

## ARTICLE

# Mutations in *PAX1* may be associated with Klippel–Feil syndrome

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**Pax genes are a highly conserved family of developmental control genes that encode transcription factors. In vertebrates, Pax genes play a role in pattern formation during embryogenesis. Mutations in Pax genes have been associated with both spontaneous mouse mutants and congenital human diseases. The mouse *Pax1* mutant phenotype *undulated* is characterised by vertebral segmentation defects reminiscent of the human disorder Klippel-Feil syndrome (KFS). To determine whether *PAX1* haploinsufficiency plays a role in KFS, we have defined the gene structure of the human *PAX1* gene and screened 63 KFS patients for mutations in this gene. Differences in the *PAX1* sequence were detected in eight patients. Two patients had a silent change within the paired box that was also seen in 2/303 control chromosomes. The other variants were missense, silent or intronic changes not represented in the control panel tested. The significance of these results and the possible role of *PAX1* in the pathogenesis of KFS are discussed.** *European Journal of Human Genetics* (2003) 11, 468–474. doi:10.1038/sj.ejhg.5200987

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## Introduction

Pax genes are a highly conserved family of developmental control genes that encode transcription factors and are present in organisms ranging from nematodes to man.<sup>1</sup> Nine Pax genes have been identified in mouse and man and are dispersed around the genome.<sup>2</sup> The Pax gene family has been classified into four subfamilies (*Pax1* and *Pax9*; *Pax2*, *Pax5* and *Pax8*; *Pax3* and *Pax7*; *Pax4* and *Pax6*), according to their genomic organisation, the sequences of the paired domains and their expression patterns.<sup>1</sup> The most conserved functional motif in all Pax proteins is the 128 amino-acid paired domain, which exhibits DNA-binding activity.<sup>3</sup> Paired-box-containing genes were first discovered in *Drosophila melanogaster* (*Drosophila paired*

segmentation gene),<sup>4</sup> where they have multiple functions during embryogenesis. Pax genes have also been shown to play a role in pattern formation during embryogenesis in vertebrates, possibly by determining the time and place of organ initiation or morphogenesis.<sup>3</sup> Although the primary developmental action of Pax transcription factors in most tissues has yet to be elucidated, they are thought to play a role in signal transduction during tissue interactions that could lead to a position-specific regulation of cell proliferation. Indeed, analysis of spontaneous and transgenic mouse mutants have shown that vertebrate Pax genes are key regulators during organogenesis of the eye, ear, nose, limb muscles, kidney, vertebral column and brain.<sup>5–12</sup>

Loss-of-function mutations in Pax genes have been associated with both spontaneous mouse mutants (*undulated* (*Pax1*),<sup>5</sup> *Splotch* (*Pax3*),<sup>6</sup> *Small eye* (*Pax6*),<sup>7</sup> *Neu* (*Pax2*)<sup>8</sup>) and congenital human diseases such as Waardenburg syndrome (*PAX3*),<sup>9</sup> aniridia and Peter's anomaly (*PAX6*),<sup>10,11</sup> renal coloboma syndrome (*PAX2*),<sup>12</sup> thyroid dysgenesis (*PAX 8*)<sup>13</sup> and oligodontia (*PAX9*).<sup>14</sup> All these

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show defects in development, and, in each case, haploinsufficiency is the pathogenetic mechanism.

The homology between mouse and human mutants has been a useful tool in defining conditions in man, that which are because of Pax gene mutations. However, the human homologue of the first mouse Pax mutant *undulated* (*un*)<sup>5</sup> remains elusive. *Un* is caused by a missense mutation in the paired box of *Pax1*, which decreases the DNA-binding affinity of the protein and alters its DNA-binding specificity.<sup>15</sup> The *un* mouse, first described in 1947,<sup>16</sup> has segmentation anomalies along the entire axial skeleton where *Pax1* is expressed. The intervertebral discs are irregular and reduced, and there are also anomalies in the development of the pectoral girdle, including the absence of the acromion of the scapula or its replacement with a ligament.<sup>17</sup> *Pax1* is also expressed in the thymus which, in *un* mice, is only half the normal size.

