applied Imaging).

The sequential 10kb probes were produced by long range PCR (using the LRPCR kit, Roche) with primers designed on the PRIMER3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) .The sequence data for the clone spanning the breakpoint was provided by the Ensembl Human Genome Browser. Prior to primer design repetitive sequences were masked using RepeatMasker (http://www.repeatmasker.org/). The primer sequences were then put through a genome blast search using the NCBI human genome browser website. (http://genome.ucsc.edu/cgi-bin/hgGateway).

Preparing LRPCR products for probes:

A 10mM dNTP mix was made up by adding together 300µl of MQH_2O,50µl of dATP(100mM),50µl of dCTP(100mM),50µl of dGTP(100mM) and 50µl dTTP(100mM). This was vortexed, spun and then stored at -20^0C.

To make the 8900kb probe 1.75µl of dNTP mix, 1.47µl of each primer, 5µl of buffer 1, 1µl of genomic or BAC DNA, 0.75µl of enzyme and 38.5µl of MQH_2O were mixed together on ice.

To make the probes 14000kb,12000kb, 9800kb, 9850kb, 12269kb, 10.1kb, Fish2, Fish 3 and 9.1kb, 2.5µl of dNTP(10mM), 1.4µl of each primer, 5µl of buffer 2(buffer 3 for

14000kb), 1µl of DNA, 37.8µl of MQH_2O, and 0.75µl enzyme were added together on ice. These preparations were then placed in a PCR machine into which the following program had been entered: a. 92^0C for 2 minutes, b. 92^0C for 10 seconds, c. 65^0C for 30 seconds, d. 68^0C for 8 minutes, e. go to step b 9 times, f. 92^0C for 10 seconds, g. 65^0C for 30 sec, h. 68^0C for 8 minutes, increasing step h. (elongation time) by 20 seconds per cycle, i. go to step f.,19 times, j. 68^0C for 7 minutes, .k. 15^0C forever. The PCR products were then purified using Quick™ 2 PCR Purification Kit (Edge Biosystems).

The DNA for these LRPCR products was quantified by making a 1/100 dilution with MQH_2O and checking the O.D. reading on a spectrophotometer (Pharmacia Biotech GeneQuantRNA/DNA calculator). The O.D. reading was used to calculate the volume of PCR product required for 1µg of DNA for use in Nick translation. The LRPCR products were labeled by Nick translation using the BioNick™Labeling System (Invitrogen) as described above for labeling the BAC probes but with the volume of MQH_2O varied to make a total reaction volume of 50µl. When preparing the slides half the slide area had probe alone the other half had the probe and the chromosome 8q telomere added to aid in identifying the chromosomes.

Results

Notes review

230 sets of patient's notes were identified from the patient lists generated from the genetics databases and hospital coding. 177 sets of notes have been reviewed. Seven individuals fulfilled the diagnostic criteria for CHARGE association. A further three individuals with coloboma + 2 other features and five with choanal atresia + 2 other features were included in the clinical review. Eight patients were identified for further genetic review as completion of the proforma suggested a likely diagnosis not previously recorded. Fifty-seven patients had another known diagnosis

The birth rate in Glasgow is approximately 10,000/yr; if the incidence of CHARGE association were 1/10,000 then we would expect to identify ten patients with CHARGE in 10 years. Our data confirms an incidence of 1/10,000 although it may be an under estimate as coding only lists the first 6 diagnoses on any hospital admission and not all individuals with suspected CHARGE are referred to genetics

Cytogenetic results

Giemsa banding of metaphase spreads at 550 band resolution had shown a *de novo* apparently balanced chromosome translocation 46,XX,t(8;13)(q11.2;q22), Figure 5. The initial FISH experiments using BAC probes, 137L15, 401H2 and 56A10 showed that the chromosome 8 breakpoint was distal to q11.2 as these three probes all localized to the derivative chromosome 8. The 242M12 probe localized to chromosome 10q and was therefore not of use in the breakpoint analysis.

Walking the chromosome using FISH with mapped BAC probes refined the breakpoints to 8q12.2 and 13q31.1 (Tables 3 & 4, Fig 6-10)

Table 3 Hybridization of Chromosome 8 clones to Twin 1

	Map Location		FISH Result	
Probe Name	Band	MB from p Telomere	Clone Present der(8)	Clone Present der(13)
RP11 137L15	q11.21	48.60Mb	YES	NO
RP11 401H2	q11.22	52.20Mb	YES	NO
RP11 56A10	q12.1	55.82Mb	YES	NO
RP11 114M5	q12.1	59.40Mb	YES	NO
RP11 414L17	q12.2	61.40Mb	YES	NO
RP11 33I11	q12.2	61.80Mb	YES	YES
RP11 35A5	q12.2	61.80Mb	NO	YES
RP3 491L6	q12.2	61.83Mb	NO	YES
RP11 317H6	q12.2	62.29Mb	NO	YES
RP11 227F6	q12.3	62.40Mb	NO	YES
RP11 45K10	q12.3	64.48Mb	NO	YES
RP11 115G12	q12.3	65.34Mb	NO	YES
RP11 366K18	q13.1	67.12Mb	NO	YES
RP11 21C5	q13.3	69.42Mb	NO	YES

Table 4 Hybridization of Chromosome 13 clones to Twin 1

	Map Location		FISH Result	
Probe name	Band	MB from p Telomere	Clone Present der(8)	Clone Present der(13)
RP11 370A2	q21.33	67.13Mb	NO	YES
RP11 309H15	q22.1	71.56Mb	NO	YES
RP11 226E21	q22.3	75.46Mb	NO	YES
RP11 421K11	q31.1	79.62Mb	NO	YES
RP11 533P8	q31.1	79.83Mb	NO	YES
RP11 115N13	q31.1	81.0Mb	YES	NO
RP11 464I4	q31.1	81.5Mb	YES	NO
RP11 366K1	q31.1	85.3Mb	YES	NO
RP11 275J18	q31.2	87.4Mb	YES	NO
RP11 388D4	q31.3	88.2Mb	YES	NO
RP11 632L2	q31.3	91.4Mb	YES	NO

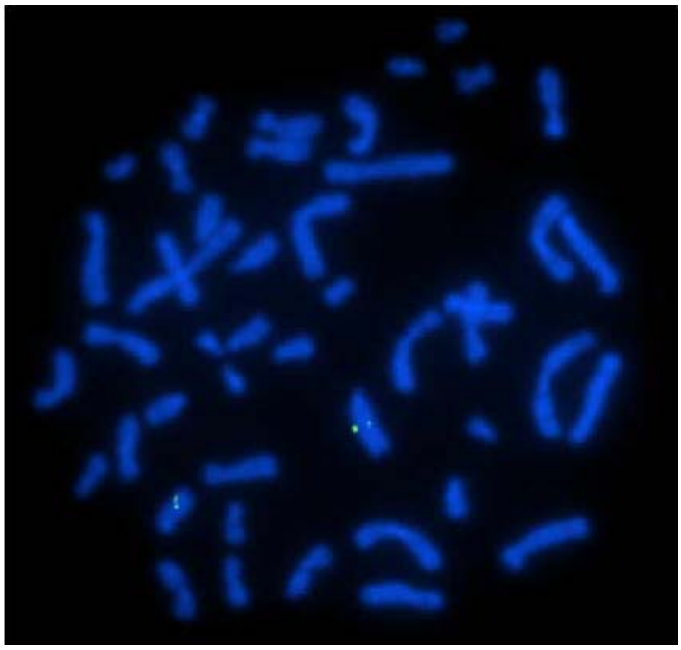

Figure 6. Hybridization of clone RP11 533P8 to twin 1 shows signals on the derivative and normal chromosome 13.

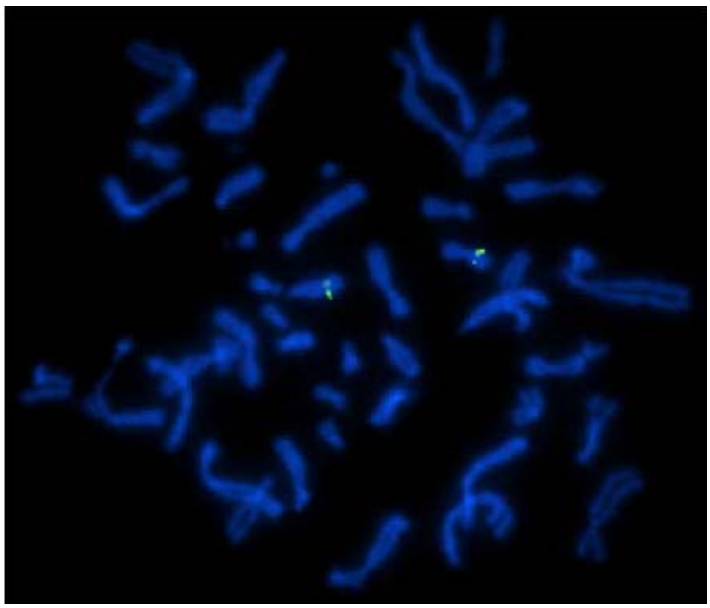

Figure 7. Hybridization of clone RP11 115N13 to twin 1 showing signals on the derivative chromosome 8 and the normal chromosome 13.

The chromosome 13 breakpoint was resolved to 1.17Mb. This region contained no obvious candidate genes.

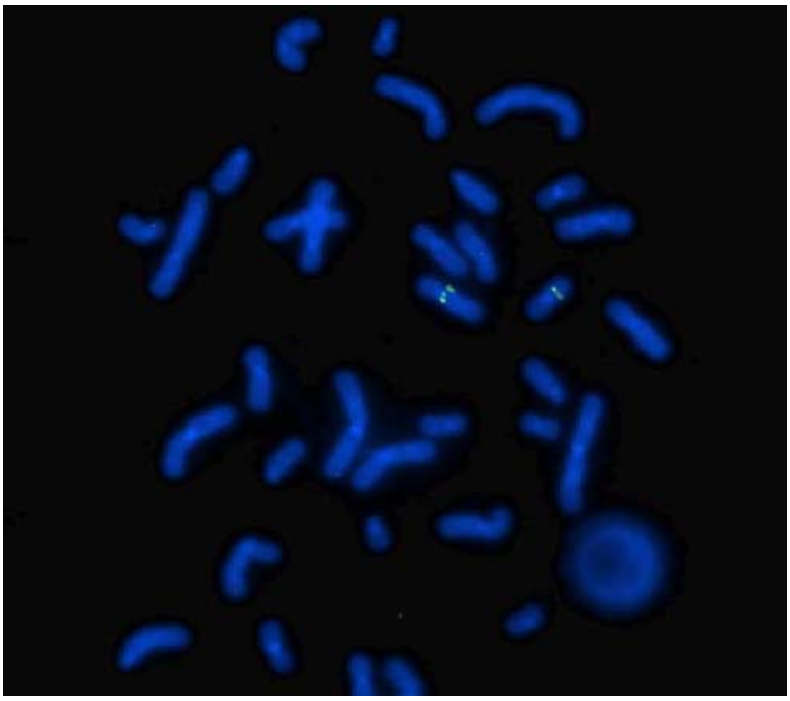

Figure 8. Hybridization of clone RP11 414L17 to twin 1 showing signals on the derivative chromosome 8 and the normal chromosome 8.

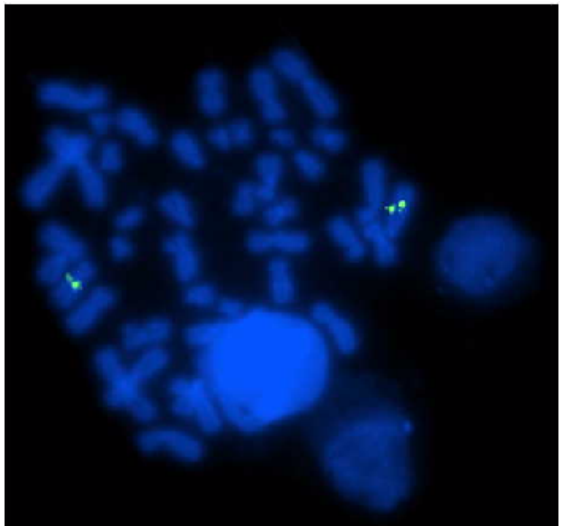

Figure 9. Hybridization of clone RP 3491L6 to twin 1 showing signals on the derivative chromosome 13 and normal chromosome 8.

Clone RP11 33I11 was found to span the breakpoint on chromosome 8 (Figure 10).

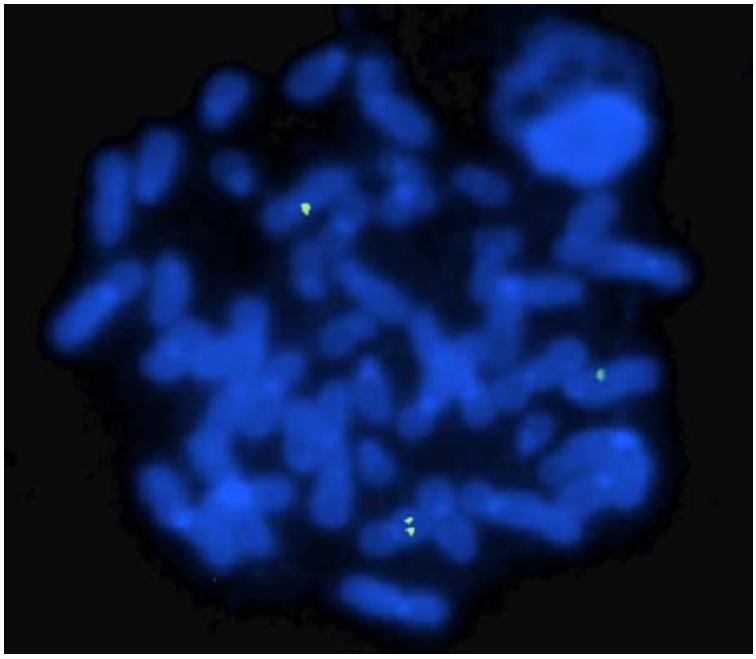

Figure 10. Hybridization of clone RP11 33I11 to twin 1 showing signals on both derivative chromosomes 8 and 13 and the normal chromosome 8.

