Sorting nexin 3 (SNX3) is disrupted in a patient with a translocation t(6;13)(q21;q12) and microcephaly, microphthalmia, ectrodactyly, prognathism (MMEP) phenotype

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ORIGINAL ARTICLE

Sorting nexin 3 (*SNX3*) is disrupted in a patient with a translocation t(6;13)(q21;q12) and microcephaly, microphthalmia, ectrodactyly, prognathism (MMEP) phenotype

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A patient with microcephaly, microphthalmia, ectrodactyly, and prognathism (MMEP) and mental retardation was previously reported to carry a de novo reciprocal t(6;13)(q21;q12) translocation. In an attempt to identify the presumed causative gene, we mapped the translocation breakpoints using fluorescence in situ hybridisation (FISH). Two overlapping genomic clones crossed the breakpoint on the der(6) chromosome, locating the breakpoint region between D6S1594 and D6S1250. Southern blot analysis allowed us to determine that the sorting nexin 3 gene (*SNX3*) was disrupted. Using Inverse PCR, we were able to amplify and sequence the der(6) breakpoint region, which exhibited homology to a BAC clone that contained marker D13S250. This clone allowed us to amplify and sequence the der(13) breakpoint region and to determine that no additional rearrangement was present at either breakpoint, nor was another gene disrupted on chromosome 13. Therefore, the translocation was balanced and *SNX3* is probably the candidate gene for MMEP in the patient. However, mutation screening by dHPLC and Southern blot analysis of another sporadic case with MMEP failed to detect any point mutations or deletions in the *SNX3* coding sequence. Considering the possibility of positional effect, another candidate gene in the vicinity of the der(6) chromosome breakpoint may be responsible for MMEP in the original patient or, just as likely, the MMEP phenotype in the two patients results from genetic heterogeneity.

Split hand/split foot malformation (SHFM) is characterised by a developmental defect of the central rays of the hands and/or feet during embryogenesis. Although usually inherited in an autosomal dominant manner, sporadic cases have also been reported.[1] The highly variable clinical manifestations of ectrodactyly can be isolated (MIM 183600) or associated with other congenital abnormalities. Examples of syndromic ectrodactylies are the EEC (ectrodactyly, ectodermal dysplasia, and cleft lip/palate) syndrome (MIM 129900), the Patterson-Stevenson-Fontaine syndrome (MIM 183700), or the ADULT (MIM 103285) syndrome. Several syndromic ectrodactylies have now been shown to be allelic forms of the same gene. Indeed, the SHFM4/EEC3 locus (MIM 605289, MIM 604292) was recently shown to be allelic with Hay-Wells syndrome (MIM 106260). The gene responsible for these conditions is *P63*, a homologue of the tumour suppressor gene *P53*, and maps to chromosome 3q27.[2–5] Nonetheless, ectrodactyly is genetically heterogeneous, as several other loci have been identified using either chromosomal rearrangements or linkage analysis. The *SHSF1* locus (MIM 183600) was mapped to chromosome 7q21.2-q21.3 by a great number of patients with interstitial deletions or translocations involving that region.[6–11] The *SHSF2* locus (MIM 313350) was mapped to Xq26 by linkage analysis in a large Pakistani kindred.[12] Another locus named *SHSF3* (MIM 600095) was mapped to 10q24-q25 by a complex rearrangement involving 10q25.2-qter and subsequent linkage analysis in families with no cytogenetically detectable rearrangement.[13–16] Moreover, other loci are presumed to be located on chromosome 2q34[17] and chromosome 7q11.2-q21.3 (EEC1)[18 19] because of chromosomal rearrangements associated with SHFM. Finally, a locus at 6q21-q22 has been suggested because of several chromosomal rearrangements associated with limb, facial, and cardiac malformations, as well as microcephaly and growth and developmental delay.[20–25] Although most of the rearrangements were interstitial deletions, two patients had de novo reciprocal translocations, t(6;13) and t(6;7), both involving chromosome 6q21.[20 22] These observations provided compelling evidence for the presence of a gene involved in limb development and other developmental pathways at band 6q21.