Human *PAX1* maps to chromosome 20p11.2.<sup>2,18</sup> Trisomy and monosomy of this locus have been associated with vertebral anomalies.<sup>19</sup> Based on the mouse studies, the human phenotype of a *PAX1* mutant would be expected to have vertebral segmentation anomalies possibly in conjunction with pectoral girdle and thymus abnormalities. Klippel–Feil syndrome (KFS)<sup>20</sup> is a condition characterised by failed segmentation of the cervical vertebrae with the clinical sequelae of a short, immobile neck and a low posterior hairline. Vertebral fusions may also occur elsewhere along the spine and other vertebral anomalies such as hemivertebrae may be present.<sup>21</sup> Some patients have a ligamentous or bony connection between the vertebrae and scapula. Other features include Sprengel's shoulder, renal, cardiac and neurological abnormalities.<sup>22–24</sup> There are three subtypes of the condition categorised according to the extent of vertebral involvement.<sup>25</sup> KFS appears to be an aetiologically heterogeneous condition with sporadic occurrence, autosomal dominant and autosomal recessive

modes of inheritance reported.<sup>26</sup> The phenotypic similarities between the *undulated* mouse and KFS syndrome encouraged us to screen KFS patients for *PAX1* mutations. In this paper, we define the genomic structure of the human *PAX1* gene and describe the sequence variations detected in a panel of 63 KFS patients screened for mutations in this gene.

## Materials and methods

### Clinical ascertainment of KF patients

A clinical study of KFS in the UK was undertaken and samples were obtained from the patients for both chromosome analysis and DNA extraction. Chromosome analysis was carried out for karyotyping and no gross abnormalities were detected. A total of 63 KFS patient samples were obtained for molecular genetic analysis. Clinical information relating to the patients was also obtained so that they could be categorised according to their phenotype.

### PCR amplification and sequencing

DNA was extracted from peripheral blood using standard automated extraction. PCR was used to amplify regions of the *PAX1* gene from patient DNA for mutation screening by single-strand conformational polymorphism (SSCP) and heteroduplex analysis, and to amplify fragments for direct sequencing. PCR reactions were carried out in a 20  $\mu$ l reaction volume using 50–100 ng genomic DNA as template and 10 pmol of each primer. The reactions were carried out in PCR buffer with a final Mg<sup>2+</sup> concentration of 3.7 mM, 0.75 mM of each dNTP and 0.5 U *Taq* polymerase. The amplifications were carried out under the following conditions: 95°C for 3 min, 30 cycles of 94°C, 55–65°C and 72°C each for 1 min and a final extension at 72°C for 5 min. *PAX1* PCR and sequencing primers and annealing conditions are summarised in Table 1.

**Table 1** *PAX 1* PCR primer sequences

Exon	Primer name	Primer sequence (5'–3')	Annealing temperature (°C)	
Exon 1	E1F	AGTCGCGAAAGGGCACGGAGGGC	60	
	267R	GGCCGTTGACGAACACACCGCCAG	60	
	223F	TCCGGCTCACTCTGTCTGGCGC	60	
	464R	TGACCACGTTGGGAGTGGTGACGC	60	
	442F	CGAGACCGGCTCCATTCTGCCCC	60	
	E1R	ATGTGGTTGTAGGGCAGCGTAGGC	60	
	X13'F	CAGCCCGGACCGTACGAGGCAAG	65	
	X13'R	CTTAATAGAAGGCTACGGAGGC	65	
	Exon 2	X2F	CAGGGCAGATTCGGAGGCACCT	65
		X2R	CTGCTTCCCTACTTAAGGTCAC	65
Exon 3	X3F	GGATTTTCTGCTGCTGACGGTC	63	
	X3R	TCCTAACCCCTCCAGCATCCAC	63	
Exon 4	X4F	CTAGGTAGTGATCCGACGCCTC	65	
	X4R	GAGTCTCCGATCCTGCCCGTG	65	