The Ensembl database identifies two genes in this region, *Q7Z6C0* (*Q66K35*) and *CHD7* (chromodomain-helicase-DNA-binding protein 7). Clone RP11 414L17, which includes most of the sequence for Q7Z6C0, is present only on the derivative chromosome 8. This clone contains *CHD7* exons 1 and 2 sequence. The remainder of the *CHD7* sequence is present in clone RP11 33I11. Thus *CHD7* was disrupted by the translocation. FISH probes produced by long range PCR from sequence data for RP11 33I11 and *CHD7* were then used to do fine mapping of the translocation breakpoint on the derivative chromosome 8. Table 5 shows the names of the probes, listed in order centromere to telomere, left to right and below the size of the PCR product. A further probe 14000kb1, which encompassed 9.1kb, failed to amplify.

Probe name	FISH3	FISH2	10.1kb	9.1kb	1200KB2	9800KB3	8900KB3	9850KB4	12269KB5
Product size (bp)	9229	9848	9167	9121	11938	9468	8185	9063	11869

Table 5. Names of the LRPCR probes and their product sizes

FISH2 failed to hybridize to the metaphase preparations. 10.1kb localized to chromosome 8 and the derivative chromosome 8 (See Figure 11). 9.1kb localized to both derivative chromosomes (See Figure 12). Probes 12000kb2 and 9800kb3 localized to the normal 8 and the derivative chromosome 13. Thus the manufactured probes using RP11 33I11 sequence data showed that the breakpoint was between exons 3 and 8 (See Figure 13).

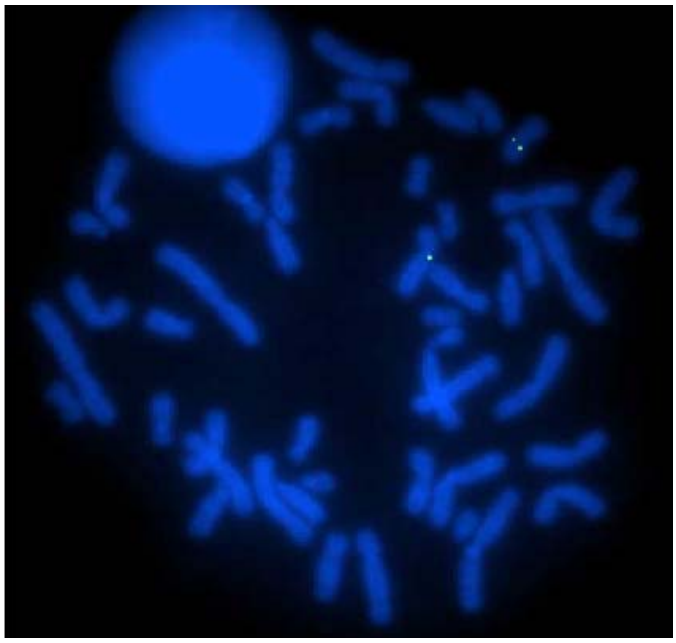

Figure 11. Hybridization of probe 10.1kb to twin 1 showing signals on the normal chromosome 8 and the derivative chromosome 8.

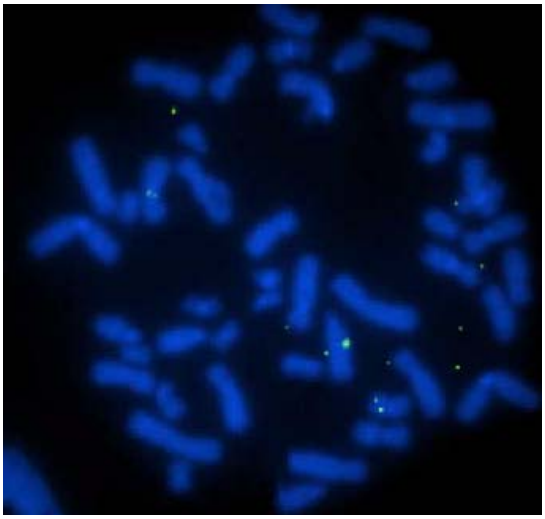

Figure 12. Hybridization of probe 9.1kb to twin 1 showing signals on the derivative chromosome 8 and the derivative chromosome 13.

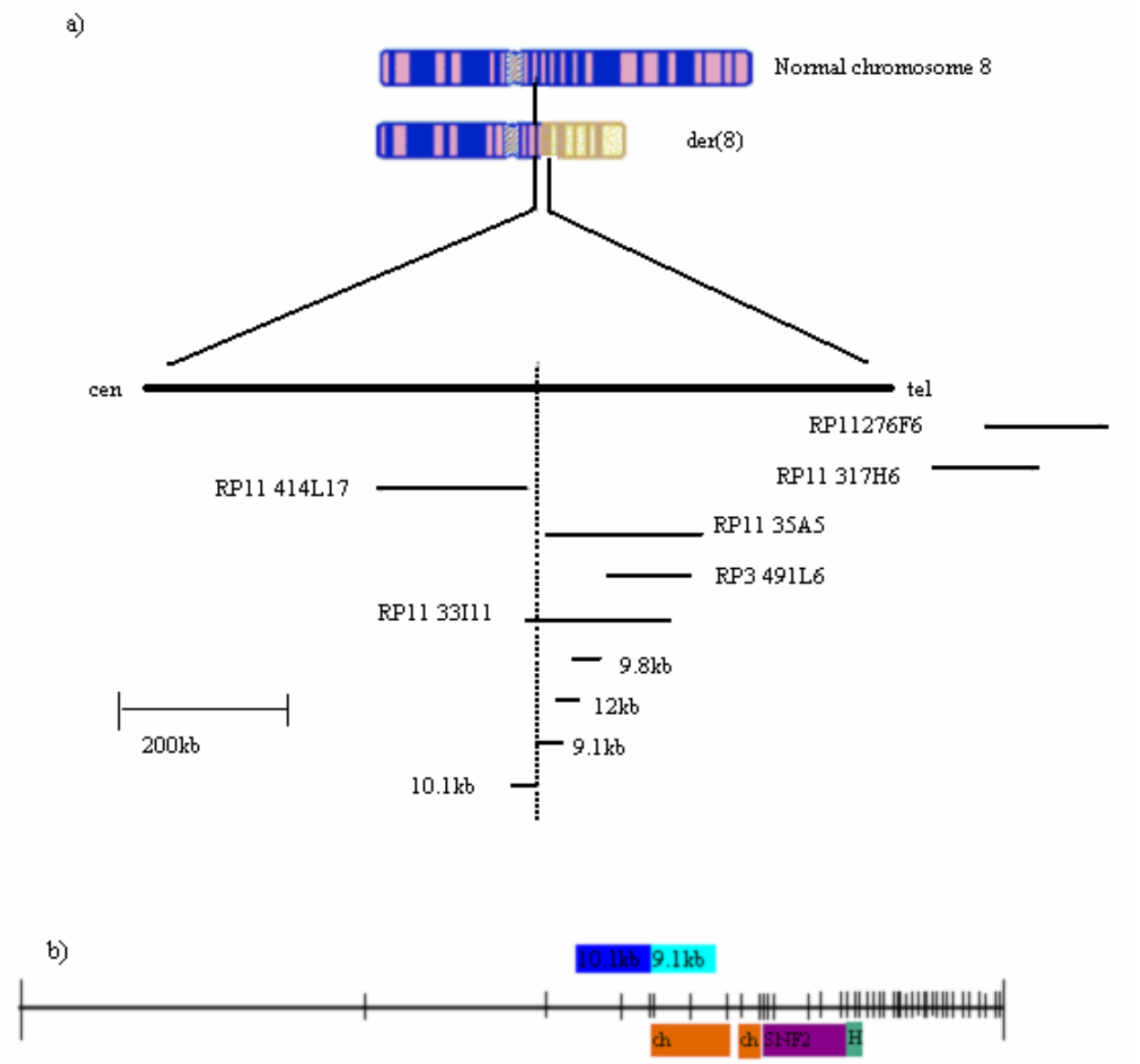

Figure 13. a) Schematic showing mapping of chromosome 8 breakpoint. Clones to the right of the dotted Line mapped to the der 13 and normal 8. b) Schematic of *CHD7* gene with positions of 10.1 and 9.1kb probes show *CHD7* is disrupted between exons 3 and 8. Short vertical Lines represent exons, chromodomain (ch), SNF2 domain (SNF2) and helicase domain (H).

Probe 10.1kb (which spans exon 4 and 5) localizes to both the normal and the derivative chromosome 8, and probe 9.1kb (which spans exons 6 and 7) localizes to

both derivative chromosomes 8 and 13 and the normal 8. The 9.1kb signal was not present on the der (8) in every cell, which would be consistent with the effect of a smaller portion of the probe sequence being present on the der (8). These findings might suggest that the breakpoint lies within the 9.1kb probe region, but the fact that the signal was not present on the der(8) in every cell prevented this from being concluded with certainty.

As the breakpoint mapping had identified that the gene *CHD7* was disrupted in our probands we then went on to perform sequence analysis of this gene in a cohort of patients with a diagnosis of CHARGE syndrome or probable CHARGE.

Materials and Methods: Molecular

Mutation analysis of *CHD7* in CHARGE and CHARGE- like cases:

CHD7 has a genomic length of 188kb with 9000 coding bases over 37 exons. The DNA sequence data for exons in *CHD7* was obtained from the UCSC Human genome Browser and ENSEMBL databases (http://genome.ucsc.edu/cgi-bin/hgGateway and http://www.ensembl.org/). The files copied from the databases were transformed into Word documents with sequence extending at least 50bp to either side of each exon. These files then had their repetitive sequences masked by entering the data into RepeatMasker (http://www.repeatmasker.org/). The masked files were used to design the primers to amplify each exon with the primer 3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer design aimed to have the primers at least 20bp outside the reading frame and aiming for a final product <500bp in length so that they could be sequenced using 36cm capillary tubing which is fitted as standard in our sequencer (ABI 3100).
Subsequently some primers were redesigned to produce longer fragments to reduce the total number of sequencing reactions required. These fragments were sequenced in a MegaBACE 1000 (GE Healthcare) using cleanup and sequencing methods 2.

The Primers were ordered from Sigma. On receipt of the primers they were resuspended in Xμl of 1x TE pH 8 as per the product sheet to make up a 100μM solution (A). The suspension was then vortexed and pulse spun. 25 μl of solution A was then added to 475μl of 1xTE pH 8, vortexed and pulse spun to make a 5μM working stock (B). The efficacy of each set of primers was first tested on control DNA samples with a PCR reaction mix containing 2.5μl each of the forward and reverse

primer, 1.5 µl of 1/5 diluted control DNA , 12.5 µl of Quiagen multiplex PCR mix and 6 µl of UV treated MQ dH_2O . These were run with the following program; denatured at 95^0C for 15 minutes then 34 cycles of 95^0C for 45seconds, annealing at 54-65^0C for 1 minute and 30 seconds, elongation at 72^0C for 45 seconds. Subsequent primers were designed using UCSC in silico PCR and NGRL SNP checker (by Jaqueline Rice). Details for the DNA sequence of the primers, the size of the fragments produced and the annealing temperatures used in each PCR reaction are shown in appendix 1. To test the success of the PCR reaction the product was run on a test gel as follows: briefly vortex and pulse spin PCR product, add 5 µl each product to 1 µl loading mix (LM), vortex and pulse spin. This mix was then run on a small 1% agar gel at 80V for 20-25 minutes with a 100kb ladder (Invitrogen). (The gel consisted of 30ml 1 x TE to which 0.3g of seagam agarose was added. This was microwaved for approximately 90seconds, then 2.5µl of Ethidium bromide was added and the gel poured). The gel was then photographed under UV light. The PCR product was then cleaned either using Shrimp Alkaline phosphatase or the Ampure system. For the former, 2 µl Shrimp Alkaline phosphatase was added to 2 µl of a 1/10 dilution of Exonuclease 1 with Exonuclease 1 buffer. This mix was vortexed and spun and added to the PCR reaction, vortexed and spun again and run on the SHRIMP program which is: 37^0C for 30 minutes, 72^0C for 15 minutes, and then ramp to 4^0C. For the Ampure system (AMPure PCR Purification (Agencourt Bioscience Corporation) the following method was used: 15µl of each PCR product was transferred into wells in a 96 well plate. The AMPure magnetic particle solution was shaken to resuspend the particles and 27µl was pipetted into each well. The plate was covered with a plate seal and vortexed for approximately 30 seconds. The plate was then incubated at room temperature for 5minutes. The plate was then placed onto the SPRIPlate® 96R magnetic plate for 5-10mins. The cleared

solution was then aspirated from each well in the reaction plate and discarded. 200µl of 70% ethanol was then added to each well. This was incubated at room temperature for 30 seconds. The ethanol was then removed by aspiration and discarded. A second aliquot of 200µl of 70% ethanol was added and the rest of this step was repeated. The plates were then allowed to air dry for ten to twenty minutes. 40µl of MQH_2O was then added to each well, the plate was covered with a plate seal and vortexed for 30 seconds.