In this study, we report the mapping of the (6;13)(q21;q12) translocation breakpoints in the patient reported by Viljoen and Smart.[20] The patient had microcephaly, microphthalmia, ectrodactyly of the feet, and prognathism (MMEP). Using FISH analysis, we localised the breakpoint on chromosome 6q21 close to marker D6S1250. Subsequent cloning of the breakpoint fragment allowed localisation of the der(13) breakpoint close to marker SHGC-102422 in the region 13q11-q12. Sequencing of the chromosome 13 breakpoint confirmed that the translocation was balanced, with no missing or duplicated material. Gene mapping at the site of the breakpoints showed that the translocation breakpoint does not appear to disrupt any gene on chromosome 13, but does disrupt a gene on chromosome 6q21, called sorting nexin 3 (*SNX3*).

MATERIALS AND METHODS

Patients

The clinical features of patient 1 with the (6;13) reciprocal translocation have previously been described in detail.[20] Briefly, the patient had ectrodactyly of the feet, microcephaly (OFC <3rd centile), prognathism, central cleft lip and palate, and severe mental retardation. With the exception of her fingerised thumbs, both hands were normal. She had bilateral microphthalmia and was totally blind. Cytogenetic analysis of the patient showed a balanced translocation, 46,XX, t(6;13)(q21;q12).[20]

Table 1 List of the oligonucleotide primers

Primer pair name	Forward	Reverse	Product size	Annealing temperature
cdnaSNX3	5′-TGATCACCAAGCCGCAGAAC-3′	5′-CAAATTAAAAGGGGGAAAGAGAAA-3′	846 bp	62°C
SNX3exon1	5′- GCGGCGGCGGCGGCTGAAC -3′	5′- GCGGGAGGGGTTTCTTGGGAGAGG -3′	269 bp	65°C
SNX3exon2	5′-TGATTTCTTTTGTTTTGCCATAC -3′	5′-TTTTGCTTTAAAGTTGCATCATAA -3′	221 bp	55°C
SNX3exon3	5′-AAATTTTCACAACCCGTCATCAT -3′	5′-AAATGGCTGAAAGAAAAGGCAACA -3′	281 bp	55°C
SNX3exon4	5′- TTTTGAACTCCCCCACCTTGTAT-3′	5′- TTTCTGTGCAGCATGCTAAAAGTT-3′	253 bp	65°C
"ex4dup"	5′-CTAACCAAAAATTCGTAAGTAT-3′	5′-GAAATGTGCTGTAAAATGTAAC-3′	308 bp	55°C
IPCR	5′-GAAGGGGCAAAAACGTGACTATT-3′	5′-TGTGCCAGAGGATGACCAG-3′	ND	65°C
NestedSNX3	5′-ATGGCTGCGAAGTGAATTAGAAAG-3′	5′-AAGCTGGAGGCAACACAATCA-3′	ND	60°C

ND, not determined.

Patient 2 has also previously been described in detail.[26] He had a phenotype very similar to the first patient and both patients were described as having microcephaly, microphthalmia, ectrodactyly, and prognathism (MMEP).

Cell lines and DNA samples

Ethidium bromide treatment of a lymphoblastoid cell line of patient 1 was used to generate prometaphase spreads for FISH analysis.[27 28] DNA was isolated by high salt precipitation[29] and diluted to a concentration of 105 ng/µl.

RNA extraction and cDNA synthesis

Total RNA was extracted from lymphoblastoid cell lines (approximately 3×10^6 cells) using TRIzol® LS (Life Technologies) according to the manufacturer's procedure. Samples were treated with DNase I/Amp (Life Technologies) for 15 minutes at room temperature and purified using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Approximately 3 µg of total RNA were reverse transcribed into cDNA using random hexamers (SuperScript™ Preamplification System, Life Technologies).

Fluorescence in situ hybridisation (FISH) analysis

PAC and BAC clones were used as FISH probes. The genomic clones were selected according to their microsatellite marker content, their genetic mapping position from the Cedar web site (http://cedar.genetics.soton.ac.uk/pub/chrom6/map), and their physical mapping position on the Whitehead radiation map (http://www-genome.wi.mit.edu/). Microsatellite markers mapping to chromosome 6q21 were used to screen the "non-redundant" (nr) database and the "high throughput genomic sequences" (htgs) database from the National Center of Biotechnology Information (NCBI), using BLASTN (http://www.ncbi.nlm.nih. gov/blast).

Human genomic BAC and PAC clones were purchased from Research Genetics (Huntsville, AL, USA) and DNA was isolated using the QIAGEN® plasmid maxi kit (QIAGEN, Valencia, CA, USA). Purified BAC or PAC DNA was labelled with digoxigenin-11-dUTP (Boehringer Mannheim Biochemical, Indianapolis, IN, USA) by standard nick translation procedures. FISH analysis was performed as described by Brkanac *et al*.[30]

Sequencing of PCR products

PCR products were gel purified for direct sequencing with gene specific primers using the Thermosequenase Cy™5 Dye Terminator kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's procedures.