### Mutation screening

Mutations were screened for using SSCP-heteroduplex analysis. PCR products were denatured in formamide dye (0.01 M EDTA, 98% formamide, trace xylene cyanol and bromophenol blue) and run on 8% polyacrylamide gels (acrylamide 49:1) at 4°C for 16 h at 350 V. The bands were visualised by silver staining. PCR products were cleaned for sequencing using Centricon 100 columns (Amicon), according to the manufacturer's instructions. Band shifts seen on SSCP-heteroduplex analysis were sequenced using a fluorescent cycle-sequencing kit (sequencing conditions were: initial denaturation for 1 min at 96°C; 30 cycles of 96°C for 30 s, 15 s at 50°C and 60°C for 4 min). Products were run on an ABI 373 automatic sequencer and analysed using ABI sequence analysis software version 3.3.

## Results

### Genomic organisation of PAX1

At the start of the project the only published *PAX1* sequence was *HuP48*,<sup>27</sup> which represented the paired box of *PAX1*. We identified the complete human *PAX1* genomic sequence by searching the Genbank database with *HuP48* and the mouse *Pax1* cDNA sequence (Accession number NM008780). The human genomic clone RP5-1065O2 (Genbank Accession number AL035562) contained the whole *PAX1* gene, allowing us to determine its genomic structure (Table 2) and predict the *PAX1* cDNA sequence (Figure 1).

*PAX1* appears to span a genomic region of approximately 10 kb, and comprises four exons, analogous to the mouse *Pax1* gene. Analysis of the genomic sequence using exon prediction and ORF software (NCBI ORF finder) suggests that the *PAX1* protein is composed of 440 amino-acid residues with a molecular weight of 45703.8 Da and a theoretical *pI* of 9.54. Comparisons between the predicted amino-acid sequence of the human *PAX1*, mouse *Pax1* (Accession number AF285175) and the *Drosophila pox meso* paired-box region (Accession number X16992) showed an extra 70 amino-acid residues 5' to the paired box in the human gene (see Figure 2). This finding is compatible with the sequence of *HuP48*, a human genomic clone taken to be derived from the *PAX1* gene.

To confirm the exon boundaries of the human *PAX1* gene as well as the existence of the extra amino-acid

residues predicted, we sequenced a number of IMAGE cDNA clones. The largest *PAX1* cDNA clone (Accession number BF237649) started at nucleotide 71 of the predicted *PAX1* cDNA sequence (Figure 2), but did not contain a methionine start codon upstream of the paired-box region, suggesting that it was not full length. The methionine codon at the amino-acid position corresponding to the start codon of the mouse gene was also absent from this cDNA clone 71 (a TCC sequence was present at nucleotide position 230–233 instead of the ATG in mouse *Pax1*), confirming that the sequence from genomic clones RP5-1065O2 and *HuP48* was correct. Analysis of the genomic clone sequences suggests that the human *PAX1* methionine start codon is predicted to lie further upstream at base 44 in the proposed *PAX1* cDNA sequence.

### Mutation analysis

Intronic primers, based on the genomic *PAX1* sequence, were designed to amplify the four exons of the gene. Three sets of primers were designed for exon 1, splitting it into three fragments of approximately 200 bp, which were more suitable for SSCP mutation screening. A total of 63 affected individuals were screened for mutations in the *PAX1* gene. SSCP band shifts were detected in eight individuals. The sequence changes were defined and healthy controls screened, by SSCP and sequence analysis, to determine whether these sequence differences were population polymorphisms.

### PAX1 mutations and clinical description of KFS patients

The clinical phenotypes of patients with the detected *PAX1* sequence changes are described below.

**Missense mutations – most likely to be pathogenic Patient 1 (932240):** This patient is a Caucasian female. X-rays demonstrated that she had only two cervical vertebrae with extensive fusion including the atlas and axis. She had six lumbar vertebrae and fusion of the first and second ribs anteriorly on the left as well as severe sensorineural deafness and developmental delay. Both parents were clinically normal.