The cleaned product was then labeled using either ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied biosystems) or DYEnamic™ ET Dye Terminator Kit (MegaBACE™)

ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit labeling:

0.2ml PCR microtubes were labeled with an identifier. To each tube was added 0.5 µl of the forward or reverse primer, 1-2 µl of the cleaned product(depending on the strength of the band on the test gel), 0.5 µl of ABI Big Dye v3.1, 2 µl of buffer and 5-6 µl of UV treated $MQdH_2O$(depending on the amount of product used) to make a total reaction volume of 10 µl.This mix was pulsed and spun and the BigDye program run: The BigDye program consists of 30 cycles of (95^0C for 10seconds, 50-60^0C for 20seconds, 72^0C for 4 minutes) and then ramp to 4^0C. The annealing temperatures varied according to the PCR annealing temperatures. For example fragment 2B had 65^0C PCR annealing temperature and 60^0C sequencing annealing temperature.

DYEnamic™ ET Dye Terminator Kit (MegaBACE™) labeling:

For each reaction 2 µl of DYEnamic ET terminator (MegaBACE) reagent premix, 2 µl of dilution buffer as supplied in kit, 1 µl of primer at 3.2 - 5 µM, cleaned PCR product

approximately 10ng/100 bases and MQH_2O to make the volume up to 10 µl were added to a well in a 96 well plate. All reagents were briefly spun to the bottom of the wells and then run in a thermal cycler for 40 cycles of ($95^oC$for 20seconds, 50^oC for 15 seconds, 60^oC for 1minute) and then ramp to 4^oC.

The labeled product was then cleaned by one of two methods, either method 1if the BIGDYE system had been used or method 2 if the Ampure system was used.

Sequence cleanup method 1:

A fresh 3100 MicroAmp Optical 96-well Reaction Plate and balance plate were collected. The plate was labeled with the next plate name for the ABI 3100 sequencer. The cleanup solution was made by adding Xµl (X=N^o of samples (+20) x 5µl) of 125mM EDTA to Yµl (Y= N^o of samples (+20) x 60 µl) of 100% EtOH. 65 µl of this mixture was added to each microtube of labeled product, mixed and transferred to a well in the reaction plate. Sample 1 goes in 1A, sample 2 in 1B, sample 8 in 1H, sample 9 in 2A and so on. The plate was then covered with a plate seal. The same number of wells in the balance plate were filled with 80µl of water. The balance plate was covered with a plate seal. Both plates were then left for 30-60 minutes at room temperature. Next the plates were spun in plate holders at 3000xg for 45 minutes at 10^0C. The plates were then removed from the centrifuge and the plate holders. The plate seals were removed and the plates tipped upside down onto pieces of tissue. Keeping the plates upside down they were placed on fresh tissues and gently tapped. The plates were then replaced upside down in the centrifuge and pulse spun to 200 x g. After removing the plates from the centrifuge 150 µl of 70% EtOH was added to each of the sample wells and 150 µl of water added to the corresponding wells in the balance plate. The plates were covered with fresh plate seals and then spun at 3000 x g

for 20minutes at 10^0C. The plates were then removed from the centrifuge and the plate holders. The plate seals were removed and the plates tipped upside down onto pieces of tissue. Keeping the plates upside down they were placed on fresh tissues and gently tapped. The plates were then replaced upside down in the centrifuge and pulse spun to 200 x g. The sample plate was then covered with a paper towel and left at room temperature to air dry for 30-60minutes.

10 µl of Hi-Di formamide was then added to each sample well tapping the bottom of the plates to get the Hi-Di to the bottom of the wells and remove any bubbles. The plate was then covered with a plate septa and wrapped in a paper towel. The plate was then left at room temperature for at least 2 hours to allow the pellet to resuspend. The plates were then put in the ABI 3100 and the samples run.

Sequence cleanup method 2(CleanSEQ Dye Terminator Removal (Agencourt Bioscience Corporation) :

The CleanSEQ magnetic particle solution was shaken to resuspend the particles. 5µl was then pipetted into each sample well in a 96 well plate containing 10µl of the sequencing reaction. 70µl of 85% ethanol was then added to each well, the plate was covered with a plate seal and vortexed for 30seconds. The plate was then left to incubate for three minutes at room temperature. The reaction plate was then placed onto the SPRIPlate®96R magnetic plate for three minutes. The supernatant was then aspirated from each well and discarded. 100µl of 85% ethanol was then added to each well and incubated at room temperature for thirty seconds. The ethanol was then aspirated out and discarded. A further 100µl of 85% ethanol was then added to each well, incubated at room temperature for thirty seconds. The ethanol was then aspirated out and discarded. The plate was then allowed to air dry for ten minutes. 30µl of

MQH_2O was then added to each well. The reaction plate was placed onto the SPRIPlate®96R for three minutes. 15µl of the solution in each well was then transferred to a new plate before loading onto the MEGABACE™ Sequencer. PCR and sequencing was performed on the 37 coding exons (2-38) which was covered by 39 fragments. Once amplified and sequenced the trace was compared to a reference trace in Mutation surveyor derived from the NCBI Genbank reference genomic DNA (NT_008183) and cDNA (NM_017780.2)

In silico analysis using existing publications, databases and bioinformatics tools were used to assess the significance of any sequence changes which were detected. Where possible parental samples were used to assist in determining the likelihood of previously unreported sequence changes being pathogenic.

Statistical Analysis

Statistical comparison of the clinical features found in mutation positive and mutation negative individuals were made using the Fisher exact test. This test was used as it produces more accurate P values with small sample sizes than the Chi squared test (Fisher 1922).

Results: Molecular

One third of the exons were optimized for sequencing using control DNAs. PCR conditions for these exons are uniform. See table 6. 4 mutations were identified in these exons. See Table 9. Modifying PCR/sequencing conditions for the remaining exons was performed by Jacqueline Rice as detailed in appendix I. MLPA on the mutation negative cases was carried out by Jacqueline Rice.

Table 6. Reaction conditions for exons using control DNA

.		Denature		Anneal		Elongation		Cycle No.
Exon No.	Amplicon length(bp)	Temp	Time	Temp	Time	Temp	Time	
4	485	94	30”	55	30”	72	1’	37
6	308	94	30”	55	30”	72	1’	37
8	374	94	30”	55	30”	72	1’	37
10-11	493	94	30”	55	30”	72	1’	37
13	452	94	30”	55	30”	72	1’	37
14	382	94	30”	55	30”	72	1’	37
15	483	94	30”	55	30”	72	1’	37
16	418	94	30”	55	30”	72	1’	37
27-28	437	94	30”	55	30”	72	1’	37
32	355	94	30”	55	30”	72	1’	37
35	451	94	30”	55	30”	72	1’	37

Results: Clinical

Samples were obtained from 45 individuals. Clinical examination was carried out by the author or clinical information was provided by completion of the proforma. The clinical details are summarized in table 7. Case reports with clinical photographs of 9 individuals to illustrate the range of features which can occur in this syndrome are in appendix II. In our cohort 20 individuals met the clinical criteria as defined by Blake *et*

al (1998). When Verloes criteria are used 8 individuals had typical CHARGE, 1 partial CHARGE and 25 atypical CHARGE, a total of 34. These figures are likely to be an under estimate, as those with an atypical diagnosis may really have typical CHARGE as the majority of individuals had not had their semicircular canals imaged, nor any endocrine studies performed. Despite this all individuals who were diagnosed as CHARGE using Blake's criteria were also diagnosed as at least atypical CHARGE using Verloes criteria.

Table 8 shows the absolute numbers and percentage of individuals in our cohort with each of the more common clinical features compared to the previous cases reported in the literature. The figures are comparable with the exception of choanal atresia which was less common in our cohort (28%vs 53%), genital hypoplasia (42% vs.71%) although this is often under reported, and cleft palate (33% vs. 56%). In contrast tracheoesophageal fistula was twice as common in our cohort (15% vs. 6.5%).

Table 8. Percentage of individuals in cohort with each of the more common clinical features compared to the previous cases reported

Malformation	Literature Cases (%)	This cohort (No (%)
Coloboma	83/105(79%	34/45 (75%)
Heart disease	76/106(72%)	35/45(75%)
Choanal atresia	53/100(53%)	13/45(28%)
Retarded growth	74/89(83%)	30/45(67%)
MR/CNS anomalies	88/100(88%)	33/45(73%)
Genital hypoplasia	61/86(71%)	19/45(42%)
Ear anomalies/deafness	94/104(90%)	37/45(82%)
Micrognathia/cleft palate	37/66(56%)	15/45(33%)
Facial palsy	46/94(49%)	17/45(37%)
Swallowing difficulty	27/66(41%)	15/45(33%)
Tracheoesophageal fistula	4/62(6.5%)	7/45(15%)

Table 7. Clinical details in cohort of 45 individuals in whom a diagnosis of CHARGE syndrome was suspected

	coloboma	CN palsy	characteristic ear	Choanal atresia	Heart defect	Genital hypoplasia	Delay, growth/ development	Orofacial cleft	TOF	Hormone deficiency	other	mutation
ZA	-	vii	+	-	-	Small uterus/ovaries	Post/mild	palate		hghg	Severe b/l mixed hl, b/l SCC abnormality, kyphoscoliosis	Nonsense n
BA	-	Vii,viii	+	-	-	cryptorchid	Post/mod	-	-	?	b/l SCC abnormality	Missense? significant
JB	?	-	+	?	Asd, vsd, dysplastic pulmonary valve, b/l superior caval vein	cryptorchid	Pre & post	-	-	?	Bronchomalacia Talipes polymicrogyria	none
NB	b/l iris,retina & optic disc	?ix,x	small	-	Pda,asd, dysplastic aortic valve	micropenis	Post/mild	palate	-	?	Probable b/l hl	Frameshift n
KB	-	-		L+	asd	-	mod	Bifid uvula, submucous palate	-	?	Otitis media	none
RC	b/l microphthalmia, retina,choroid, disc coloboma	viii	+	+	Avsd,rvot	-	Post/profound	lip	-	?		Frameshift n
JC	b/l fundus, optic disc	-	+	-	Tof,dorv,vsd,ps,pda	Micropenis, cryptorchid	Post/mod	-	+	-	Otitis media hypocalcaemia	Nonsense pr
JD	b/l choroid, L disc	Ix,x	+	-	pda	Micropenis, cryptorchid	Post/mild	-	-	Low testosterone	Hypocalcaemia, LSNHL	Frameshift n

Table 7. Continued

	coloboma	CN palsy	Characteristic ear	Choanal atresia	Heart defect	Genital hypoplasia	Delay, growth/ development	Orofacial cleft	TOF	Hormone deficiency	other	mutation
ZD	b/l. retinal	-	-	-	-	Small uterus ?ovaries present	Post/delay	palate	-	?	u/l snhl u/l duplex renal collecting system	Nonsense n
CD	b/l retinal	Viii,ix, x	+	-	Vsd/pda	-	Post/mod	-	-	?	Radial ray defect L arm, ?tracheomalacia	Nonsense n
CF	b/l optic disc	-	+	-	Pda, wpw	Micropenis, cryptorchid	Post/?mild	b/l Cl/p	+	-	Profound b/l snhl, otitis media, vertebral anomalies	Frameshift n
BF	L choriod	R vii	+	L atresia	Pda, pfo,*	Hypoplastic labia majora	Post/mild	-	-	?	Severe b/l mixed hl, agenesis R kidney	Nonsense n
RF	L optic disc	?vii	+	L atresia	-	cryptorchidism	Pre & post/mild	-	-	hghg	Severe b/l snhl, facial asymmetry, vertebral malformation	Nonsense n
EG1	R iris b/l retinal	R vii, ?b/l ix&x	+	-	Pda,asd	-	Post/mild	-	-	?	Gord, hl?type	Frameshift n
EG2	b/l retinal, L iris, microphthalmia	Ix,x	+	-	Pfo,pda, asd, Raa	-	Post/severe	b/l cl/p	-	?	b/l snhl Otitis media, tracheomalacia	Splice site n
SG	Iris	-	-	?L stenosis	Pulmonary atresia & vsd	-	-/-	Sub mucous cleft	-	?	Imperforate anus	fail
EH1	R retina	Ix,x	-	-	R aortic arch	-	-/mild	-	+	-	Otitis media, preauricular tags, facial asymmetry	none

Table 7. Continued

	coloboma	CN palsy	Characteristic ear	Choanal atresia	Heart defect	Genital hypoplasia	Delay, growth/ development	Orofacial cleft	TOF	Hormone deficiency	other	mutation
EH2	R retinal	?vii	+	-	Vsd, dorv, asd, pda	-	Pre, post/	-	-	?	Small thymus	none
AH 1	b/l retinal	-	+	-	Tetralogy of Fallot	-	-/?	-	-	hypocalcaemia	immunodeficiency	Missense? significant
AH 2	R retinal	-	+	-	-	-	Post/mild,mod	-	+	?	Hearing aid	none
KJ	?L optic disc	Rvii, ?ix,x	+	-	Asd/vsd & rvot	-	Post/yes	-	-	?		fail
EL	-	-	prominent	-	ps	-	No/mod	-	-	-	Microcephaly Malrotation of bowel	none
TL	b/l retinal	Vii ,ix,x	+	-	Asd	-	Post/mod	-	-	?	Snhl, sleep apnoea	Frameshift n
JM1	-	viii	+	-	Pda,Vsd, coarctation	-	post/mod	-	-	?	Hypoplastic SCCs	Nonsense n
CM	-	Rvii, Lviii, ix,x	+	-	Asd, vsd, pda	cryptorchid	No/mod	palate	-	?	Micrognathia, tracheostomy, dislocated hip, hypoplastic SCCs	Nonsense pr
LM	R iris	b/l vi	-	-	Avsd	-	Pre&post/-	-	-	-	Oesophageal atresia, abnormal thumbs(cannot abduct)	none
JM2	+	?	?	?	Vsd,Pda	?	?	?	+	?		Nonsense pr
BM	b/l	-	?	+	Pda	Micropenis	Pre & post/?	-	+	?	Immune deficiency	Nonsense n

