Prevalence of \( \text{SDHB}, \text{SDHC}, \) and \( \text{SDHD} \) germline mutations in clinic patients with head and neck paragangliomas

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**Background:** Paragangliomas are rare and highly heritable tumours of neuroectodermal origin that often develop in the head and neck region. Germline mutations in the mitochondrial complex II genes, \( \text{SDHB}, \text{SDHC}, \) and \( \text{SDHD} \), cause hereditary paraganglioma (PGL).

**Methods:** We assessed the frequency of \( \text{SDHB}, \text{SDHC}, \) and \( \text{SDHD} \) gene mutations by PCR amplification and sequencing in a set of head and neck paraganglioma patients who were previously managed in two otolaryngology clinics in the USA.

**Results:** Fifty-five subjects were grouped into 10 families and 37 non-familial cases. Five of the non-familial cases had multiple tumours. Germline \( \text{SDHD} \) mutations were identified in five of 10 (50%) familial and two of 37 (5%) non-familial cases. \( \text{R38X}, \text{P81L}, \text{H102L}, \text{Q109X} \), and \( \text{L128fsX134} \) mutations were identified in the familial cases and \( \text{P81L} \) was identified in the non-familial cases. Both familial and non-familial cases had multiple tumours. \( \text{P81L} \) and \( \text{R38X} \) mutations have previously been reported in other PGL families and \( \text{P81L} \) was suggested as a founder mutation. Allelic analyses of different chromosomes carrying these mutations did not show common disease haplotypes, strongly suggesting that \( \text{R38X} \) and \( \text{P81L} \) are potentially recurrent mutations. Germline \( \text{SDHB} \) mutations were identified in two of 10 (20%) familial and one of 33 (3%) non-familial cases. \( \text{P131R} \) and \( \text{M71fsX80} \) were identified in the familial cases and \( \text{Q59X} \) was identified in the one non-familial case. The non-familial case had a solitary tumour. No mutations could be identified in the \( \text{SDHC} \) gene in the remaining four families and 20 sporadic cases.

**Conclusions:** Mutations in \( \text{SDHD} \) are the leading cause of head and neck paragangliomas in this clinic patient series. \( \text{SDHD} \) and \( \text{SDHB} \) mutations account for 70% of familial cases and ~8% of non-familial cases. These results also suggest that the commonness of the \( \text{SDHB} \) \( \text{P81L} \) mutation in North America is the result of both a founder effect and recurrent mutations.

**Abbreviations:** CB, carotid body; PGL, paraganglioma; STRP, single tandem repeat polymorphism
mitochondrial complex II subunits is unknown. In a recent study, SDHD mutations were identified in 30 of 32 (~94%) PGL families and in 20 of 55 (~36%) isolated cases in The Netherlands. Twenty-four of the 32 (75%) Dutch PGL families were the result of a single founder mutation. However, given the genetic (locus) and non-genetic heterogeneity in the aetiology of paragangliomas, it is unknown whether SDHD plays such a prominent role in other populations. It is conceivable that a fraction of paraganglioma patients without a family history could be carriers of germline SDHD mutations, because of imprinting and age dependent penetrance that obscure the familial nature of the PGL1 tumours. Accordingly, previous analyses of patients without a family history of head and neck paragangliomas and those with phaeochromocytomas (that is, adrenal paragangliomas) showed germline mutations in the SDHD gene. To determine the relative frequencies of SDHD, SDHC, and SDHB mutations in the aetiology of head and neck paragangliomas, we performed mutation analysis in a group of patients ascertained from two clinic patient populations in the United States.

**MATERIALS AND METHODS**

**Head and neck paraganglioma patients**

The subjects were patients diagnosed with head and neck paragangliomas, originating in the carotid body, jugular foramen, vagal nerve, temporal bone, or middle ear, at the Department of Otolaryngology at the University of Pittsburgh School of Medicine, Pittsburgh, PA and at the House Ear Institute, Los Angeles, CA. The recruitment of the subjects was performed either for the ongoing PGL linkage studies or through a more recent study aimed at establishing the proportion of heritable cases of paragangliomas by clinical criteria. The latter study followed a procedure that involved mail questionnaires and structured telephone interviews and has been described in detail elsewhere. Family 33 has been independently ascertained from Canada and only one family member was available for the research study. Both studies were approved by the Institutional Review Board of the University of Pittsburgh.