A band shift was detected on heteroduplex analysis in patient 1. Sequencing showed a C>G base change at position 224 in the cDNA sequence resulting in a missense

**Table 2** Genomic organisation of *PAX1*

Exon	5' terminus	Size of exon (bp)	3' terminus	Size of intron (bp)
1	5'UTR ATGCGCCGCGCTCCACTCCG	844	GAGCAAACAGgtcagttgtggcgcc	1502
2	cctctctctctcagGGGCCCTGGC	143	ATACACTCAGgtaaccaggaggcag	521
3	ctctctatccccacagTCGGCCTCCA	223	AGCCGAGAAAGgtgaggagcgcagggg	5026
4	gtgcttctcccagTGGCTGACAG	330	ACTTGGTTTTAGactgcccga 3'UTR	

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1  aagtacagcgcgctaaagtgcgaaagggcagcggagggtaca
44  atgcgcgcgctccactccgcggtcctctcgcgccttggccact
M R R A P L R G S S A P L P T
89  ccaagccagaccagcgggttggccttggactccgagctgtctg
P S Q T Q A V C P W T P S C L
134  gggaccacaggtcaccttgggaagtgcgcctgggtgagtcct
G T H R S P L E V R L G A V P
179  cgctccgcgtggggacccttggtaaccgcgggtgttttctcc
R S A W G P L A N P P G V F S
224  ccctccggtcactcttctgctggcgcacccgagagcagctat
P S G S L L S G A S *A E Q T Y
269  ggcgaggtgaaccagctgggggtgtgttcgtcaacggcgcgcc
G E V N Q L G G V F V N G R P
314  ctgcccacgccatcccttgcgcatgtggagctggcgagctg
L P N A I R L R I V E L A Q L
359  ggcatccgacctgtgacatcagtcggcagctccgcgtatccac
G I R P C D I S R Q L R V S H
404  ggctgctgagcaagaatggcgctacaacgagaccggctcc
G C V S K I L A R Y N E T G S
449  attctgccccggccatgggggagcaagccccgcgtaccact
I L P G A I G S K P R V T T
494  cccaactgtgtaacgacatccgggactacaagcagggaccct
P N V V K H I R D Y K Q G D P
539  ggcatcttggctggagatccgcgaccggctgtggcgcaggc
G I F A W E I R D R L A D G
584  gtctgtgacaagtacaatgtgcctcgtgagctccatcagcgc
V C D K Y N V P S V S S I S R
629  atctgcgcaacaagatggcagcctggcgagccggccttac
I L R N K I G S L A Q P G P Y
674  gaggcaagtaagcagcccgctcgcagcctacgctgccctacaac
E A S K Q P P S Q P T L P Y N
719  cacatctaccagtaaccctaccccagctcccgctgcgcccagggc
H I Y Q Y P Y P S P V S P T G
764  gccaaagatgggcagcccccgggtcccgggcagcggcgggccac
A K M G S H P G V P G T A G H
809  gtcagcctcccgctcatggccctcggcacactcggctcagcaac
V S I P R S W P S A H S V S N
854  atcctgggcatccggagctttatggagcaaacagggccctggct
I L G I R T F M E Q T G A L A
899  gggagcgaaggcagccttactctcccaagatggaagactgggcc
G S E G T A Y S P K M E D W A
944  ggcgtgaaccgcagcgccttccccgccaccccagctgaatggg
G V N R T A F P A T P A V N G