Table 7. Continued

	coloboma	CN palsy	Characteristic ear	Choanal atresia	Heart defect	Genital hypoplasia	Delay, growth/ development	Orofacial cleft	TOF	Hormone deficiency	other	mutation
RM	-	-	-	-	CAVSD	cryptorchid	-/?	Cl/p	-	-	Immune deficiency, dandy walker malformation	none
CO	L choroid	RVII	-	-	pda	-	Post/moderate	-	-	Hypo-parathyroid	hemivertibrae	Frameshift n
EP	b/l choroid, R iris & lens	Lvii	+	-	Pda,pfo	-	Post/mod	-	+	?	b/l conductive hl	Frameshift pr
JP	R iris	?	Right anotia	u/l stenosis	Tetralogy of fallot	-	Pre& post/ mild	-	-	?	Klippel feil , facial asymmetry, abnormal SCCs	none
LP	R iris & retina,L microphth	-	protruding	+	vsd	-		-	-	?	Pyloric stenosis	none
IP-S	b/l retinal	R vii	+	-	-	-	?/?	palate	-	?		Splice site pr
MR	+	vii	+	stenosis	+	-	Pre/?	-	-	?		none
JR	?L optic disc	-	R lop	R atresia	-	cryptorchid	-/mod	-	-	-	b/l snhl,,otitis media, poor balance	None
HR	b/l retinal involving optic disc	L vii	+	-	Asd secundum	-	-/mild	-	-	?	L snhl	Nonsense n
FS	b/l retinal	Vi,vii,ix,x	+	+R	Pda, Asd	cryptorchid	Pre&post/mild	Cl/p	-	hghg	Severe hl ?type, hypoplastic SCCs	Nonsense n
DT	-	Ix,x	+	+	Pavd,ps, asd,pda	Left undescended testes	Post/mild	-	-	Low testosterone	b/l snhl, otitis media, pyloric stenosis, caecal volvulus	Nonsense, pr

Table 7. Continued

	coloboma	CN palsy	Characteristic ear	Choanal atresia	Heart defect	Genital hypoplasia	Delay, growth/ development	Orofacial cleft	TOF	Hormone deficiency	other	mutation
JT	retinal	ix,x	+		unspecified ,pda	small	?	Cl/p	+		b/l hl	Nonsense, pr
SW	b/l iris & retina	ix,x	+	-	Pulmonary atresia, vsd	Hypoplasia labia minora	-/Mod,severe	Cl/p	-	-	Microcephaly Small kidneys Hl ?type	Nonsense pr
GW	-	-	+	-	Asd/pavd	-	Post/mild,mod	Palate	-	?	microcephaly	none
KW	b/l retinal & optic disc	?ix,x	+	Partial atresia	-	-	Post/mild	-	-	?	Hypoplastic SCCs, abnormal malleus and incus	Nonsense n
JW	b/l chorioretinal	Vi,vii,viii,ix,x	+	-	-	Cryptorchid, micropenis	Post/mild	-	-	?	Anosmic, hypocalcaemia, hydronephrosis	Frameshift pr
LZ	R optic disc	Lvii	+	-	Pda,pfo,mr	-	Pre,post/severe	-	-	?	Laryngomalacia, b/l snhl	Frameshift n

b/l = bilateral, CN = cranial nerve, Cl/p = cleft lip and palate, GORD = gastroesophageal reflux, hghg = hypogonadotrophic hypogonadism, hl = hearing loss, n = novel, pre = prenatal, post = postnatal, pr = previously reported, SCC = semicircular canal, SN = sensorineural, u/l = unilateral, L = left, R = right, + = present, - = absent *anomalous origin of right subclavian artery from descending aortic arch

17/20 individuals who met Blake's criteria were found to have a mutation. A detection rate of 85%. The detection rate for those with typical CHARGE was 63% (5/8), partial CHARGE 100% (1/1) and atypical CHARGE 88% (22/25). The average detection rate when analyzing individuals who met Verloes criteria was 82% (28/34).

No mutations were identified in individuals who did not have at least atypical CHARGE. Two sequence changes of uncertain significance were found. One in an individual who met diagnostic criteria and one who did not. This is still undergoing evaluation. If one uses only the Blake criteria we would not have tested 11 individuals who were found to have a mutation. If one is using Verloes criteria to make decisions with regards to mutation analysis it is important to arrange imaging of the semicircular canals in individuals who have neither coloboma or choanal atresia. 4 of the 7 in our cohort who had semicircular canal hypoplasia (and a mutation) would not have met diagnostic criteria with out this investigation. Table 9 delineates the mutations in this cohort. . Figure 14 is a schematic illustrating the type of mutation and their position within the gene. 28 mutations were identified of which 20 were novel. 8 of the mutations had been previously reported in other studies. The majority of the mutations were nonsense (16) or frameshift (10) and therefore predicted to cause premature truncation of the protein. There were 2 splice site mutations and 2 missense sequence changes of uncertain significance. The mutations are spread throughout the gene without any hotspots.

Table 9. Results of sequence analysis and MLPA in cohort

Name	Sequence/amino acid change	N/PR	Parental test result	MLPA result
ZA	c.484C>T p.Q162X	N	Not tested	
BA	c.7579A>C p.M2527L	N	Not tested	
JB	No mutation		Not tested	
NB	c.1528delC p.Q510SfsX54	N	Not tested	
KB	No mutation		Not tested	negative
RC	c. 4337_4340delAAAA p.E1446VfsX14	N	Not tested	
JC	c.7879C>T p.R2627X	PR	Not tested	
JD	c.4422_4423insA p.E1475RfsX4	N	*De novo*	
ZD	c.1474C>T p.Q492X	N	Not tested	
CD	c.1969A>T p.K657X	N	*De novo*	
CF	c.1505_1508delCTGG p.P502LfsX61	N	Not tested	
BF	c.2180T>G p.L727X	N*	Not tested	
RF	c.1735C>T p.Q579X	N	Not tested	
EG1	c.5757_5769delCTATCAGCGCAGC p.A1919AfsX7	N	Not tested	
EG2	c.2836-1G>T	N*	*De novo*	
EH1	No mutation		N/A	
EH2	No mutation		N/A	
AH1	Sequence change? significance		Not tested	
AH2	No mutation		N/A	
EL	No mutation		N/A	
TL	c.2627delT p.F877LfsX11	N	Not tested	
JM1	c.2839C>T p.R947X	N*	Not tested	
CM	c.3655C>T p.R1219X	PR	Not tested	
LM	No mutation		N/A	
JM2	c.5428C>T p.R1810X	PR	Not tested	
BM	c.2505T>G p.Y835X	PR*	Not tested	
RM	No mutation		N/A	
CO	c.4527delT p.F1509LfsX37	N	Not tested	
EP	c.8962dupG p.D2988GfsX2	N	*De novo*	
JP	No mutation		N/A	
LP	No mutation		N/A	
IP-S	c.5405-17G>A	PR	*De novo*	
MR	No mutation		N/A	
JR	No mutation		N/A	negative
HR	c.7282C>T p.R2428X	N	Not tested	
FS	c.7291C>T p.Q2431X	N	Not tested	
DT	c.7252C>T p.R2418X	PR	Not tested	
JT	c.7957C>T p.Arg2653X	PR	Not tested	
SW	c.4015C>T p.R1339X	PR	Not tested	
GW	No mutation		N/A	
KW	c.934C>T p.R312X	N	*De novo*	
JW	c.5961delT p.P1987PfsX2	N	*De novo*	
LZ	c.6265_6266ins19 p.E2089AfsX15	N	Not tested	

N/A not applicable, N novel, PR previously reported, * found by author

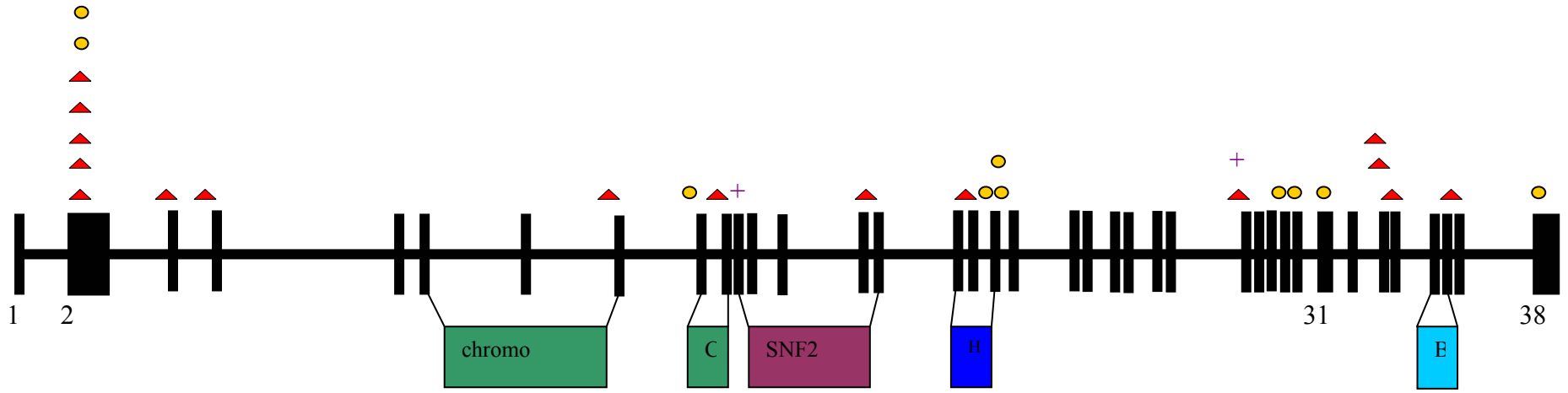

Figure 14. Schematic illustrating the types of mutation and their position in the gene. ▲ nonsense mutation, ● frameshift mutation, + splice site, c/chromo =chromodomain, SNF2 = SNF2 domain, H = Helicase domain, B = Break domain. Domains drawn using amino acid postions from Pfam and NCBI protein accession number NP_060250.2.

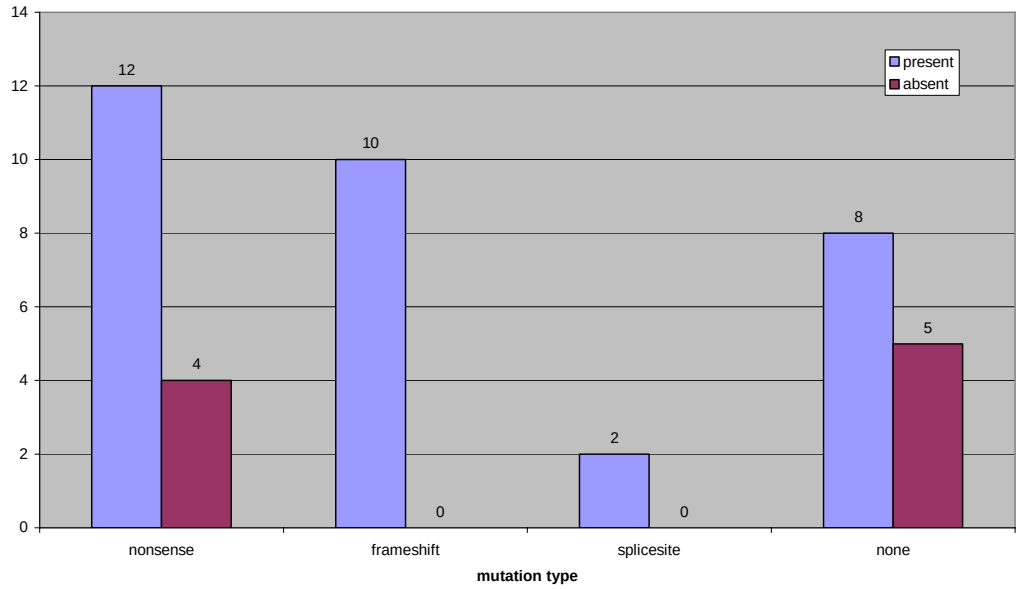

Figure 15. Clinical features 1. Coloboma

Coloboma were present in 24/28 individuals with a mutation (86%) and 8/13 of those who did not have a mutation (61%), (P = 0.113).See figure 15. In those with a mutation a coloboma of one eye was present in four cases; both eyes in 19 cases and one did not specify details. The iris was affected in only five cases that all also had retinal involvement.