Southern blot analysis

SNX3 cDNA was amplified by RT-PCR from mRNA prepared from fresh lymphocytes, using cDNASNX3 primers (table 1). *SNX3* exons 3 and 4 and their flanking intronic sequences were amplified from genomic DNA using primers designed for mutation analysis (table 1). BAC clone RP11-427M20 (GenBank accession No AC079380) was used as a basis for "ex4dup" primers (table 1) and probe design. PCR products were gel purified using QIAquick gel extraction kit; 25 ng were labelled with 50 µCi at 3000 Ci/mmol [α ^{32}P]-CTP (NEN, Boston, MA, USA), using the random prime labelling system Redprime™ II (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Southern blot analysis was performed as described previously.[31]

Densitometry

Densitometric analysis of Southern blot filters hybridised with *SNX3* probes was performed as previously described.[32] A single copy probe from chromosome 4, D4S12, was hybridised to each filter as a control. Hybridisation signals of both D4S12 and *SNX3* gene specific probes were measured using the Molecular Dynamics 300A Computing Densitometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Calculations of the *SNX3*/D4S12 probe ratios allowed comparisons between the controls and the patients, facilitating detection of deletions.

Inverse PCR

Inverse PCR was performed as described previously[31] using "IPCR primers", corresponding to the "complementary" primers of *SNX3* exon 4 (table 1).

SNX3 IMAGE clone

A *SNX3* Unigene cluster, Hs.12102, from the NCBI database (http://www.ncbi.nlm.nih.gov/unigene) allowed identification of *SNX3* IMAGE clones No 1084159 and No 1977743 that were purchased from Research Genetics (Huntsville, AL, USA). IMAGE clone No 1977743 was sequenced using M13 universal Cy™5 labelled primers with the Thermosequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Internal primers "nestedSNX3" (table 1) were designed and used for sequencing of the entire 1.6 kb insert. Alignment between the known *SNX3* cDNA sequence (GenBank accession No NM_003795) and the IMAGE clone No 1977743 sequence was performed using the program Windows 32 SeqMan 4.05© (DNASTAR Inc, Madison, WI, USA).

Mutation detection by dHPLC analysis

Individual exons of the *SNX3* gene, along with flanking intronic sequences, were amplified and tested by dHPLC analysis as described by Han *et al*.[33]

RESULTS

Mapping of the 6q21 breakpoint

In order to localise the breakpoint in band 6q21 of the t(6;13) translocation, we conducted a systematic series of FISH analyses. As shown in fig 1A, we could narrow the breakpoint region to less than 1 cM using seven BAC/PAC probes. In order to cover the area between clone RP3-429G5 (positive for marker D6S1594) that mapped to the der(6) and clone RP3-354J5 (positive for marker D6S1250) that mapped to the

