ORIGINAL ARTICLE

Disruption of the neuronal PAS3 gene in a family affected with schizophrenia

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Schizophrenia and its subtypes are part of a complex brain disorder with multiple postulated aetiologies. There is evidence that this common disease is genetically heterogeneous, with many loci involved. In this report, we describe a mother and daughter affected with schizophrenia, who are carriers of a t(9;14)(q34;q13) chromosome. By mapping on flow sorted aberrant chromosomes isolated from lymphoblast cell lines, both subjects were found to have a translocation breakpoint junction between the markers D14S730 and D14S70, a 683 kb interval on chromosome 14q13. This interval was found to contain the neuronal PAS3 gene (*NPAS3*), by annotating the genomic sequence for ESTs and performing RACE and cDNA library screenings. The *NPAS3* gene was characterised with respect to the genomic structure, human expression profile, and protein cellular localisation to gain insight into gene function. The translocation breakpoint junction lies within the third intron of *NPAS3*, resulting in the disruption of the coding potential. The fact that the bHLH and PAS domains are disrupted from the remaining parts of the encoded protein suggests that the DNA binding and dimerisation functions of this protein are destroyed. The daughter (proband), who is more severely affected, has an additional microdeletion in the second intron of *NPAS3*. On chromosome 9q34, the translocation breakpoint junction was defined between D9S752 and D9S972 and no genes were found to be disrupted. We propose that haploinsufficiency of *NPAS3* contributes to the cause of mental illness in this family.

Schizophrenia is a complex disease, manifesting symptoms which include changes in perception (hallucinations), inferential thinking (delusions), motivation (avolition), and thought and speech (alogia). These clinical signs are also compounded by a spectrum of negative symptoms that include decrease in social interaction, cognitive impairment, and attentional impairment. The diagnostic criteria for this disease have been established by the International Classification of Diseases, 10th edition,[1] and the Diagnostic and Statistical Manual of Mental Disorders, 4th edition.[2] Many subtypes of schizophrenia have been proposed. Those affected with schizophreniform psychosis exhibit the psychotic symptoms but no deteriorating course. The population prevalence of schizophrenia is estimated to be 1-2%, with an age of onset that is usually in the late teens to early 20s in males and with a five year lag in onset in females.[3] Late onset schizophrenia occurs after 40 years of age in 10% of diagnosed cases and is more prevalent in females. Schizophrenia is also diagnosed during early childhood. In general, patients display common clinical and neurophysiological findings at variable ages of onset.

The genetic basis of schizophrenia is suggested by the observation of a higher risk among family members of patients than those in the general population.[4] Evidence from adoption and twin studies also showed a higher risk among relatives of affected families than in the general population.[4] Owing to the complex nature of this disease, no specific mode of inheritance can be established. Mathematical modelling has, however, favoured polygenic models of inheritance involving several susceptibility genes acting additively, and possibly in conjunction with environmental factors, to result in schizophrenia. Evidence for genetic heterogeneity is supported by several genome wide screens for causative loci, by linkage analysis and association studies.[5–7] These studies suggested that several chromosomes including 1q, 4q, 5p, 6p, 6q, 8p, 9q, 10p, 13q, 14q, 15q, 22q, and Xp contain major, or susceptibility, genes for schizophrenia.[5–7] Many of these locations have not been supported by independent studies. We report here a family with schizophrenia and a translocation (9;14) chromosome with a breakpoint junction in a novel bHLH-PAS transcription factor on chromosome 14.

MATERIALS AND METHODS

Subjects

Diagnoses were established by an experienced psychiatrist (WJM) using SADS-L, PAS-ADD structured assessment, and hospital case note review. The proband (cell line L6874) has severe learning disability and a clinical diagnosis of schizophreniform psychosis, established in her late teens, according to DSM-IV criteria. She shows periods of almost continuous agitated outbursts, emotional lability, and stereotypic posturing movements. Visual and auditory hallucinations are noted from apparent preoccupation with stimuli that cannot be detected in her environment, manifest as periods of staring at objects or walls during which she cannot be distracted, and repeated cries and screams with no internal or apparent external causes. The phenomena were controlled with a combination of neuroleptic medications (depot piportil and chlorpromazine). Repeated attempts at medication reduction have met with symptom resurgence. Although there is no cyclical pattern to the disturbance and no episodes of depressive disorder, which is easier to diagnosis in those with severe mental retardation, the possibility of an underlying comorbid affective disorder inherited through the father cannot completely be ruled out. Her mother (cell line number SMOM) is physically normal with mild or borderline intellectual impairment. She attended special schooling for children with developmental delay. A history of schizophrenic illness began in her late thirties, and included symptoms of formal thought disorder, ideas of self-reference, bizarre delusions, and somatic hallucinations. This was accompanied by severe social dysfunction that required admission to a psychiatric hospital. There was no evidence of affective disorder. She met the DSM-IV symptom and duration criteria for schizophrenia, which was responsive to standard oral neuroleptic (phenothiazine) medication. The

Table 1 Primers, PCR conditions, and cycles used for mapping within the *NPAS3* and *KIAA0391* genes