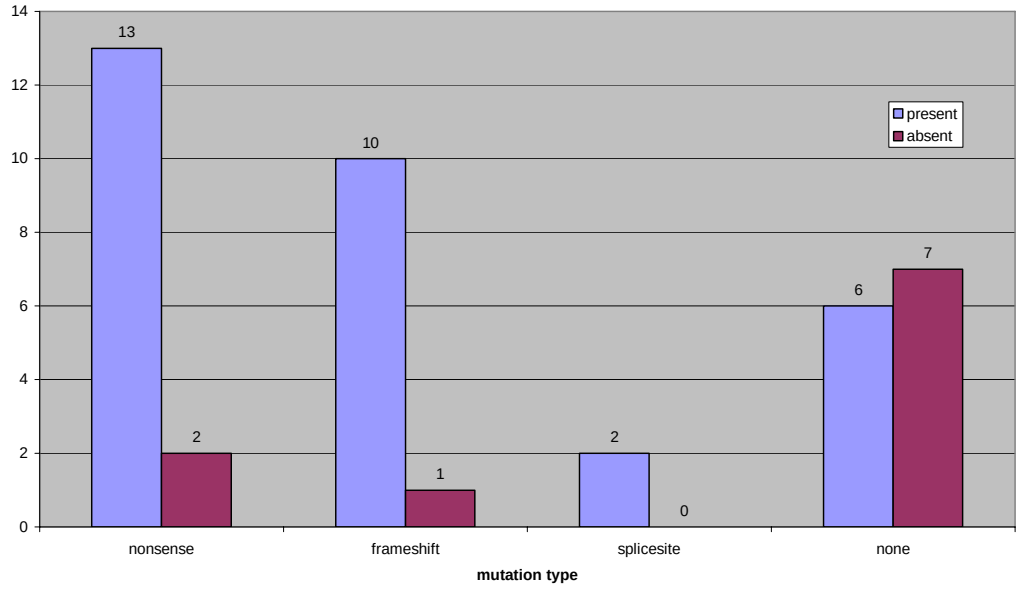

Figure 16. Clinical features 2. Cranial nerve palsies

Cranial nerve palsies were present in 25/27 (93%) of those with mutations. Only 6/13 (46%) of those with no mutation had a cranial nerve palsy, see figure 16. (P = 0.002). In those with a mutation the eighth nerve was most frequently affected with bilateral involvement in twelve cases, unilateral left sided involvement in four cases and unspecified in four cases. The ninth/tenth cranial nerves were involved in thirteen cases. The seventh cranial nerve was involved in twelve cases, one bilateral, five right sided, three left sided and four not specified. The sixth nerve was affected in two cases. The first and twelfth nerves were affected in one case (the same patient).

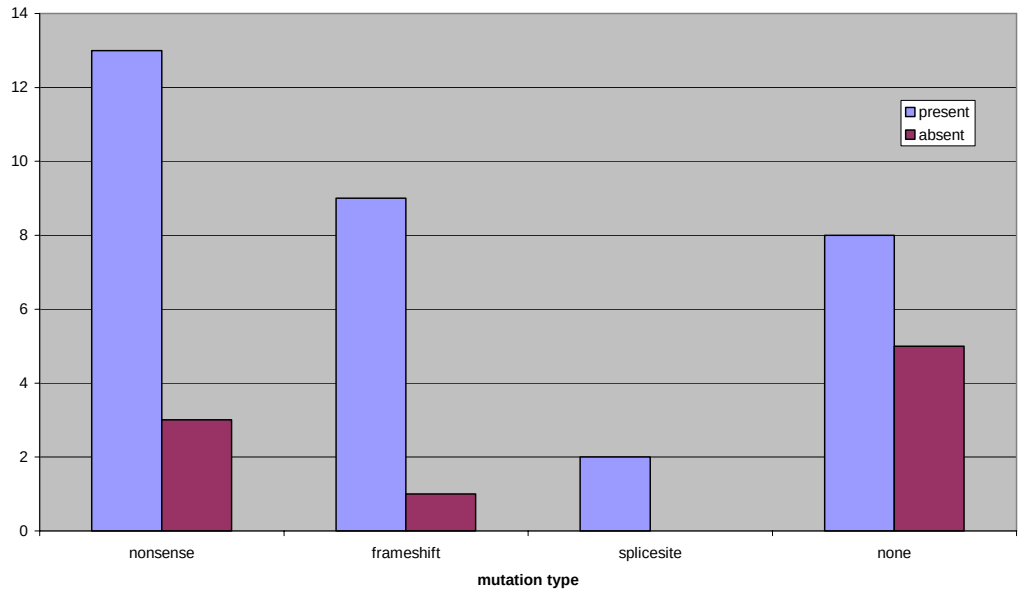

Figure 17. Clinical features 3. External ear anomalies

Figure 17 illustrates the number of individuals with external ear abnormalities in relation to the presence of a mutation and its' type. 13/16 (81%) with a nonsense mutation had an external ear abnormality. Three individuals with a nonsense mutation are shown as not having an external ear malformation, this is because the external ear section of the proforma was not completed in two cases and in the third the ears are said to be normal. 9/10 (90%) of individuals with a frameshift mutation had an external

ear malformation. Both individuals with a splice site mutation had an external ear malformation. Thus an external ear malformation was present in at least 24/28 (86%) individuals with a mutation. In comparison only 8/13 (61%) of those without a mutation had an external ear malformation. (P = 0.02, calculated using figures from complete proforma ie. 24/26 vs. 8/13). The data shows that if an individual does not have an external ear malformation then they are unlikely to have a mutation. The ear malformation ranged from the typical protruding, simple, cup-shaped ear to very subtle abnormalities, absent ear lobe and triangular concha.

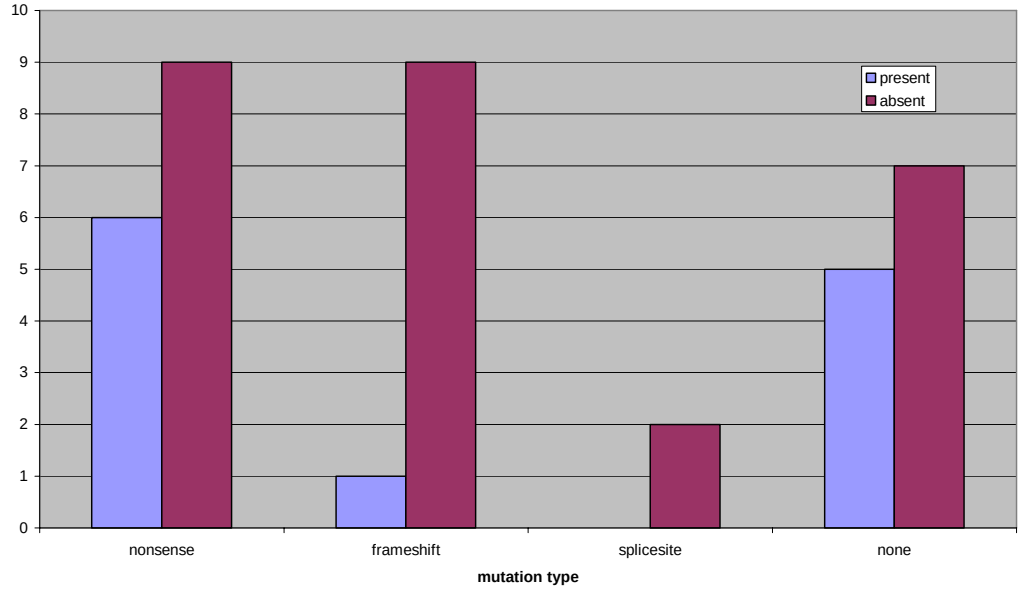

Figure 18. Clinical features 4. Choanal atresia.

7/27 (26%) of individuals with a mutation had choanal atresia (in one case with a mutation this part of the proforma was not completed). In comparison 5/12 (42%) 0f individuals without a mutation had choanal atresia. (P = 0.455). See figure 18 for a comparison of the presence of choanal atresia vs. the type of mutation. The choanal atresia was unilateral in three cases, bilateral in one case, and details not specified in three cases.

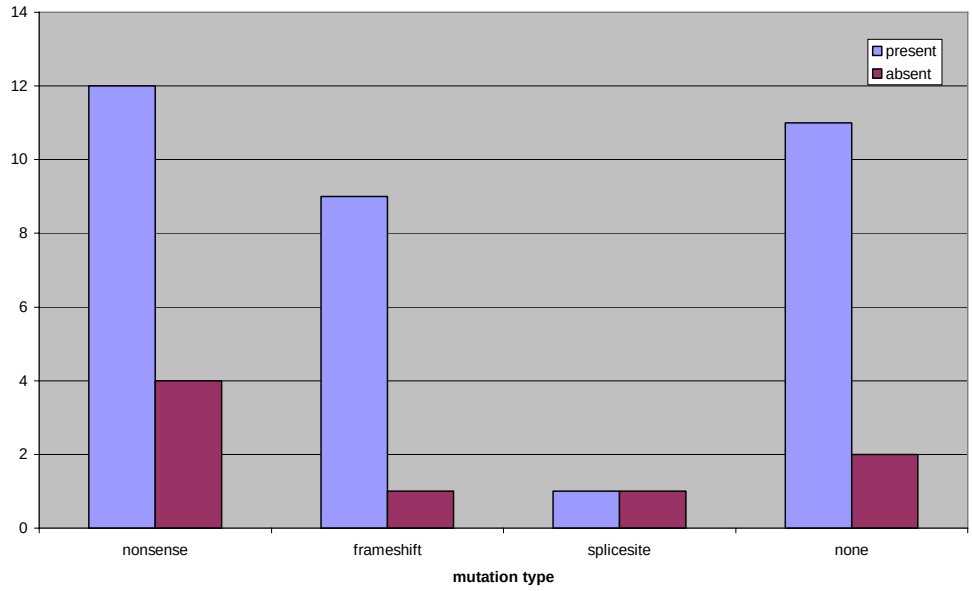

Figure 19. Clinical features 5. Heart defects.

Figure 19 shows in histogram form the number of individuals with a heart defect plotted with the type of mutation. For all individuals with a mutation where the type of heart defect was specified, 16/22 had a complex heart defect and 6 had a single heart defect. These single defects comprised 4 PDA and 2 ASD.

Of those with a nonsense mutation and a complex heart defect 9/10 had a PDA, 3 had an ASD, 5 a VSD, 3 pulmonary stenosis/atresia, 1 PAVD, 1 DORV and 1 anomalous origin of the right subclavian artery and 1 coarctation of the aorta.
Of those individuals with a frameshift mutation and a complex heart defect 4 had a PDA, 2 an ASD, 1 AVSD, 1 RVOT, 1 abnormal aortic valve and 1 mitral incompetence.

The individual with a splice site mutation had a complex heart defect involving a PDA, ASD and a PFO. Table 10 shows the frequency of each of the different types of heart defect in our cohort.

Table 10. Heart defects/Vascular anomalies in mutation positive cases

Type of heart defect	present in X% of those with mutations
PDA	64
ASD	29
VSD	18
PS	11
AVSD	4
PAVD	4
DORV	4
Tetralogy of Fallot	4
Abnormal aortic valve	4
Anomalous origin right subclavian artery	4
Mitral incompetence	4

10 cases in our cohort who did not have a mutation had a cardiac defect. 4/9 had a complex lesion. Of the 5 with a single defect there was 1 ASD, 1 VSD, 2 AVSD, 1 PS and 1 right aortic arch. For those with a complex defect, 3 had an ASD, 2 a VSD, 1 Tetralogy of Fallot, 1 DORV, 1 PDA, 1 PAVD, 1 dysplastic pulmonary valve and 1 bilateral superior caval vein.

The 3 individuals who met diagnostic criteria for CHARGE, who had heart defects were of the same type as in those who had mutations, 1 VSD, 1 ASD + VSD + dysplastic pulmonary valve and 1 Tetralogy of Fallot.

There was no significant difference in the frequency of heart defects between the mutation positive and mutation negative individuals, P = 1.0.

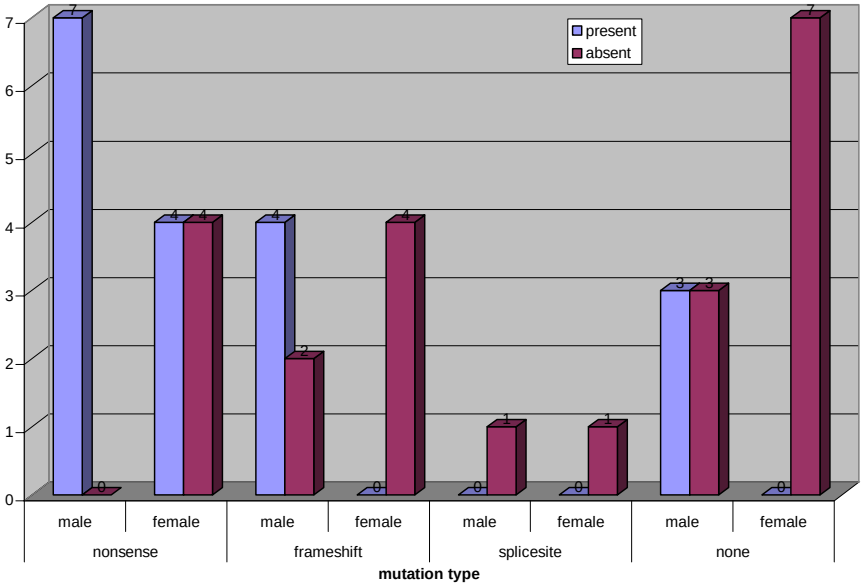

Figure 20. Clinical features 6.Genital anomalies

Figure 20 illustrates that15/27(56%) of individuals with a mutation had genital hypoplasia. Four boys had cryptorchidism, two had micropenis and five had both cryptorchidism and micropenis. Two girls had a small uterus, one had hypoplastic labia majora and one had hypoplastic labia minora. The true figure for genital hypoplasia is likely to be higher as this is underreported in girls. 3/13 (23%) individuals without a mutation had genital hypoplasia. Comparing the frequency of genital anomalies in mutation positive verses mutation negative cases the P value was 0.09. Urological anomalies were not common in this cohort. One case had agenesis of the right kidney, on case had bilateral small kidneys, one case had hydronephrosis and one had unilateral renal duplex collecting system.