**Classification of subjects on the basis of family history**

The subjects were classified as familial if two or more subjects of an extended pedigree were diagnosed with paragangliomas as documented either by hospital records or by the family history reported in the questionnaires. On the basis of positive family history, 18 subjects were classified into 10 families. A total of 37 subjects who were recruited to the study had no family history. Five of the 37 non-familial cases had multiple tumours. Pedigrees for families 1, 3, 4, 9, and 13, which had multiple affected subjects, have been described previously. Maternal transmission was observed in family 9. None of the five new families (fig 1) showed maternal disease transmission.

**Genotyping and haplotype analysis**

DNA was isolated either from peripheral blood by standard phenol-chloroform extraction (PGL linkage study) or from cheek swabs using a commercial kit (Puregene D-500A) following the manufacturer's protocol (clinical study). The genotyping was performed only in the DNA obtained from peripheral blood. The simple tandem repeat polymorphisms (STRPs) used in genotyping and the haplotype analysis have been described elsewhere, except for the new polymorphic tetranucleotide repeat marker, D11S5030, which was located approximately 40 kb telomeric to the 3′-SDHD. This STRP had five alleles in 20 unrelated chromosomes with a calculated
Mutation analysis

**SDHD** mutation analysis was performed by SSCP analyses and direct sequencing as described previously. The primer sequences for **SDHD** coding exons of *SDHD* were 5′-CTGATCTGGTGGTTGGGAA-3′ and 5′-CTGGCTGAGGGCTACGCTA-3′; exon 2, 5′-CTAGTGGTGCTTGATGAC-3′ and 5′-GGAGAGCGACCTCGGGT-3′; exon 3, 5′-GTAGTGGTGCTTGATGAC-3′ and 5′-AGAGGT-GAGGCTACGCTA-3′; exon 4, 5′-CTAGTGGTGCTTGATGAC-3′ and 5′-AGAGGT-GAGGCTACGCTA-3′. The primer sequences for **SDHB** exon 1 and exon 2, and the amplicon sizes were F: 5′-GGAGAGCGACCTCGGGTT-3′, R: 5′-GTGGTGCTTGATGAC-3′; exon 3, 5′-CTGGCTGAGGGCTACGCTA-3′ and 5′-CTCTCGCTCTCTCACTCT-3′; exon 4, 5′-GTGGTGCTTGATGAC-3′ and 5′-AGAGGT-GAGGCTACGCTA-3′. The preamplified samples were diluted 20-fold and subsequently amplified with the originally described primers.

To sequence the six coding exons of **SDHC** and the eight coding exons of **SDHB**, the DNA extracted from the cheek swabs was first preamplified using Degenerate Oligonucleotide PCR (DOP) technique following a commercial protocol (Roche Molecular Biochemicals). The DOP PCR amplification was performed for 50 cycles. Each cycle was composed of the following steps: one minute at 94°C, two minutes at 37°C, four minutes at 55°C, 30 seconds at 68°C. The reaction was finalised with five minutes’ incubation at 74°C. An aliquot of the DOP PCR product was subsequently used to amplify the gene exons. The primer sequences for **SDHB** exon 1 and exon 2, and the amplicon sizes were F: 5′-GGAGAGCGACCTCGGGTT-3′, R: 5′-GTGGTGCTTGATGAC-3′; exon 3, 5′-CTGGCTGAGGGCTACGCTA-3′ and 5′-CTCTCGCTCTCTCACTCT-3′; exon 4, 5′-GTGGTGCTTGATGAC-3′ and 5′-AGAGGT-GAGGCTACGCTA-3′. The preamplified samples were diluted 20-fold and subsequently amplified with the originally described primers.

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**RESULTS**

**Mutation analyses of the paraganglioma patients**

Mutations in **SDHD** were detected in five of the 10 (50%) PGL families and two of the 37 (5%) subjects without any family history (table 1). The identified mutations were predicted to truncate or dramatically alter the formation of the **SDHD** protein product, cybS. Family 3 and the disease causing mutation H102L have been reported previously. The mutations in family SP64 and family 27 introduce premature stop codons that remove most of exon 4 from the mature cybS. R38X and P81L were identified in family 1 and family SP52, respectively. Subjects SP59 and SP77 who had no family history also carried the P81L mutation. Both subjects had multiple tumours in the head and neck region. The mutations R38X and P81L were previously reported in other families linked to the 11q23 locus and their potential impact on cybS has been discussed.

Mutations in **SDHD** were identified in two of the 10 (20%) PGL families and in one of the 33 (∼3%) subjects (including the two cases with **SDHD** P81L mutations) without any family history. P131R and M71fsX80 mutations were identified in families 9 and 13, respectively. Both mutations segregated with the disease phenotype in three affected subjects in family 9 and in two affected subjects in family 13. The P131R mutation alters an amino acid residue conserved among five eukaryotic species and was not detected in 200 normal chromosomes by PCR amplification of exon 4 and 5′UTR restriction enzyme digestion, which recognises the mutant allele. The M71fsX80 mutation of family 13 is predicted to cause a very early truncation of the **SDHB** protein product. A premature stop codon mutation, Q59X, was identified in one subject without a positive family history. This subject did not have multiple paraganglioma tumours.