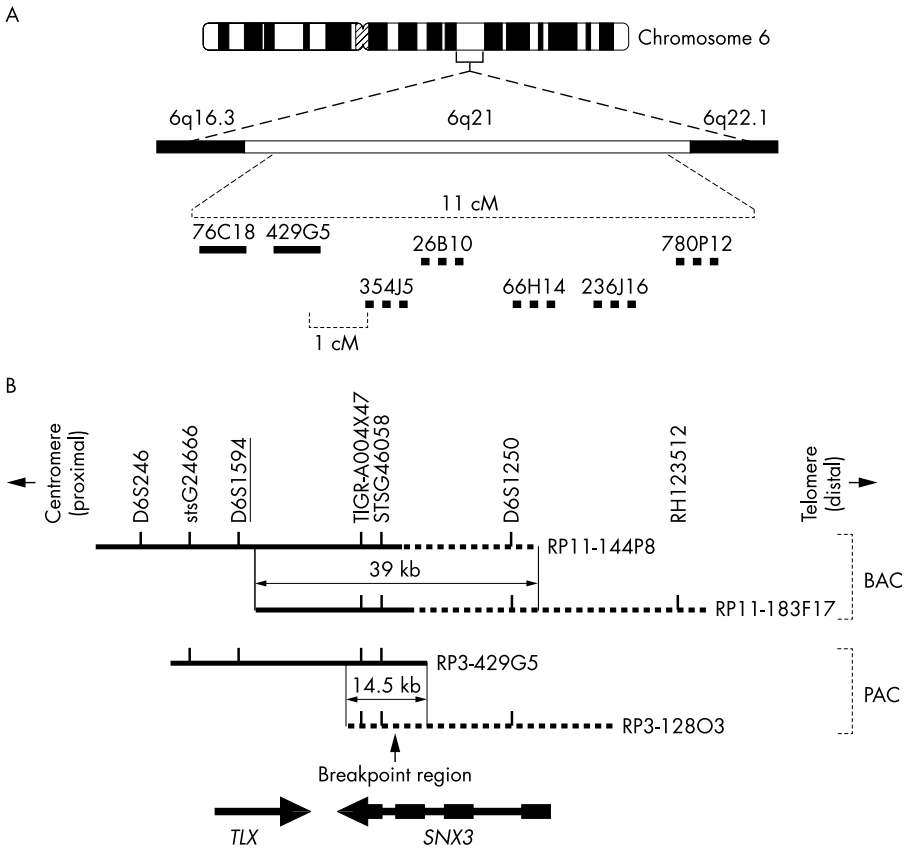

Figure 1 (A) Mapping of the breakpoint region from the t(6;13) translocation at chromosome 6q21. Ideogram of chromosome 6 and the chromosome band 6q21 showing the results of the first three rounds of FISH analysis which narrowed the breakpoint region from 11 cM to 1 cM. Clones hybridising to the der(6) chromosome are represented by solid lines and clones hybridising to the der(13) chromosome are represented by dashed lines. (B) Physical map of the breakpoint region showing the four clones mapping to the breakpoint region. Arrows indicate the transcript orientation of the two genes tailless (*TLX*) and sorting nexin 3 (*SNX3*) relative to the centromere. The diagram is not drawn to scale.

der(13) chromosome, we constructed a BAC/PAC contig map (data not shown). This contig was then used for further FISH analysis of the region of interest. In summary, two clones mapped proximal, nine mapped distal, and two clones (RP11-183F17 and RP11-144P8) hybridised to both derivative chromosomes in addition to the normal chromosome 6 (fig 1B). Since these two clones overlapped by 40 kb, the breakpoint location was narrowed to this overlapping region. Interestingly enough, FISH analysis using clones RP1-128O3 and RP3-429G5, which covered the same region, did not result in signals on both derivative chromosomes (fig 2). This might be explained by the fact that they only overlapped by 14 kb.

Molecular cloning of the 6q21 breakpoint

In order to detect a candidate gene mapping to the 40 kb breakpoint region, a genomic sequence analysis was performed by searching the NCBI database (http://www.ncbi.nlm.nih.gov/), which identified two already published genes: tailless (*TLX*), which is located in clone RP3-429G5, centromeric to the overlapping region, and sorting nexin 3 (*SNX3*), which exhibited homology to both RP3-429G5 and RP1-128O3 and was thus included in the overlapping region that spanned the breakpoint (fig 1B). The genomic structure of the *SNX3* gene was determined by alignment between the known *SNX3* cDNA sequence (GenBank accession No NM_003795) or the IMAGE clone No1977743 and the PAC clone RP1-128O3 using BLASTN 2 sequences (http://www.ncbi.nlm.nih.gov/gorf/bl2.html) (table 2). The *SNX3* gene is composed of four exons that encompass 50 kb. Southern hybridisation with a *SNX3* cDNA probe had shown a different pattern between the translocated patient and the controls. To determine precisely which exon was contained in the shifted DNA fragments, we hybridised with probes containing first exon 3 (fig 3A) and then exon 4 (fig 3B). Since both exons 3 and 4 hybridised to two different fragments in patient 1, but normally hybridise to a single DNA fragment for all the enzymes tested, the translocation breakpoint occurred somewhere between exons 3 and 4. Isolation of the 1.4 kb *Pst*I fragment (fig 3B) by Inverse-PCR using exon 4 specific primers allowed us to sequence the chromosome 6 breakpoint. A BLASTN search (http://www.ncbi.nlm.nih.gov/blast/) against the "non-redundant" database confirmed the presence of *SNX3* exon 4 and its adjacent intronic sequence in the shifted fragment and showed that the breakpoint occurred within a SINE/Alu repeat, 222 bp upstream from exon 4 (fig 4). This result was consistent with the fact that clones RP3-429G5 and RP1-128O3 did not give a split signal when hybridised to the derivative chromosomes. The signal was too weak to be detected since only 7 kb of RP3-429G5 hybridised to der(13) and only 7.8 kb hybridised to der(6) with probe RP1-128O3.