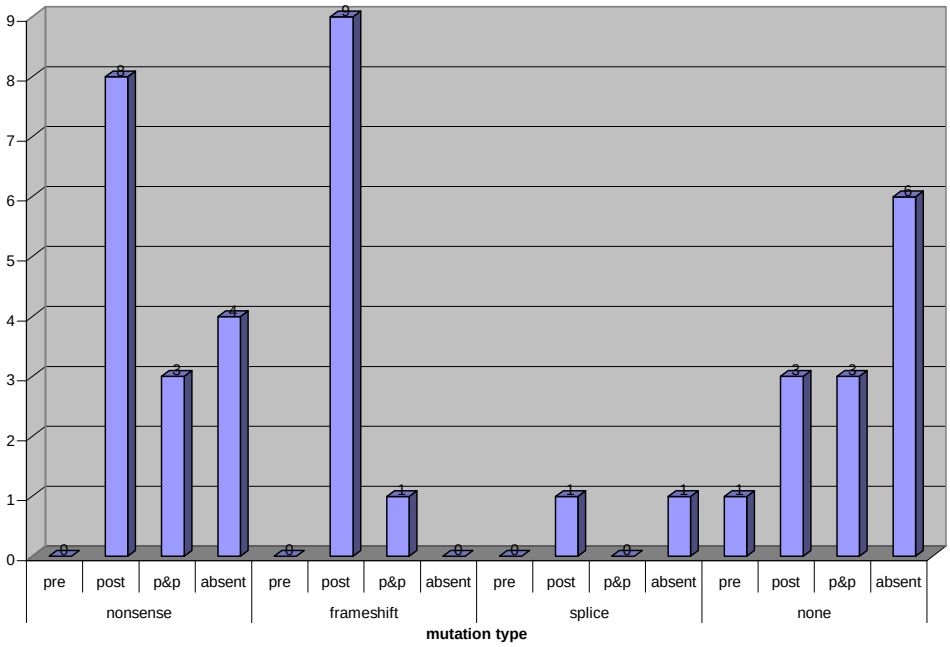

Figure 21. Clinical features 7. Growth retardation

22/27(81%) individuals with a mutation had retarded growth vs. 7/13(54%) of those without a mutation (see Figure 21), P = 0.128.

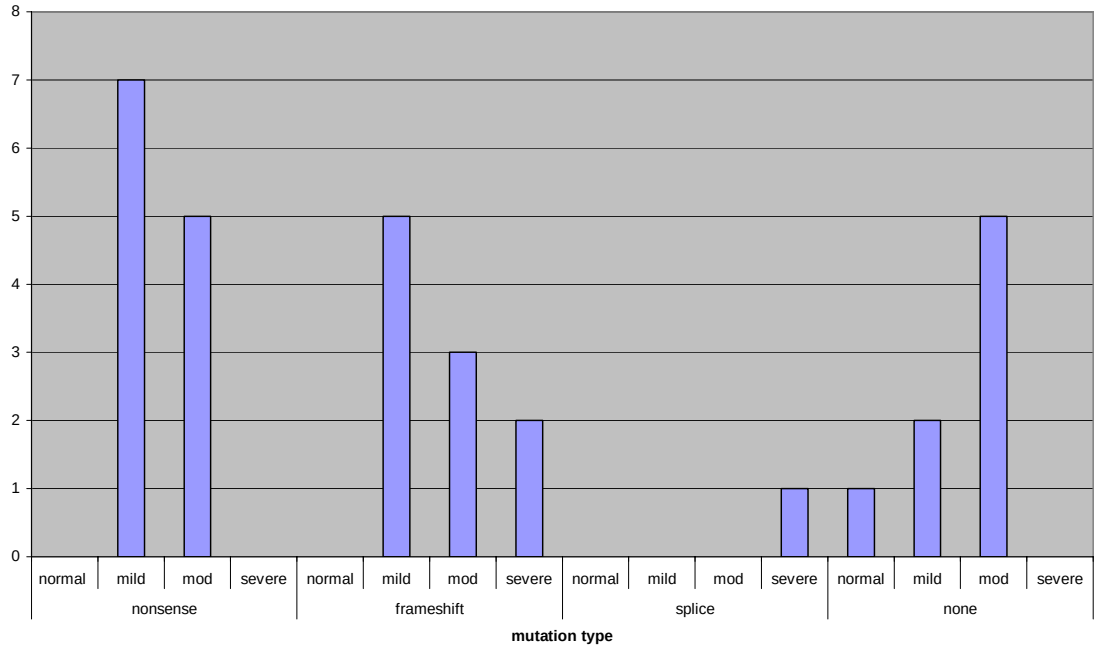

Figure 22.Clinical features 8. Developmental delay.

All 23 individuals with mutations in whom it was commented upon had developmental delay, 12 mild, 8 moderate and 3 severe. In those cases without a mutation, 1 had normal intelligence, 2 mild delay and 5 moderate delay (See Figure 22), P = 0.26.

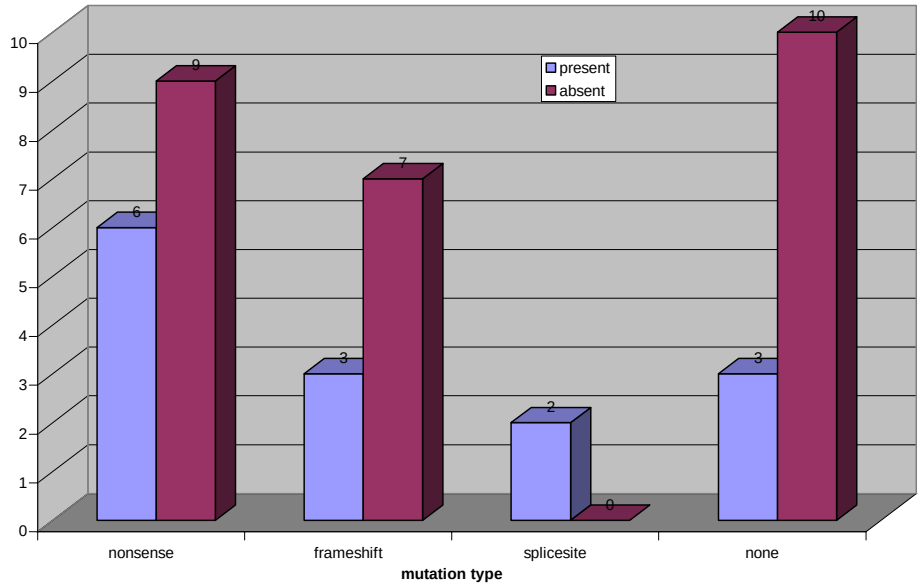

Figure 23. Clinical features 9. Orofacial cleft

We identified mutations in 28 individuals, of these 11 had an orofacial cleft (39%). These included one cleft lip, five cleft palate, three cleft lip and palate and two bilateral cleft lip and palate. In those cases without a mutation 3/13 had a cleft (23%), P = 0.48. Six cases had tracheoesophageal fistula, (21%). 2/13(15%) cases without a mutation had this malformation. One patient had pyloric stenosis and a caecal volvulus, 3/27(11%) had vertebral anomalies.

The paragraphs above compare the clinical features in those individuals with mutations with those without mutations in *CHD7*. If one compares the phenotype of individuals with nonsense mutations at the start of the gene with those at the end of the gene these are not significantly different, see Table 11. This is as expected as the introduction of a

premature stop codon will result in nonsense mediated decay of the mRNA and haploinsuffiency of the CHD7 protein.

Table 11.Clinical features in individuals with a nonsense mutation in Exon 2 vs. 34/36.

Clinical feature	Mutation in exon 2	Mutations in exons 34/36
Coloboma	4/5	3/4
Cranial nerve palsy	4/5	4/4
Ear anomaly	4/5	4/4
Choanal atresia	1/5	2/4
Heart defect	2/5	4/4
Genital hypoplasia	3/5	3/4
Delay	5/5	3/4
Orofacial cleft	2/5	2/4
TOF	1/5	1/4
Hormone deficiency	1/5	2/4
Semicircular canal defect	2	1

Numerator number of individuals with feature. Denominator number of individuals with mutation in the exon.

There is also no phenotypic difference between individuals with frameshift mutations in relation to the position of the mutation within the gene. The splice site mutation in exon 11 found in EG2 causes deletion of that exon and a frameshift. The splice site mutation in exon 26 found in IP-S causes an in frame insertion of five amino acids. One might expect him to be more mildly affected but in fact he has typical CHARGE syndrome.

DISCUSSION

CHARGE syndrome is a condition of previously unknown etiology. The majority of cases have been sporadic and several different pathogenic mechanisms have been suggested. Evidence supporting a genetic cause includes the isolated reports of multiplex families with presumed autosomal dominant (Mitchell *et al*., 1985) and autosomal recessive inheritance (Awrich *et al*., 1982; Pagon *et al*., 1981). There are also several reports of concordant monozygotic twin pairs (Farquhar *et al*., 2002). There is phenotypic overlap with some chromosomal syndromes such as partial trisomy 13 and 4p-. A number of single cases with chromosome abnormalities have also been reported with features consistent with a diagnosis of CHARGE syndrome/association to a greater or lesser degree. These include trisomy 18 (Lee *et al*., 1995), der (9)t(9;13), der(6)t(4;6) (Sanlaville *et al*., 2002), ring chromosome 14 (Fledelius, 1996) and a case with a balanced translocation t(6;8)(6p8p;6q8q) (Hurst *et al*., 1991). Genome scans of 10 patients looking for loss of heterozygosity did not detect any deletions (Lalani *et al*., 2003, 2005). CGH of twenty seven individuals identified two different chromosomal abnormalities but again no regions which were consistently deleted/duplicated (Sanlaville *et al*., 2002). Concurrently with this study Vissers *et al* (2004) used array comparative genome hybridization in individuals diagnosed with CHARGE and found a deletion overlap in two affected individuals at 8q12. They sequenced each of the nine predicted genes in this region in CHARGE patients and identified *CHD7* mutations in10/17 individuals. *CHD7* is a gene with previously unknown function. It is a member of a relatively newly described family of nine proteins which are evolutionarily conserved. These nine proteins are subdivided into three groups (Shur and Benayahu, 2005). CHD7 is one of the third group of chromatin remodeling enzymes which includes CHD 6, 8 and 9 that are involved in the

control of gene expression through chromatin modification. They contain two N-terminal chromodomains (*chro*matin organization *m*odifier), a central SNF2 related helicase/ATPase domain and a DNA binding domain, BRK at the C-terminus. The proteins form part of a complex that is involved in the acetylation of histones. Acetylation and methylation of histones is important in controlling the transcriptional activity of genes through conformational changes to chromatin (Strahl *et al*., 2000; Brehm *et al*., 2004). These alterations are made by chromatin modifying complexes. Recently the yeast CHD1 has been found to be part of the SLIK chromatin modifying complex which interacts with Lys 4 methylated histone H3. The chromodomain 2 (CD2) of CHD1 appears to be important in recognizing the substrate. PSI –Blast sequence analysis of CD2 in the Swiss-Prot database identified several proteins with significant similarity to CD2 including the second chromodomain in *CHD7* (Pray-Grant *et al*., 2005).

The CHD gene family are all thought to control gene expression by chromatin modification and hence regulate transcription.

Expression studies have shown that CHD7 is expressed in immune, nervous, muscle, secretory and other tissues (GeneCards). The gene is 188Kb in length with 38exons. The start codon is in exon 2.

Following the identification of mutations in *CHD7* as a major cause of CHARGE syndrome further expression studies have been carried out. Bosman *et al* (2005) sequenced Chd7 on Mouse chromosome 4 in *Whirligig* mice who have truncations of the lateral semicircular canal. 9 mutations were identified. Analysis of the coding sequence and protein of mouse Chd7 was found to have very high identity and similarity to human CHD7 (94.9 and 97% respectively). Like *CHD7* it has two chromodomains which are involved in binding to methylated histones at the N-

terminus, a central SNF2-like ATPase and helicase domains thought to be involved in DNA unwinding and two C-terminal domains SANT involved in binding to histone tail and BRK involved in DNA binding. Expression studies in mice found that Chd7 is expressed in the organs affected in CHARGE syndrome patients, that is eye, olfactory epithelium, ear, kidney and vascular system. Expression is widespread during foetal development with high expression levels in several epithelial cell types (olfactory, lung and gut), the vestibulo-cochlear, facial, olfactory and dorsal root ganglia and several specific areas in the brain. There was lower expression in mesenchymal cell types (Bosman *et al*., 2005). Lalani *et al* (2005) also found Chd7 was widely expressed with higher expression in cardiac outflow tract, truncus arteriosus, facio-acoustic preganglionic complex, hindbrain, forebrain, mandibular component of first branchial arch, otic vesicle, optic stalk, optic vesicle and olfactory pit. Microarray expression studies comparing mutation positive individuals with mutation negative individuals showed significant differences in CHD7 levels (Lalani *et al*., 2005), suggesting that mutation negative individuals have a different etiology for their malformations. Expression studies in human embryos show that at d20-24 CHD7 is ubiquitously expressed with distinct signal in the neural tube, by d26 it is expressed throughout the central nervous system and neural crest mesenchyme of the pharyngeal arches. By d33 it is expressed in the cephalic mesenchyme, pharyngeal arches, brain, otic vesicle and limb bud mesenchyme with a more intense signal in the spinal cord and dorsal root ganglia. By d34 there is intense expression in the CNS and expression in the otic vesicle is now restricted to the dorsal part. By d47expression is strong in the neural retina and rhombencephalon, moderate in the semicircular canals, forebrain, pituitary, olfactory bulbs and nerves. By 9 weeks it is expressed in the nasal epithelia, neural retina, optic nerve sheath and pituitary.