989  ctagagaacctgccttagagggcagacatataactcagctcg
L E K P A L E A D I K Y T Q S
1034  gctccaccctctcgcgctggcggttctccccgcctgcgcc
A S T L S A V G G F L P A C A
1079  taccggcctccaaccagcagcggcgtgtacagcgcgcccgggcggc
Y P A S N Q H G V Y S A P G G
1124  ggctacctcgccccggcccgctggcgccctgcgaaggtcct
G Y L A P G P P W P P A Q G P
1169  cctctggcgcccccgggcgcgctagctgtgcatggcgggaa
P L A P P G A G V A V H G G E
1214  ctgcggcagcaatgaccttcaagcatcccagccgagaggggcc
L A A A M T F K H P S R E G A
1259  ctggctgggagcgaagtggctgacaggaagcctcccagctccggc
L A G S E V A D R K P P S S G
1304  agcaagggccggagcgcctcagtagcttacacggactgcccatc
S K A P D A L S S L H G L P I
1349  cgggctcgacctccaggggcagctctccccgagcccgagcccg
P A S T S *
1394  gagggaacggcagggcgaccggcgacaggtctgcgcgggcggc
1439  cccggcaatcgccagggcaggatcggaggactcgggaggaggga
1484  agcca gtgcccggcccggggtgcaacggccagccagcccgagg
1529  ccagccctgctctgcccggaccaccacactccttatgtgt
1574  ctgggtttttaggcttctctgaactgggttttagactgcgctac
1619  cctcctcacaatccttgcctgacgtggcctcctctcctctgcca
1664  gttctcaaatttcttttggctcactccttgcctcctcctctcct
1709  cctctcctcctgtttcctccccctcttcttctcactcctccc
1754  tcccttcccttctcttccctcctcctcctcctcctcctcctcctc
1799  tttccccgacctccagggtccctctgccccttccactcctttt
1844  cctgctccgacctctgctccagctccgcttcttccccctctcc
1889  tcttctagctcctctatagctatcagcccttttctcgtgctcat
1934  cctgtcgttctctctctctctctgctctggttctactgtcaaggg
1979  ccctagttcccccttggtttactgcttcccccaaccctcctcagtc
2024  cctgagagccctagagccatctcgggtgacagctcctctctggca
2069  cctgggactttgtagcctcactcctctgagccaccagccctaa
2114  tggaggggggctcaaacctaggaagtggacagaaaagccacagcc
2159  tttattttgatattttttgtaatacaaacctcaaaaaacctc
2204  catgaactgtcctctcccgtcagcccccgagcattatgtctgga
2249  aatctggtttcttctcagagccagtgcccgccgggtctctctt
2294  cctctgagatgatgtcaccacaaagaagactgggtaggagta
2339  gggagcacaggggtgtgtgtgtttcagtgcttagaaaaactggg
2384  ttgctcgcaggtgtctcctcccaccctccactcgcgaacttg
2429  ctagggaatccgaccagtggttaaacacatcagcacttttgggt
2474  tcaggggtcagcacatttggtaatttaaacacagactgcttcca
2519  ggtacaaaaggttcaattggactctcgggttgcaagggatgaggt
2564  taaaaaaaaaaaaacaaaacaaacaaacaaacaaacaaacaaac
2609  ccagaaaagtatctctagagccagccgcaagagctgtgagctgct
2654  tccaagttaggcgagctgggtggtaccctggacataatgcaat
2699  tctgtagttagagcaatccctggcgagagcctgacatcgctg
2744  ttaaaggtggctgcctctgataaagggaaagcgtctgaaaggtgt
2789  gtggtgaggtgagcaag