Molecular cloning of the 13q12 breakpoint

The BLASTN search against the "htgs" database also identified a BAC clone RP11-347L8 (GenBank accession No AL137250) containing several markers specific to chromosome 13q12. This placed the breakpoint on chromosome 13 close to marker SHCG-102422. Since the BAC clone sequence was deposited in the database, we could design primers from the flanking sequences of each chromosome at the breakpoints and amplify the der(13) chromosome breakpoint region. Sequencing of the PCR product showed that no material was deleted

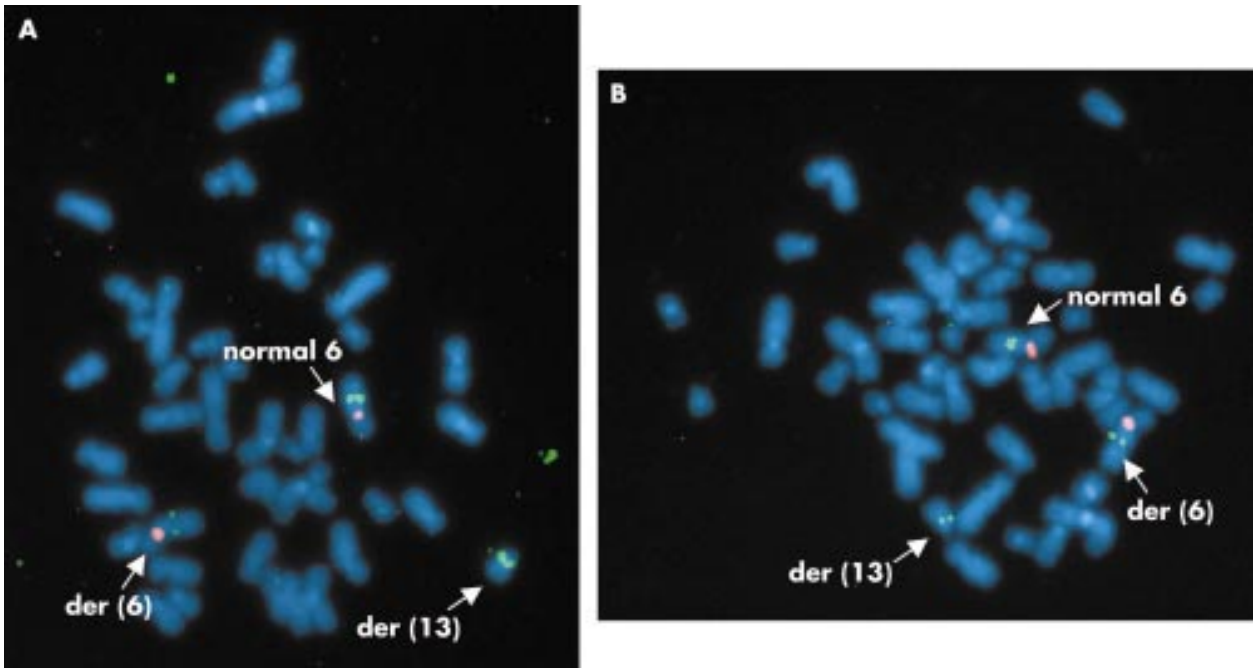

Figure 2 Fluorescence in situ hybridisation (FISH) images of the BAC clone RP11-183F17 (A) and of the BAC clone RP11-144P8 (B) on metaphase chromosomes of the patient with a 6;13 translocation. Red signals identify chromosome 6 centromeres. Note a stronger green signal on the der(13) chromosome compared to the der(6) chromosome for probe RP11-183F17, while probe RP11-144P8 exhibits a stronger green signal on the der(6) chromosome compared to the der(13) chromosome.

and that a two base duplication (TA) from the chromosome 6 Alu repeat sequence was present at both breakpoint sites (fig 4).