In this study I have confirmed that *CHD7* is a cause of CHARGE syndrome by mapping the breakpoints of a balanced translocation in twin girls affected by CHARGE syndrome. The breakpoint on chromosome 8 disrupted *CHD7* between exons 3 and 8. Sequence analysis of a cohort of 45 individuals with clinical features suggesting a possible diagnosis of CHARGE syndrome identified a pathogenic mutation in 28 of the 43 in which analysis was successful, a detection rate of 65%. When clinical diagnostic criteria are applied to this cohort we identified a mutation in 28/34 (82%) of individuals who had at least atypical CHARGE syndrome. The majority of the mutations are predicted to cause truncation of the protein (nonsense and frameshift mutations, 26/28 (93%), there were two splice site mutations, one causing a deletion of exon 11 resulting in a frameshift and premature stop codon, the other causing insertion of 15 base pairs in frame. Two missense sequence changes of uncertain significance were also identified. In 7 of our families we were able to confirm that the mutations were *de novo.* Parental samples were not available from the remaining 21.The mutations were spread throughout the gene with no mutation hotspots. 20 of the 28 mutations were novel, previously unreported mutations. 8 of the mutations had been previously reported by other groups carrying out mutation analysis of affected individuals. As with this study they have found mutations throughout the gene. Most mutations are unique with the exception of R1339X, R1819X, W2332X, R2653X(Lalani *et al*., 2005)W1994X, R2050X(Jongmans *et al*., 2006), R2284X, I1028V,R1069X,D1596G,R1820X,R2319C(Vourela *et al*., 2007), R494X, R947X, Q1214R, R1810X(Wincent *et al*.,2008). In this study R2627X, R1219X, R1819X, Y835X, 5405-17G>A, R2418X, R2653X and R1339X were the mutations which had been reported before. In most studies no genotype/phenotype correlations have been found (Aramaki *et al*., 2006; Jongmans *et al*., 2006). However Lalani *et al* (2005)

found a correlation between the presence of a mutation and cardiovascular malformation, coloboma and facial asymmetry (Lalani *et al*., 2005). When diagnostic criteria are applied to the individuals being tested for mutations the positive predictive value of the clinical diagnostic criteria (Blake *et al*., 1998) is 95% (Jongmans *et al*., 2006). In our cohort the positive predictive value of using Blake's diagnostic criteria is 85% which is comparable with that of Jongmans. Table 12 shows the percentage of individuals with each of the more common clinical features in four recently reported studies and in this study.

Table 12. Comparison of clinical features with the presence of a mutation in four recent studies.

	Aramaki 2006	Lalani 2005	Jongmans 2006	Wincent 2008	Current study	average
No. Sequenced	24	110	107	30	43	
No. with mutation	17(71%)	64(58%)	69(65%)	20(66%)	28(65%)	
Semicircular canal/cochlea hypoplasia	6/6(100%)	21/22(95%)	21/21(100%)a	8/9(89%)	5/5 (100%)	97%
Coloboma	15/17(88%)	55/62(89%)	33/47(70%)	18/20(90%)	24/28(86%)	83%
Microphthalmia	-	-	10/47(21%)	-		
Congenital heart disease	13/17(76%)	54/59(92%)	31/47(66%)	15/20(75%)	22/28(79%)	79%
Choanal atresia/stenosis	5/17(29%)	34/57(60%)	17/47(36%)	8/20(40%)	7/27(26%)	42%
Cleft lip/palate	8/17(47%)	18/60(30%)	17/47(36%)	4/20(20%)	11/28(39%)	34%
Developmental delay	17/17(100%)	-	24/32(75%)	11/15(73%)	23/23(100%)	86%
Postnatal growth retardation	14/17(82%)	-	21/32(63%)	17/19(89%)	22/27((81%)	78%
Genital hypoplasia /gonadotrophin deficiency	13/17(76%)	29/53(55%)	13/15(87%)	12/20(60%)	15/27(56%)	62%
External ear abnormality	17/17(100%)	59/62(95%)	47/47(100%)	18/20(90%)	24/28(86%)	95%
Hearing loss	-	54/59(92%)	37/41(90%)	14/15(93%)	22/27(81%)	89%
Facial asymmetry	-	36/56(64%)	10/47(21%)	9/19(47%)	13/27(48%)	46%
Laryngomalacia	14/17(82%)	-	1 tracheomalacia	-	3/28(11%)	
TOF	3/17(18%)	10/55(18%)	8/47(17%)	-	6/28(21%)	18%

a. only 21 individuals had MRI scans.27/41(57%) showed evidence of vestibular anomaly.

Face

The facial appearance of patients with CHARGE syndrome is square shaped with bitemporal narrowing. The nasal bridge is wide, the mouth is small with down turned corners of the upper lip (Blake *et al*., 1998 and see photographs in case reports, appendix II). Abnormalities of the ear and cranial nerve palsies cause facial asymmetry. Cleft lip and palate may also contribute to the dysmorphic features.

Ear abnormalities/hearing loss

Most patients with CHARGE syndrome have hearing loss. This may be conductive, sensorineural or mixed loss. It may also be progressive. In our cohort 81% of mutation positive cases had hearing loss. This was a slightly lower percentage than in the other mutation studies (90-93%). (Lalani *et al*., 2005; Jongmans *et al*., 2006; Wincent *et al*., 2008)

It is important that hearing loss is treated with hearing aids/ cochlear implants to maximize the communication abilities. CT abnormalities found in people with CHARGE include ossicular malformations, abnormal/absent semicircular canals, malformation of the pars superior and inferior, mondini deformity, absence of the oval window and stapedius muscle, narrow bony internal auditory canals, venous malformations and aberrant course of the facial nerve (Lemmerling *et al*., 1998; Satar *et al*., 2003; Stjernholm *et al*., 2003). However Cochlear implants have been shown to be successful in people with CHARGE (Woolley *et al*., 1998; Papsin, 2005; Lanson *et al*., 2007) although it is especially important to do a proper evaluation of the vascular system in the area. A recent report by Song *et al* (2008) described a case with a skull base vascular anomaly with an enlarged collateral emissary vein superficial to the mastoid area which was draining the internal jugular veins.

Semicircular canal abnormalities are found in nearly all individuals with CHARGE who have a mutation. Only 5 mutation positive cases in our cohort had had temporal bone imaging. All 5 had semicircular canal hypoplasia which supports the finding in other studies. Semicircular canal abnormalities are now included in the major diagnostic criteria for CHARGE syndrome which would suggest that temporal bone imaging should be performed in all individuals in whom this diagnosis is being considered. However it is important to liaise closely with the radiologists when requesting this investigation or abnormalities may be missed. One of our cases, KW was initially reported as having abnormalities of the malleus and incus and normal semicircular canals. A review of the scans was requested and it was noted that she had rudimentary superior semicircular canals with absent lateral and posterior semicircular canals.

Coloboma

Coloboma with or without microphthalmia occurs in 70-90% of cases.(Lalani *et al*., 2005; Aramaki *et al*., 2006; Jongmans *et al*., 2006; Wincent *et al*., 2008)

They may be unilateral or bilateral. They may be small and as only a small proportion involves the iris it is important to arrange formal fundoscopy in patients in whom the diagnosis of CHARGE syndrome is a possibility. The percentage of individuals with coloboma in our cohort was concordant with that found in the other studies. There was no significant difference in the frequency of coloboma in our mutation positive cases (24/28, 86%) compared with the mutation negative cases who met the diagnostic criteria (5/6, 83%). As with other studies the majority of our cases had bilateral coloboma. Other abnormalities in the eye include hypoplasia of the optic nerve, anophthalmia and refractive errors. These structural problems cause defects in vision

which may result in strabismus and nystagmus. Atresia of the lacrimal ducts, eyelid coloboma and ankyloblepharon have also been reported (Tellier *et al*., 1998; Chestler and France, 1988; Blake *et al*., 1990). Vision in CHARGE syndrome ranges from normal in the majority to blindness.

Congenital heart disease

Congenital heart disease was present in 66-92% of those with a mutation (Lalani *et al*., 2005; Jongmans *et al*., 2006; Aramaki *et al*., 2006; Wincent *et al*., 2008). In our study 79% of cases with a mutation had congenital heart disease (22/28) compared to 66 % (4/6) of the mutation negative cases who met diagnostic criteria. The most common malformation was a PDA alone or in combination with another defect. Tetralogy of fallot was less common in our series than in other studies with a frequency of 4% in comparison to 33% (Cyran *et al*., 1987; Tellier *et al*., 1998). AVSD, PAVD and DORV also occurred at a frequency of 4%.

Choanal atresia.

Choanal atresia/stenosis is present in 29-60% of those with a mutation (Lalani *et al*., 2005; Jongmans *et al*., 2006; Aramaki *et al*., 2006; Wincent *et al*., 2008). In our study 26 % (7/27) of cases with a mutation had choanal atresia/stenosis compared to 66% (4/6) of the mutation negative cases who met diagnostic criteria. Choanal atresia is usually of the bilateral osseous type (Sanlaville and Verloes, 2007), but may be membranous and unilateral. In our cohort it was unilateral in at least 43% of cases, although the figures are small with only 7 affected individuals. There may be a bias in reporting bilateral atresia unless imaging of the choanae is performed in all cases with CHARGE as unilateral atresia may be asymptomatic.

Orofacial cleft

Orofacial clefts were present in 20-47% of those with a mutation (Lalani *et al*., 2005; Jongmans *et al*., 2006; Aramaki *et al*., 2006; Wincent *et al*., 2008). In our cohort 39% (11/28) of cases with a mutation had orofacial clefting compared 0% (0/6) of the mutation negative group.

Developmental delay

Development in people with CHARGE may range from near normal to profound retardation. In the mutation studies delay was found in 75-100% of those with a mutation (Jongmans *et al*., 2006; Aramaki *et al*., 2006; Wincent *et al*., 2008). Our study produced similar figures with delay in100% (23/23) of individuals for whom this information was complete.

Growth retardation

Most patients with CHARGE syndrome have a normal birth weight. Post natal growth retardation is common. There are many interacting factors which may cause growth retardation. Cardiovascular, respiratory and feeding problems may all contribute. There is also growth hormone deficiency in some cases. Growth retardation was found in 63-89% of cases with a mutation (Jongmans *et al*., 2006; Aramaki *et al*., 2006; Wincent *et al*., 2008). In our cohort 81% (22/27) of the children were growth retarded which is concurrent with the other studies. People with CHARGE syndrome can continue to grow in height well into their twenties (Blake *et al*., 2006).

Genital hypoplasia/hypogonadism

Genital hypoplasia with or without hypogonadism occurs in 55-87% of mutation positive individuals (Lalani *et al*., 2005; Jongmans *et al*., 2006; Aramaki *et al*., 2006; Wincent *et al*.,2008). It was found/reported in 56% (15/27) of our cohort which is towards the lower end of this range. 78% (11/14) boys and 44% (4/9) girls had genital hypoplasia. Genital hypoplasia is more difficult to identify in girls and this can lead to under reporting. Accurate data for the frequency of hypogonadism is also difficult to obtain as despite short stature and genital hypoplasia many people with CHARGE syndrome do not routinely have endocrine investigations at present.

Cranial nerves

Cranial nerve abnormalities now form one of the major diagnostic criteria for CHARGE syndrome. Reviews of the cranial nerve abnormalities which occur in CHARGE by Byerly *et al* (1993) showed that abnormities of the seventh, eighth and ninth/tenth nerves were common whilst the third and sixth nerves were only occasionally involved. Tellier *et al* (1998) stated that 78% of patients had cranial nerve dysfunction affecting at least one nerve, usually the seventh and eighth. Examining the data more closely from these and other studies the seventh nerve is involved in 32-48% of cases (Blake *et al*., 1990; Byerly *et al*., 1993; Edwards *et al*., 1995,2002; Hartshorne *et al*., 2005; Stromland *et al*., 2005; Tellier *et al*., 1998). The figures in our cohort are consistent with this as 48% of individuals had a seventh nerve palsy.

The eighth nerve is involved in 49-68% of cases (Blake *et al*., 1990; Byerly *et al*., 1993; Edwards *et al*., 1995, 2002; Tellier *et al*., 1998). In our cohort 81% had sensorineural or mixed hearing loss. These were individuals in whom we had identified a mutation. The previously quoted studies predated the availability of mutation

analysis and may represent a more heterogeneous diagnostic group. The percentage of individuals with hearing loss reported in the studies where mutation analysis has been carried out show that 90-93% are affected, although these do not distinguish between those with sensori neural and those with conductive hearing loss (Lalani *et al*.,2005; Jongmans *et al*., 2006; Wincent *et al*.,2008).

Previous reports indicated that the ninth/tenth cranial nerves are affected in 31-79% of cases (Byerly *et al*., 1993; Hartshorne *et al*., 2005). In our cohort 48%(13/27) mutation positive cases had swallowing problems indicating involvement of the ninth/tenth nerves.

As cranial nerve abnormalities are now recognized as a major features in CHARGE Blake *et al* (2008) carried out a study to look specifically at cranial nerve abnormalities five, seven, eight and nine/ten (Blake *et al*.,2008). They reported that 92% of cases had at least one cranial nerve involved, 72% of cases had more than one nerve involved and 18% had all four cranial nerves involved. It is postulated that these nerves may be affected more frequently than the other cranial nerves as they are different from the other motor nerves in that they have associated sensory ganglions derived from the interaction of the neural crest mesenchyme and ectodermal placodes.

Abnormalities of the first cranial nerve causing olfactory deficits are thought to be common in CHARGE. Chalouli *et al* (2005) found olfactory deficits in 14/14 in the group they studied. In our cohort absent sense of smell was only reported in one case, JW, however many of our cases were too young or had too severe learning difficulties to enquire about problems with this sense. To date in the U.K. it has not been routine practice to perform scans of the arrhinenchephalon and olfactory bulbs in these patients.

Cranial nerves two, three, four and six are difficult to assess because of the confounding effects that coloboma and eighth nerve abnormalities cause.
Abnormal function of the twelfth nerve has been anecdotally noted (Blake *et al*., 2008) but not previously reported in the medical literature. Our case JW has dysfunction of the first,sixth,seventh,eighth,ninth/tenth and twelfth cranial nerves representing the more extreme end of the spectrum. He also has cutaneous allodynia. Despite this he manages in a main stream school with support.
Lalani *et al* (2005) found a correlation between the presence of a mutation and cardiovascular malformation, coloboma and facial asymmetry (Lalani *et al*., 2005). In our study cranial nerve palsy and orofacial cleft was positively associated with finding a mutation. Choanal atresia was more common in the mutation negative group.