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**Figure 1** Predicted human *PAX1* cDNA sequence. The putative stop codon (nucleotide bases 1364–1366) is underlined. The paired-box region is highlighted in bold type (nucleotide bases 254–673) and the conserved octapeptide sequence (nucleotide bases 839–862) is in bold type and italics. The polyA tail is shown in bold type and underlined. Amino-acid 71 (\*A underlined) corresponds to the start codon in mouse *pax1* cDNA.

mutation (CCC>GCC; P61A) 38bp upstream from the paired-box region. This mutation resides in the extra amino-acid sequence not present in published mouse cDNA sequences. This mutation was also detected in the mother of the proband, who does not show clinical signs of KFS, but not in the paternal sample. A total of 303 chromosomes tested negative for this mutation, suggesting it is not a common polymorphism.

**Patient 2 (931369)** This female Caucasian was diagnosed with KFS type II, vertebrae fusion of C4/5, at age 10 years. Her only clinical features were a low hairline and a slight head tilt to left.

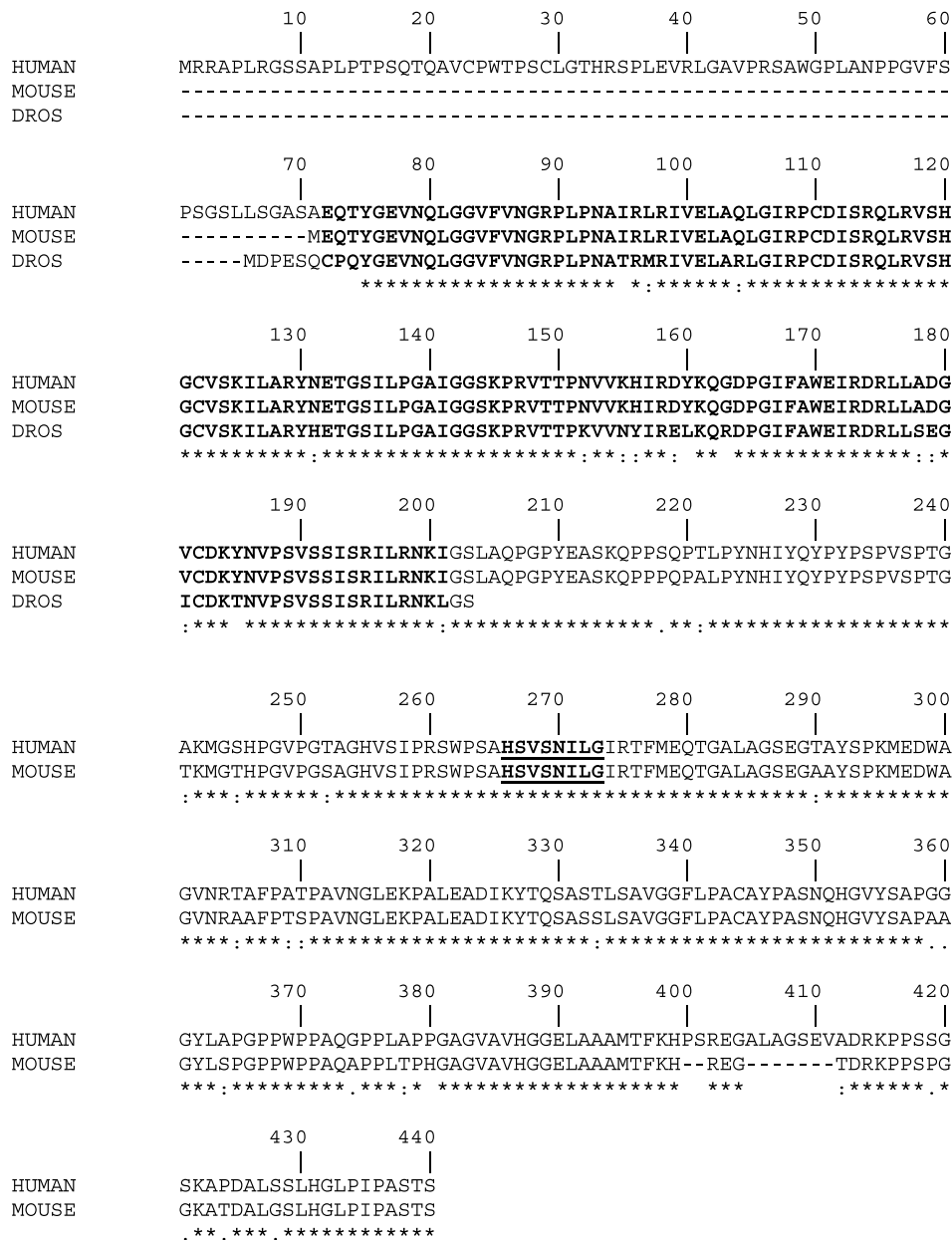
Exon 2 gave an SSCP shift, and sequencing showed a G>C base change at position 890 in the cDNA sequence, resulting in a missense mutation (GCC>CCC; A283P). This sequence change was not detected in 100 controls

(200 chromosomes) tested, indicating that it is not a common polymorphism.