Characterisation of the human *SNX3* gene

A 1201 bp mRNA sequence of the *SNX3* gene (GenBank accession No NM_003795) was reported by Haft *et al*,[34] which encoded for a protein of 162 amino acids. Although *SNX3* was widely expressed (NCBI Unigene database, Hs.12102), it was more strongly expressed in heart, skeletal muscle, and spleen.[34] A *SNX3* Unigene cluster, Hs.12102, comprising 241 ESTs entries, included an IMAGE clone No 1084159 from a colon tumour library. However, this clone of 2.4 kb could not be characterised any further as it failed to grow, but sequencing of another IMAGE clone (No 1977743) from a brain library, with a 1.52 kb insert, showed a novel isoform, which we named *SNX3.2*. The isoform appears to have originated from use of a cryptic splice site within exon 1 that eliminates 22 amino acids (fig 5). In addition, the 3'UTR was extended by 288 bp with another putative polyadenylation site and the 5'UTR was extended by 135 bp upstream of the known sequence. Hybridisation of Southern filters with exon 4 of *SNX3* had shown an extra fragment in the controls and patients with all three enzymes that was not expected from the genomic sequence of clones RP1-120O3 or RP3429G5 (fig 3B). Hybridisation of CEPH random subjects with *SNX3* exon 4 showed the same fragments and a polymorphism of at least three alleles (data not shown). A BLASTN search against the htgs database (NCBI) showed 95% homology over 117 bp between exon 4 probe and BAC clone RP11-427M20. This 117 bp sequence was the only homology found between the *SNX3* cDNA and this BAC clone. This clone was mapped to chromosome 4 by identification of several markers including D4S1575, using the electronic PCR from NCBI. The size of one of the extra fragments was consistent with the predicted restriction map from BAC clone RP11-427M20. According to the database sequence, the exon 4 homologous sequence on chromosome 4 was interrupted by a LINE element, which may cause this region to be unstable, possibly generating the three fragments shown by Southern hybridisation.

Mutation analysis of patient 2

To further our analysis of the *SNX3* gene, we screened patient 2[26] for alterations of the gene as he had the MMEP phenotype and did not have any cytogenetically visible rearrangements. Amplification of all four *SNX3* exons and their flanking sequence was used for dHPLC analysis and sequencing. No differences in dHPLC patterns were detected between a control and patient 2. Also, we sequenced the four exons of the *SNX3* gene for patient 2 and failed to detect any point mutation. Southern analysis detected an extra fragment with the *SNX3* exon 4 probe, which varied in size depending on the

Table 2 Splice site sequence and organisation of the *SNX3* gene

Exon	Exon size (bp)	Intron	Intron size (kb)	Donor site	Acceptor site
1	267*	I	37.64	GATgtgagcaac	ttgtttcagACA
1′	198	I′	37.71	AAGgtgaggcaa	"
2	95	II	8.33	AAGgtaagaatg	acattttagGTC
3	124	III	2.24	CAAgtaagtgctc	attctcagGGT
4	740†				
4′	1028†				

*Correspond to a cryptic splice site in exon 1.
†The size of the last exon differs according to a different polyadenylation site.