Feeding and breathing problems.

Laryngomalacia is common in CHARGE affecting 8-37% of patients (Stack and Wyse, 1991; Morgan *et al*., 1993; White *et al*., 2005). It is thought to be a common cause of upper airway obstruction. Laryngomalacia usually resolves spontaneously by 2 years. Between 10-22% (Friedman *et al*., 1990) will need surgery which usually has a successful outcome. However this is not always the case in children with CHARGE and this may be due to the presence of other causes of upper airway obstruction such as pharyngolaryngo hypotonia perhaps due to ninth and tenth cranial nerve palsies. Naito *et al* (2007) carried out a fiberoptic laryngoscopic study of 10 patients with CHARGE which showed that all the patients had structural abnormalities of the larynx, arytenoids and vocal cords. This is a further reason, in addition to the gastroesophageal reflux which occurs in 80% of patients that children with CHARGE are at risk from aspiration. The vocal cord abnormalities may be a contributory factor in the failure to

develop speech in some people with CHARGE. In one mutation study 14/17(82%) mutation positive children had laryngomalacia (Aramaki *et al*., 2006).In our cohort 3/28(11%) of the children had laryngomalacia. It is possible that the discrepancy between these two figures is due to the lack of investigation rather than a true difference in the frequency of laryngomalacia. The complex feeding problems that occur in CHARGE means that 90% of the children will need tube feeding at some time. This may be prolonged. In the study by Dobbelsteyn *et al* (2007) of 39 children with CHARGE only half of the children were fed orally by the age of 5 years. One third of the children still had problems eating some food textures at age 11(Dobbelsteyn *et al*., 2007).

Tracheoesophageal fistula/oesophageal atresia

Tracheoesophageal fistula/oesophageal atresia (TOF/OA) occurs in 1/3500 live births. In mutation studies 17-18% of cases have been found to have TOF/OA (Lalani *et al*., 2005; Jongmans *et al*., 2006; Aramaki *et al*., 2006). In our study this malformation occurred with similar frequency. 21% (6/28) of cases had TOF/OA.

Immune dysfunction

Immunological abnormalities due to anomalies of the 3rd and 4th pharyngeal pouch complex causing absence or hypoplasia of the thymus and/or parathyroid glands may be under diagnosed in CHARGE syndrome. Writzl *et al* (2007) describe two patients with hypocalcaemia and a SCID phenotype. On reviewing the literature they found a further 12/15 individuals with CHARGE syndrome and an immunological abnormality with SCID. Salanville *et al* (2006) found that 7/10 abortuses with CHARGE had thymic abnormalities at post mortem. Sedlacek *et al* (2007) reports a further case of

CHARGE with SCID. In our cohort two individuals had a SCID phenotype (Gennery *et al*., 2008). More mild immunological abnormalities of T lymphocytes and humoral immunity also occur. Fuentes-Paez *et al* (2007) describe a case with hyper-immunoglobulin M syndrome. Mild immunological abnormalities are likely to be under reported. Patients with CHARGE have other causes of frequent infections and therefore immune studies are frequently omitted.

CHARGE syndrome is usually sporadic. However there have been a few reports of familial CHARGE syndrome and since the identification of the gene it has been possible to confirm this molecularly. Jongmans *et al* (2006, 2008) describe two affected brothers whose mother was found to be a somatic mosaic (Jongmans *et al*., 2006), two sib pairs with apparently *de novo* mutations, a sib pair with mosaicism in the father who is unaffected and two families where the mother and child are affected (Jongmans *et al*., 2008). Delahaye *et al* (2007) describe two families. The first, two boys and their affected mother with a p.S834F missense mutation. The second family, two boys with clinical CHARGE syndrome and their father whose only abnormality was a cup shaped ear had p.R157X (Delahaye *et al*., 2007). Lalani *et al* (2006) report a pair of monozygotic twins, a *de novo* sib pair and an affected mother and daughter. The probands who led to this study were monozygotic twins with a balanced *de novo* translocation disrupting *CHD7*. As with the other familial cases the phenotype of different affected family members was not consistent. It is therefore not perhaps surprising that no genotype-phenotype correlations have been made in CHARGE syndrome.

Whole gene/exon deletions are not common in CHARGE. To my knowledge they have only been reported on four occasions, two individuals by Vissers *et al* (2004) and one each by Arrington *et al* (2005) and Udaka *et al* (2007).

Limb anomalies

Limb anomalies were not recognized initially as a significant part of the phenotype of CHARGE syndrome. There were occasional reports of limb anomalies as a rare finding but these were usually mild. Findings included a distal transverse crease which extends between the 2nd and 3rd finger web, 2-3 cutaneous syndactyly, tapered fingers, 5th finger clinodactyly and camptodactyly with absence of the distal phalanx of the 5th finger in one individual and nail hypoplasia, triphalangeal thumb and ectodactyly. There may be limited supination, talipes, sandal gap, tibial hemimelia and dimpling (Davenport *et al*., 1986; Oley *et al*., 1988; Meinecke *et al*., 1989; Prasad *et al*., 1997; Dhooge *et al*., 1998; Jongmans *et al*., 2006; Sanlaville *et al*., 2006). A report by Brock *et al* (2003) suggested that limb anomalies occurred in approximately 30% of patients with CHARGE. These were usually mild. However there have now been further papers reporting significant limb anomalies in individuals with mutations or deletions of *CHD7*.Tibial hypoplasia (Aramaki *et al*., 2006) bilateral ectrodactyly of the upper limbs (Sanlaville *et al*., 2006), bifid femora and fibula aplasia in a child with a presumed deletion of *CHD7* (Asamoah *et al*., 2004) and three cases described by Van de Laar *et al* (2007). The first case had unilateral monodactyly and ulnar hypoplasia. The second case had bifid right femur, bilateral tibial aplasia, dysplastic fibular and four digits on each foot. The third case had tibial aplasia on the right and hypoplasia on the left. Our case CD adds to this spectrum of more severe anomalies. She has an absent radius in the left arm and only three digits in the hand. CHD 7 is expressed in the limb bud mesenchyme during embryogenesis (Sanlaville *et al*., 2006) and therefore abnormalities would be predicted to occur. The limited number of cases with

significant limb abnormalities reported to date might be explained by the failure to recognize CHARGE syndrome as a possible diagnosis in patients with these anomalies. Our case CD is an example of this, before review by the author it was thought unlikely that CHARGE was the correct diagnosis because of the presence of the limb anomaly.

CHARGE syndrome remains a clinical diagnosis. It is likely that there are other genes involved although it is likely that *CHD7* is the major gene as mutations have been identified in 85-95% of individuals who meet the diagnostic criteria (current study, Jongmans *et al*., 2006). The diagnosis of CHARGE syndrome cannot be excluded on the basis of negative analysis of *CHD7*.

Conclusion

We have confirmed that mutations in *CHD7* are the cause of CHARGE syndrome in the majority of cases who meet diagnostic criteria. The mutation detection rate in our cohort was 82%.

There are no hotspots for mutations in *CHD7* and no genotype/phenotype correlations have been identified.

Coloboma, external ear anomalies, congenital heart defects, genital abnormalities, growth retardation, developmental delay and orofacial clefting were all present at higher frequencies in mutation positive cases, however the presence of a cranial nerve palsy was most positively associated with identifying a mutation.

We would recommend that mutation analysis of *CHD7* is worthwhile in any individuals with at least atypical CHARGE as defined by Verloes (2005) criteria. In addition these criteria should be used in preference to Blake's as they are more inclusive allowing a diagnosis to be made in more patients.

Imaging the semicircular canals and hormone studies should be performed in any individuals in whom the diagnosis of CHARGE syndrome is being considered both to aid in making the clinical diagnosis and to allow the appropriate treatment.

Further Work

It would be interesting to do expression studies of *CHD7* in the cases in our cohort who did not have a mutation in the gene, to ascertain whether they have reduced/altered expression of *CHD7* or whether another gene was likely to be the underlying cause of their condition.

Prospective studies of children with CHARGE syndrome looking at the management of their physical problems would be of value. This would include immunological studies in all children with a diagnosis of CHARGE syndrome which is probably an under recognized part of the phenotype. In those with certain types of immune abnormality, treatment might reduce morbidity and the effect on intellect which occurs with hypoxemia related to frequent infections. Using this same rationale, congenital heart malformations should be aggressively treated, and those individuals with obstructive airway problems due to tracheobronchomalacia should be treated with CPAP.

This might identify whether aggressive management or the lack thereof has a major effect on the intellectual outcome or whether the presence of a brain malformation is the only predictor of this.

CHD7 is possibly at the top of a gene pathway and it will be fascinating in the future if the genes with which it interacts, and its relationship to other malformation syndromes can be elucidated.

Electronic-database information

The URLs for this paper are as follows:

UCSC Human Genome Browsers, http://genome.ucsc.edu/cgi-bin/hgGateway

Ensembl Human Genome Browser, http://www.ensembl.org/

CHORI BACPAC Resources, http://www.chori.org/bacpac/

PRIMER3 website, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

RepeatMasker, http://www.repeatmasker.org/

Automated Splice Site Analyses https://splice.cmh.edu/

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Appendices

Appendix I. Details of the primers, fragment sizes and the annealing conditions used in DNA amplification

Exon	Exon Size(bp)	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon size(bp)	Fra	Annealing temp ^{o}C
2A	1839	gagggcaaacacctcagtgaagt	ctgctgtccacaaaggattgcc	503	1	60
2B		tggcagtggtggcggtca	ctgtctggctccgagaactaaagtta	548	2	65+DMSO
2C		gttgcccacagtcccagattct	ctgctggcagtttctggggcc	567	3	65+DMSO
2D		gggataccaatggaagttggc	gcacaatccctgggacaggg	596	4	60
3	431	gaaacatcagccactaactttca	cccctcatttcataggctgta	599	5	53
4	142	gcattttgcacaaatgtactatgtgtgg	ggggaggtcttgtgtacttcatacatgg	485	6	54
5	138	gccactgtcttgggtttttg	ccaacattaggtggatgttcc	290	7	54
6	66	cagtgacttaaaaggtgtggaggtgg	ttagacaggattagaaatattactggaag	308	8	54
7	56	ccaaatttggggtcaggttgttgtg	atacacagaagttagtcaacttttacacc	568	9	54
8	115	gctcagcagccttaatgggtaattaagc	gttaccaatatgcaagttgacagcac	374	10	54
9	84	gccaaatgtaagttttatattgc	gctctgaccaagaccaggtc	302	11	60
9		**aacttttttttttcccttttg**	internal			
10	138	gagcatgcttttccttaatgtg	ctccctggaactctccgatt	579	12	60
11	122	atcgaggagtttgagaaactaatg	acacacttatatcaatctctgtattaat	449	13	60
12	244	tttgggtatgcatttgtgggtacaatgg	ccttcccaagtcaccaagac	436	14	60
13	177	ataacttgaaaacagaatgtatgtcacc	ccagagaatatatcacaatatttgacaagc	452	15	60
14	144	cctgattcctatactttgcatagggtag	ggtgggaaaaactgtaatataatcacac	382	16	54
15	256	tggatgtttaatgaatgagataatcctg	aggaatgactatacaccatgaaatcc	483	17	54
16	211	attcttgttcataagcaggagtttggtg	ttttaggtggactgcttggacccc	418	18	54
17	196	cgccaataaaccctatttgct	cgcaacattaatttatgggtctgactgg	446	19	60
18	168	taccatcacattggaatgag	ggctgagtaatttcttactattagtta	346	20	60
19	180	tgcagcatttgtttagtctgc	attcccaatgcatcttgtaagc	352	21	60
20-21	111,206	cggagcaaatacataaacaaaa	ggggtgtcacacaaattcaa	600	22	53
22	200	ctggtacctgacttaaagtaaagc	ccccttggcacaggagc	418	23	60
23	160	tgctacagggtcacaaagc	gttgaccctccaaatctgc	439	24	60
24	90	caggatgatggatgaacagc	cgaaggacaaatactgcattc	330	25	60
25	104	atgtttatcgtgggagagag	gagtcctttggaactatcctc	319	26	60
26	130	gttgtggcagtgctgtgatt	tgtgtactgcagggtaagaactg	300	27	60
27-28	73,63	agattattactctttcctacccacccc	ccacgtgaacaatgactgctcagtgcac	437	28	54
29	224	ccctttcccacactgtcatt	gagcctttctttggtggtca	455	29	53
30	209	ccacccccaaataactacca	tctgtaacacagaagggctca	443	30	55
31A	672	aacaaagttctatacaaaaagacgag	ccagtgggttcaaggaag	443	31	60
31B		ggttggtgctgctaaacacg	ccgtgctgccagaaagc	549	32	60
32	161	ccatgtagtaggtactcaataaaatggagc	caagctagaaagatttcctctaccctaatc	355	33	60
33	228	cattttatgctcttttgcatcttgatgg	gggctggcttttagaaataaggaaca	509	34	60
34	444	tgttccttatttctaaaagccagccc	ggctttcatacaatgctgctgagagaaac	636	35	60
35	222	gttcccaaacaactagacattgtttctag	ctgtcagggatttctatgttgtaagg	451	36	54
36-37	141,105	ttgaagatgatctgacagttctctttgg	gatgtattatgtcaattcttttaagctaag	678	37	54
38A	1889	gttcaccacagaggctcacattgagatc	tcgtcttcattctcatttcc	562	38	53
38B		aggagaaccggaagacag	gcactgcacaatacttaatgac	685	39	53

NB 9Fseq aacttttttttttcccttttg