**Patient 3:** This patient was diagnosed overseas as KFS, and had the typical clinical phenotype.

A G>A sequence change at base 908 in the *PAX1* cDNA sequence was detected in this patient, which results in a missense mutation in exon 2 (GGC>AGC; G289S). This change was not detected in 100 controls (200 chromosomes) screened, suggesting that it is not a common polymorphism.

**Intronic changes Patient 4 (921897):** This Pakistani male was diagnosed at birth with KFS. He had a right Sprengel shoulder, mirror movements, small stature and torticollis. X-rays showed a gross complex anomaly of lower cervical and upper thoracic spine, multiple hemivertebrae and



**Figure 2** Comparison of human and mouse *PAX1* amino-acid sequence and *Drosophila pox meso* paired-box sequence. The paired box is shown in bold type and the conserved octapeptide is underlined (Clustalw alignment).

hypoplasia of the vertebral bodies with a short neck. Mother (921896) and maternal grandmother both had short necks but both had normal neck X-rays. The mother later had a son with spina bifida.

An SSCP shift was detected in the mother of patient 4, but was not present in either of her sons. Sequencing identified a G>A base change in intron 2, 10bp from the donor splice junction of exon 2 (exon 2+12). This mutation was not detected in 60 individuals (120 chromosomes) tested.

*Patient 5 (932917)*: This patient is a female of Caucasian/Algerian origin. She had fusion of several cervical vertebrae and was diagnosed at birth with KFS type 1. Other phenotypes included a heart murmur, VSD, scoliosis, Sprengel shoulder, mirror movements, short stature (third centile), posteriorly rotated ears, high palate, webbed neck and torticollis. Karyotype analysis did not detect any chromosome abnormalities.

The SSCP shift detected in patient 5 was an A>C base change in intron 3, 10bp from the donor splice junction of

exon 3 (exon 3+12). This mutation was not detected in 60 individuals (120 chromosomes) tested.

**Silent mutation Patient 6 (931423):** This female Caucasian had a number of congenital abnormalities. Polyhydramnios was noted in pregnancy, she had an imperforate anus with fistula, oesophageal atresia, unilateral renal agenesis with vesicoureteric reflux in the remaining kidney and radial ray dysplasia. Subsequent investigation had shown an absent uterus and vagina. She also had bilateral preauricular tags and was diagnosed with VATER association. Her development was normal but she had bilateral conductive hearing loss needing speech therapy. She also had mirror movements, a low hairline, torticollis, webbing of the neck and scoliosis. X-rays showed a block vertebra in her neck and multiple vertebral anomalies in the mid-thoracic region with at least six lumbar vertebrae and a fusion bar between the two lowermost vertebrae, which have ribs on them. Hemivertebrae were present from T4–T7 and the sacrum was hypoplastic. Her spinal abnormalities placed her in the type III KFS group.

The base change (C>G, at base 976) in patient 6 is a silent mutation in exon 2 (CCC>CCG; P311P), which creates a new *Bgl*I restriction site. Digestion of the exon 2 PCR product with *Bgl*I in individuals with the wild-type sequence gives three bands (170, 65 and 35 bp), whereas the heterozygote containing the P311P mutation gives an additional band of 135 bp. Only patient 6 gave the four-band mutant pattern (data not shown). This sequence change was not detected in 100 controls (200 chromosomes) screened in this way, indicating that it is not a common polymorphism.

**Rare polymorphism Patient 7 (932345):** This patient is a male Caucasian with a cleft palate, a left radial nerve palsy and mild talipes of the right foot and a KF anomaly. His low hairline, webbed neck and good neck movement suggested he had KFS type II.

**Patient 8 (931978):** This male Afro-Caribbean had the clinical triad of KFS as well as a Sprengel shoulder, mirror movements and strabismus as a child.