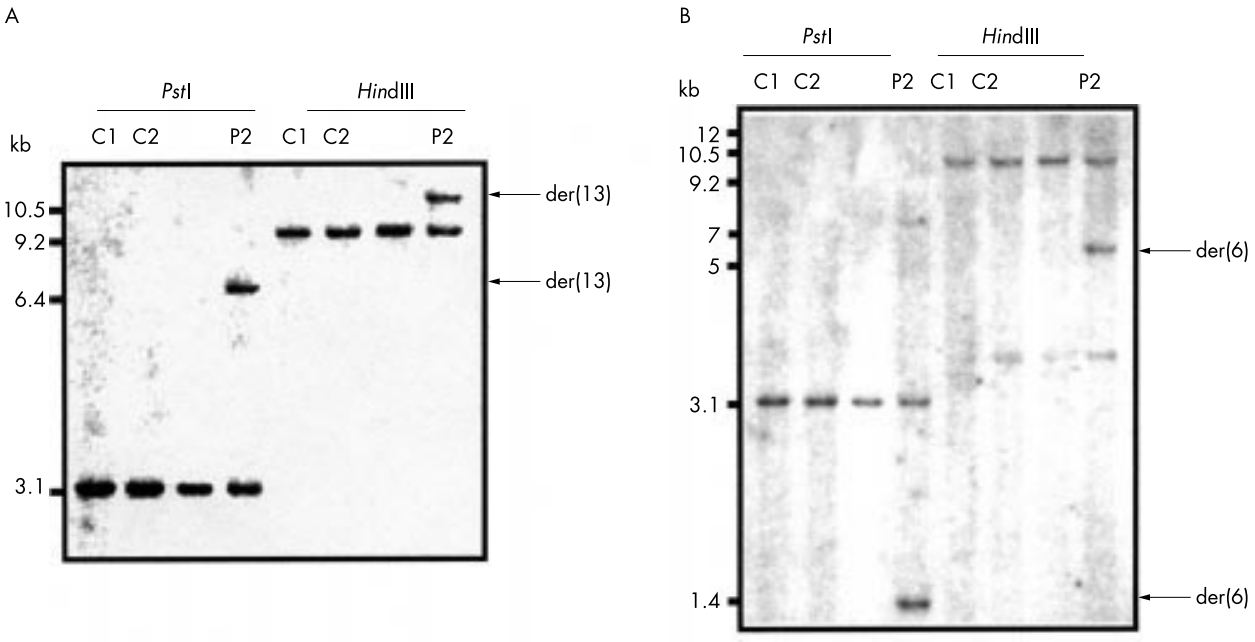

Figure 3 Southern blot analysis of genomic DNA from two controls, C1 and C2, and patient 1 with the t(6;13) translocation (P2). (A) The filter hybridised with a *SNX3* exon 3 probe. (B) The filter hybridised with a *SNX3* exon 4 probe. Exons 3 and 4 normally hybridise to a single band of 3.1 and 9.2 kb with *Pst*I and *Hin*dIII enzymes, respectively. The arrows indicate the altered restriction fragments owing to the t(6;13) translocation. These are only present in the patient P2.

enzyme used for digestion. However hybridisation with a chromosome 4 specific probe "ex4dup" showed that all of the extra fragments originated from chromosome 4 and were present in normal subjects (data not shown).

DISCUSSION

In this study, we were able to map both breakpoints of a translocation t(6;13)(q21;q12) in a patient with MMEP. Using FISH analysis, we could map the breakpoint region on chromosome 6 between D6S1594 and D6S1250, while the der(13) breakpoint was subsequently mapped close to SHCG-102422. Molecular cloning of both breakpoints confirmed the cytogenetic karyotype previously reported by Viljoen and Smart[20] and also confirmed that the translocation was balanced.

The sorting nexin 3 (*SNX3*) gene was found to be disrupted within intron 3 by the der(6) breakpoint. Therefore, we suspect that the disruption of the *SNX3* gene underlies the MMEP phenotype in patient 1. However, no alteration of the *SNX3* gene could be detected in patient 2, a sporadic case with the MMEP phenotype but with no cytogenetically detectable

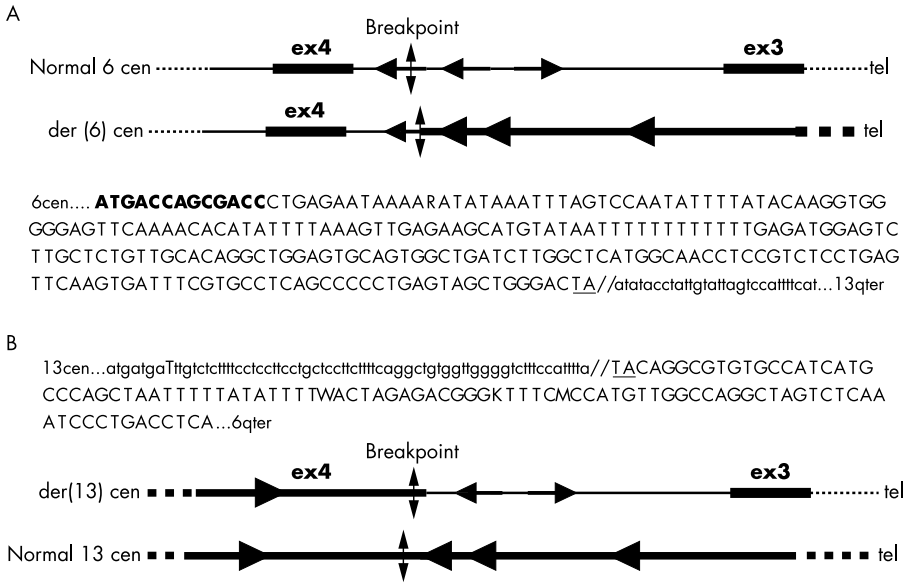

Figure 4 Diagrams of the sequence analysis of the t(6;13) translocation breakpoints. (A) The normal chromosome 6 and the der(6) chromosome and (B) the normal chromosome 13 and the der(13) chromosome are represented with the chromosomal organisation of the repeat sequence, symbolised by arrows, and *SNX3* exons 3 and 4, symbolised by boxes. The upper case lettering corresponds to chromosome 6 sequence and the lower case lettering to chromosome 13 sequence. The underlined sequence corresponds to a 2 base duplication from the chromosome 6 Alu repeat sequence at the chromosome junction. The bold sequence corresponds to *SNX3* exon 4.