No mutations could be identified in the **SDHC** gene in the remaining PGL families 4, SP48, SP56, and in 20 subjects (including the three cases with multiple tumours) who had no family history and no mutations in the **SDHD** and **SDHB** genes. Availability of multiple affected subjects in family 4 enabled us to show that the markers at chromosome 1q23 and chromosome 1p36 did not segregate in the affected subjects, thus confirming the exclusion of the **SDHD** and **SDHB** genes by linkage. However, several markers at the **SDHC** containing region on chromosome 1q23 were cosegregated with the disease phenotype, thus precluding the exclusion of **SDHC** by linkage (data not shown). Additional members of families SP48 and SP56 were not available to assess the segregation of the three PGL loci further.

**Haplotype analyses of the disease chromosomes carrying the P81L and R38X mutations**

Previously, haplotype analyses had shown extensive haplotype sharing in four families with P81L mutations, suggesting the presence of a common ancestral mutation in these US
The same mutation was subsequently identified in three other families from the USA, one family from the UK, and one family from Australia. Similarly, the R38X germline mutation was independently reported in two families from the USA and in one subject with extra-adrenal paraganglioma. To test whether P81L and R38X cases were the result of founder mutations, we performed haplotype analyses using polymorphic markers located very close to the SDHD gene.

A visual inspection of the haplotypes comprising nine STRPs distributed within an approximate 450 kb region around the SDHD gene did not show a single haplotype for each mutation (fig 2). The identical alleles observed for the markers which immediately flank the SDHD gene were common among normal control chromosomes: D11S5015 allele 162 had a frequency of \( \sim 35\% \) and D11S5019 alleles 168 and 170 had frequencies of \( \sim 50\% \) and \( \sim 45\% \), respectively. Therefore, these alleles shared among families cannot be readily concluded to be identical by descent. Even under the conservative assumption that the observed alleles are identical by descent, five independent ancestral recombination events within an approximate 200 kb interval between D11S5017 and D11S5030 would be required to explain the P81L haplotypes observed in the American founder families, family 5, family 33, and subject SP77. Similarly, the hypothesis that family 11 and family 1 derive from a common ancestral haplotype would require the occurrence of two recombination events. Previous analysis of a total of 632 meioses within an approximately 2 Mb interval between D11S897 and D11S1647, a region that also contains the SDHD region analysed here, uncovered 11 recombination events (0.87 cM/1 Mb) without any significant sex specific difference. Assuming random distribution of the recombination breakpoints within this interval, the probability of a recombination event occurring within the 200 kb interval tested in this study is estimated to be 0.87/5 = \( \sim 0.17\% \). Thus, the very low probability of a single recombination event within this small interval strongly suggests that the observed haplotypes do not originate from single ancestral haplotypes, although the possibility of very old mutations cannot be entirely excluded.

**DISCUSSION**

To determine the prevalence of SDHB, SDHC, and SDHD germ-line mutations in the aetiology of head and neck paragangliomas, we tested a total of 18 subjects (10 families) with a family history and 37 subjects without a family history, all of whom were previously evaluated in two otolaryngology clinics. We identified mutations in SDHD in five of the 10 (50%) families and two of the 37 (\( \sim 5\% \)) subjects without a family history. These two subjects were among the five cases that showed multifocality without a family history, suggesting that tumour multifocality is predictive of hereditary PGL. Mutations in SDHD were identified in two of the 10 (20%) PGL families and in one of the 37 (\( \sim 3\% \)) subjects without any family history. The one non-familial case had a single tumour. No mutations were identified in the SDHC gene in the remaining four PGL families and in 20 non-familial cases without a family history and without mutations in the SDHD and SDHB genes. Thus, mutations in SDHD are the leading causes of paraganglioma tumours among the three mitochondrial complex II genes tested in this study. These findings also indicate that mutations in SDHD and SDHB account for 70% of head and neck paragangliomas with a positive family history and for \( \sim 8\% \) of those without a positive family history. The remaining familial cases could be the result of mutations that are not detectable by exon amplification and sequencing in the tested genes or there may be other genes involved in the aetiology.
Germline SDHD mutations were predicted to occur in non-familial paragangliomas because the familial nature of the tumours could be obscured by de novo and imprinting. In fact, the frequent (~36%) deletion of SDHD founder mutations among non-familial cases in The Netherlands suggests that some of the apparently sporadic cases do inherit their disease genes from their fathers. In our study, however, it was unclear whether P81L mutations in the non-familial paraganglioma cases were inherited or arose de novo through the recurrent mutational mechanism. Other family members were not available to distinguish between these two competing hypotheses. We detected the P81L mutation in three new cases and the R38X mutation in one new case. Because both mutations had previously been reported and P81L was further implicated as a founder mutation among US families, we tested for the presence of a single founder chromosome for each mutation by the analyses of nine STRPs located within an approximately 450 kb region around the SDHD gene. Haplotype analyses showed no evidence for an extensive haplotype sharing for each mutation, strongly suggesting that P81L and R38X are potentially recurrent mutations in the SDHD gene. Both mutations result from CpG to TpG transitions that are likely to be triggered by the deamination of methylcytosine. We conclude that both a founder effect and recurrent mutations are likely to be responsible for the high prevalence of P81L in The Netherlands. Thus, the P81L mutation may be recommended as the first mutation to test by PCR and restriction enzyme analysis in patients with paraganglioma tumours.