A rare polymorphism was identified in the paired-box region in both patients 7 and 8. The sequence change (G>A) at base 526 of the *PAX1* cDNA sequence is silent (AAG>AAA; K161K). Two of 303 control chromosomes tested also had this change, suggesting that it is not pathogenic and represents a rare polymorphism. Interestingly, this base change corresponds to the normal mouse *Pax1* cDNA sequence.

## Discussion

It is interesting that the predicted human *PAX1* gene product has a significant number of additional amino acids upstream of the paired box compared to the mouse *Pax1*

and the *Drosophila pox meso* sequence. This is unusual as Pax family genes usually show high homology, particularly between human and mouse, and the paired-box domain is located very near the N-terminus in most Pax proteins. This deviation from the mouse sequence could reflect an additional or different function of the human *PAX1* protein, which could be affected by mutations in this region. If that were true, it is not self-evident that mutations in this region would produce a human phenotype similar to the *undulated* mouse.<sup>15</sup>

Mutation screening of our patients with KFS identified two with a polymorphism within the paired box (analogous to the mouse normal sequence), and six patients with variants that were not seen in controls. Although we did not detect any nonsense, frameshift or evident splicing mutations, the missense mutations could potentially have a pathogenic role. Of the missense mutations, those in exon 2 (A283P, G289S) lie outside the paired box but affect amino acids that are conserved in the mouse sequence. The mutation P61A lies in the run of amino acids upstream of the paired box (not present in the mouse sequence), and was detected in both the proband (patient 1) and her clinically unaffected mother, but not in any control individuals tested. The lack of clinical signs in the mother could be explained by reduced penetrance of KFS, which has been reported in other families with KFS.<sup>28</sup> Silent and intronic mutations have the potential to be pathogenic if they activate cryptic splice sites, leading to exon skipping or the inclusion of intronic sequence into the mature transcript. Pathogenesis by silent mutations has been described in other diseases, for example, limb girdle muscular dystrophy.<sup>29</sup> Unfortunately, since *PAX1* is expressed primarily during development and only in the thymus in adults, we are unlikely to access mRNA from these patients to investigate anomalous splicing.

We also cannot exclude the possibility that all these changes are rare nonpathogenic variants, which probably applies in the case of the mother of patient 4 (who may have a mild form of KFS-short neck and deafness) with an intronic mutation not transmitted to her classically affected KFS son. Alternatively, the variable penetrance seen in some families could be explained by modifying effects of another unlinked locus or by digenic inheritance as in retinitis pigmentosa, where mutations in both *ROM1* and *eripherin/RDS* are necessary to cause disease in some individuals.<sup>30</sup> To support this hypothesis, a missense mutation in a conserved region of the *PAX1* paired box has been described in a foetus with a neural tube defect.<sup>31</sup> The mutation Q42H (Q115H in our sequence) was inherited through an unaffected mother and grandmother. A phenotype reminiscent of extreme spina bifida occulta in humans is also seen in mice doubly mutant for *undulated* and *Patch*.<sup>32</sup>

Our study shows that mutations in the *PAX1* coding sequence are not the cause of all cases of KFS, but this does

not exclude *PAX1* as a candidate gene for some cases. KFS is clearly a highly heterogeneous condition. All three diagnostic signs for the syndrome (failure of segmentation of two or more vertebrae, a short neck with limitation of head movement and a low posterior hairline) are present in fewer than 50% of patients. KFS has also been described as a manifestation of foetal alcohol syndrome,<sup>33</sup> and a phenotype similar to *undulated* is provoked in mice by maternal treatment with the anticonvulsant valproic acid.<sup>34</sup> The possibility remains that *PAX1*, alone or in conjunction with other genetic or environmental factors, plays a role in the pathogenesis of KFS. Future work will concentrate on identifying and screening more candidate genes implicated in the *PAX1* pathway or in other aspects of skeletal development. Gross chromosomal abnormalities and association with other syndromes may provide vital clues as to the location of candidate gene(s) involved in the pathogenesis of KFS. In fact, a familial KFS gene locus on 8q has been identified (*inv(8)(q22.2q23.3)* – segregated with congenital vertebral fusion), which provides a good starting point for candidate gene approaches.<sup>35</sup>

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