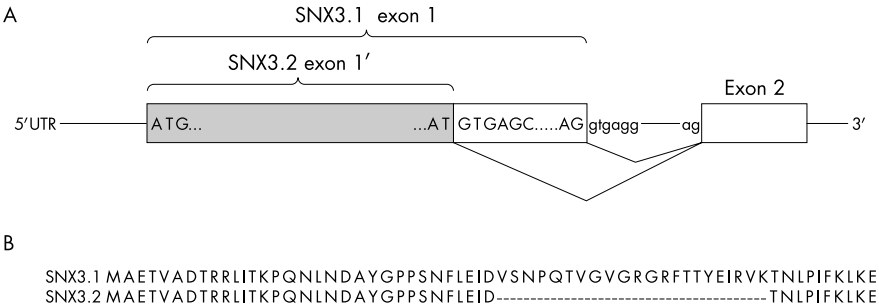

Figure 5 Alternative splicing of *SNX3* exon 1. A diagram of *SNX3* indicates the two splice sites, resulting in alternative splicing of exon 1 (*SNX3.1* isoform) or exon 1′ (*SNX3.2* isoform). (B) Translation of the two isoforms showing the missing 24 amino acids in isoform *SNX3.2*.

rearrangement. It is possible that a mutation in the promoter region of the *SNX3* gene could still be responsible for the phenotype, as we have not screened this portion of the gene. Also, an inversion of the gene could have been missed by Southern analysis if one or both breakpoints had occurred within an intron or involved the promoter region only. Both scenarios could result in haploinsufficiency as in the patient with the translocation.

When a chromosome breakpoint does not directly disrupt the disease causing gene, it is thought to alter the gene expression by a phenomenon called position effect, for which the mechanism is still unknown. Several hypotheses have been suggested and reviewed by Kleinjan and van Heyningen.[35] (1) The chromosomal rearrangement could separate the promoter from a distant regulatory element. (2) The chromosomal rearrangement may juxtapose a gene with a regulatory element from another gene. (3) The chromosomal rearrangement may bring a gene and its regulatory element close to another gene generating a competition for the regulatory element between the two genes. (4) And finally the rearrangement could give rise to position effect variegation (PEV), a phenomenon first described in *Drosophila*[36] and later reported in mammalian systems.[37 38] Several translocation cloning projects have shown that a gene causing a disorder is not always disrupted within the coding sequence[35] or that, even though a gene is disrupted, it can still be another gene mapping proximal or distal to the breakpoint that causes the phenotype.[39] Therefore, the MMEP phenotype in patient 1 could in fact be cause by the impaired expression of another gene or by contiguous genes in the vicinity of the der(6) breakpoint. The necessity of altering the expression of two genes in order to underlie the MMEP phenotype would be consistent with its rarity. Patient 2 could possibly carry an inversion involving contiguous genes, while in patient 1 *SNX3* is disrupted by the breakpoint, and perhaps another gene is affected by position effect.

SNX3 is a member of the sorting nexin family, composed of at least 14 other members (NCBI database).[34 40] None of the sorting nexin family members has yet been associated with any human disorder. Sorting nexin family members are identified by a conserved phox homology (PX) domain of about 100 amino acids, first characterised in NADPH oxidase, p47phox and p40phox.[41 42] Function of the SNX3 protein remains to be characterised to determine its putative role in development. The PX domain is highly conserved through evolution, therefore suggesting a similarity in biological function between species.[34] The yeast Grd19p is the most closely related in sequence to the human *SNX3*. Grd19p was shown to maintain late Golgi enzymes in their proper locations by retrieving mislocalised molecules from the prevacuolar compartment and Grd19p is not required for vacuolar protein sorting, unlike the other studied yeast sorting nexin proteins.[43] Study of the SNX3 function may help in deciding if *SNX3* is or is not a good candidate gene for this disorder. SNX3 knockout mice would be most useful in providing this information.

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