The mutations in SDHB and the pedigrees in which they are identified contrast with those of SDHD. Six different mutations have now been described in SDHD without evidence for a founder mutation. This finding contrasts with SDHD gene mutations that show strong founder effects. Taschner et al. found that two founder mutations in the SDHD gene accounted for 30 of 32 PGL families in The Netherlands and we found that the P81L founder mutation accounts for more than half of the PGL1 linked families ascertained in the USA. Accordingly, the pedigrees with the SDHB mutations are relatively small with only a few affected subjects. This finding also contrasts with the presence of many extended SDHD mutant families which had enabled linkage mapping of the PGL1 locus before the gene was identified. Thus, under the assumption that the de novo mutation rates of the two genes are comparable, the mutations in SDHD may be associated with better phenotypic fitness than those in SDHD. The presumed fitness difference between the two genes can be explained in part by the genomic imprinting at SDHD, which effectively reduces the overall disease penetrance. Unlike SDHD, the presence of paternal transmission in family 13 and maternal transmission in family 9 suggests that the inheritance of SDHD does not show any parent of origin effects.

Finally, the contribution of SDHC to the aetiology of paragangliomas remains unconfirmed. No coding mutations were discovered in SDHC. However, the chromosome 1q21 genomic region where SDHC resides could not be excluded by linkage in one of the multiplex families. This finding suggests that either unconventional mutational mechanisms, which could not be detected by the methods we used in this study, are operative in SDHC or that there is a distinct nearby gene that also contributes to the aetiology of PGL. Further studies are needed to suggest additional genes that also contribute to the genetics of hereditary paragangliomas.

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REFERENCES

ECHO

ARC syndrome is not so rare

ARC syndrome has a wider clinical spectrum than previously thought. Its clinical features cover abnormal morphology, abnormal platelets, recurrent fevers, diarrhoea, and failure to thrive, as well as the classic picture of arthrogryposis, renal tubular acidosis, and cholestasis.

ARC syndrome is associated with consanguinity and is generally supposed to be a rare autosomal disorder. Since the first description in 1973, 11 pedigrees have been reported. Now observations are available from a review of six further cases from three paediatric centres in the UK over 10 years.

In common with previous findings, clinical features proved somewhat variable. However, typical findings of arthrogryposis, renal Fanconi syndrome, and cholestasis were present in all but one patient, who had nephrogenic diabetes insipidus. Unusually, despite severe cholestasis, serum γ glutamyltransferase values were normal for all patients. Further features included abnormal morphology, such as lax skin, low set ears, arched palate; scaly skin; recurrent febrile illnesses or sepsis; diarrhoea; and failure to thrive—all of which have been reported sporadically for ARC syndrome. Abnormally large platelets were also a common feature.

These observations indicate that ARC syndrome has a broader clinical picture and is likely to be less rare than previously thought. Indeed, a syndrome showing these features and reported as new in 1990 is probably ARC syndrome. Two consistent features from these latest cases—normal serum γ glutamyltransferase values and occurrence in Pakistani immigrant families, where marriages between blood relatives are common—may give clues to a way of identifying a candidate gene.

Please visit the Journal of Medical Genetics website [www.jmedgenet.com] for link to this full article.

Since the study a multicentre project to locate the ARC gene locus has started in Birmingham and Utrecht. (Contact: Dr P J McKiernan, Pat.Mckiernan@bhamchildrens.wmids.nhs.uk)