Marker	Map position	Primer sequence (5′→3′)	PCR product size (bp)	$MgCl_2$ (mmol/l)
SDK5	*NPAS3* exon 1	F-TTGAAGAGGTCCTTCACATC R-CGGTTATAGCAGCATGATTT	142	1.5
AEX2	*NPAS3* exon 2	F-TGACCAGAGCATTATGGCATT R-AAATTGGTTCAGCAGCAAGG	184	1.5
I2DK	*NPAS3* intron 2	F-CCTGCTGGACATCAGTGCTA R-TCCCTGTAGAGGCAAGCAGT	232	1.5
I3DK	*NPAS3* intron 2	F-GGCAACAAGAGCAGAACTCC R-GGGCTTCCCTGGTAAAGAAC	208	1.5
I4DK	*NPAS3* intron 2	F-GGATGCAGAAGCAGATAGGC R-GCTCAGTCCACTCCGAACTC	232	1.5
I5DK	*NPAS3* intron 2	F-GTTTGGTAAGCAGGCCACAT R-CCTACCCAGGCCAAACACTA	189	1.5
I6DK	*NPAS3* intron 2	F-GTGAGTGCTCAGGTTGCGTA R-CCATACAGTGAGGCCTGGTT	199	1.5
I7DK	*NPAS3* intron 2	F-AGGCCTCCCTTAACTGTGGT R-CCACCACCCTCTGAAACAGT	225	1.5
I8DK	*NPAS3* intron 2	F-AGGTTTGAGAGGGGTGAGGT R-CATGTCCAAGGGGGATTATG	211	1.5
66M11SL	*NPAS3* intron 3	F-AATGTGATGCCCTGATGGAT R-TGGGTTTTGATTGACAGCAA	156	1.5
66M11SR	*NPAS3* intron 3	F-TGCAGTCAACAGGTGCTTTC R-CTTTTCCTCCCACTGCTGAG	150	1.5
SDK1	*NPAS3* intron 3	F-TTTCCTGCAGTCCCTGGAT R-GCTTCATAATCGCTGCCAAG	100	1.5
SDK2	*NPAS3* intron 3	F-CTGACAGGCAGCAGTGTCTT R-TCTCCGACTGAGAGGAGGAA	137	1.5
1075M22SL	*NPAS3* intron 5	F-ACAAGCCCTTACCCTTTTGG R-GGGCACTCCTTTTTCCTTTC	144	1.5
1078I14SR	*NPAS3* intron 6	F-AACTGGTAATCACCCCACCA R-GCACTGAGCAAAGCTTGACA	152	1.5
SDK6	*KIAA0391* exon 3	F-GGGCTGTGGAAAAACCATAG R-CTGGTCACCTCCATCTATCACA	100	1.5
K4DK	*KIAA0391* intron 3	F-TCCCCTCTCAAAAGGTGAT R-CATTCTCAGGATGGCTGGT	194	1.5
K3DK	*KIAA0391* intron 3	F-AGCACTGTTGGGGACATAG R-TTCTGGGTTAGGGGAGGAG	207	1.5
K2DK	*KIAA0391* intron 3	F-TCCAAGAGCCAACAAAATC R-TGCTGGCCTTAAAAGCCTT	234	1.5
K1DK	*KIAA0391* intron 3	F-GGGCCTTGAGTGAGACTCTG R-CGTTTCCAAAAGCAGAGGAG	128	1.5
SDK7	*KIAA0391* exon 4	F-AACTTAAGAGATTTGAGAACTTCA R-TTCACGAACTTTAGGAAACA	98	1.5

The PCR cycle is 94°C-four minutes, 30 cycles of 94°C-30 seconds, 57°C-30 seconds; followed by 72°C-five minutes final extension.

father, who had died, was diagnosed with DSM-IV bipolar I disorder, with an onset in his twenties. The disorder followed a classical pattern with recurrent episodes of mania alternating with major depressive disorder that required hospitalisation. Cranial imaging on these subjects could not be obtained. Both proband and mother had a 46,XX, t(9;14)(q34;q13) karyotype. The father had a normal karyotype. The cytogenetic findings on this family have been previously reported.[8] An older sib of the proband, with severe mental delay, was known to carry the translocation. However, this sib was separated from the family early in life, and no cell line or information on mental status is available.

Mapping on flow sorted chromosomes

Transformed lymphoblast cell lines were flow sorted for the aberrant chromosomes as previously described.[9] A total of 300-500 copies of the normal or aberrant sorted chromosome 14 were subjected to DOP-PCR amplification before use as a template for mapping, as previously described.[9] A panel of 16 proximal chromosome 14q and 16 chromosome 9q34 specific markers, selected from the Whitehead Institute for Genome Research STS YAC physical map (www-genome.wi.mit.edu), were used for mapping in standard 20 µl PCR reactions that included 40 ng human genomic DNA or flow sorted aberrant chromosomes. The marker D14S49 mapped within the second intron of the *NPAS3* gene. The marker D14S1014 mapped within the third intron of the *KIAA0391* gene. The PCR primers, conditions, and cycles for these markers are reported by Research Genetics. Primer pairs, listed in table 1, were designed from the sequences of BAC clones R1075M22 (Genbank accession number AL157689), R1078I14 (Genbank accession number AL161851), and R66M11 (Genbank accession number AL133305), using primer premiere 3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), to map precisely the chromosome 14q breakpoint junction within *NPAS3*. The genomic sequence of BAC clone R173D09 (Genbank accession number AL121594) was used to design primer pairs for mapping within the *KIAA0391* gene (Genbank accession number NM_014672).

Rapid amplification of cDNA ends (RACE)

5′ and 3′ RACE were performed on a Marathon ready human fetal brain cDNA library (21-30 weeks' gestation, 10 pooled white male and female) (ClonTech) as specified by the manufacturer. Primers for 5′ (F-5′ tgccttgtcgagctggctggtaa 3′, F-5′ gctggctggtaatggctgcagag 3′) and 3′ (F-5′ acggagccagctcagcatcttcc 3′) RACE reactions were designed from partial cDNA sequences belonging to *NPAS3*, with melting temperatures between 65°C and 72°C using Primer Premiere 3. Standard 50 µl PCR reactions were performed with the Advantage *Taq* polymerase mix (ClonTech) as specified by the manufacturer. The RACE products were cloned with the pCR4-TOPO TA kit

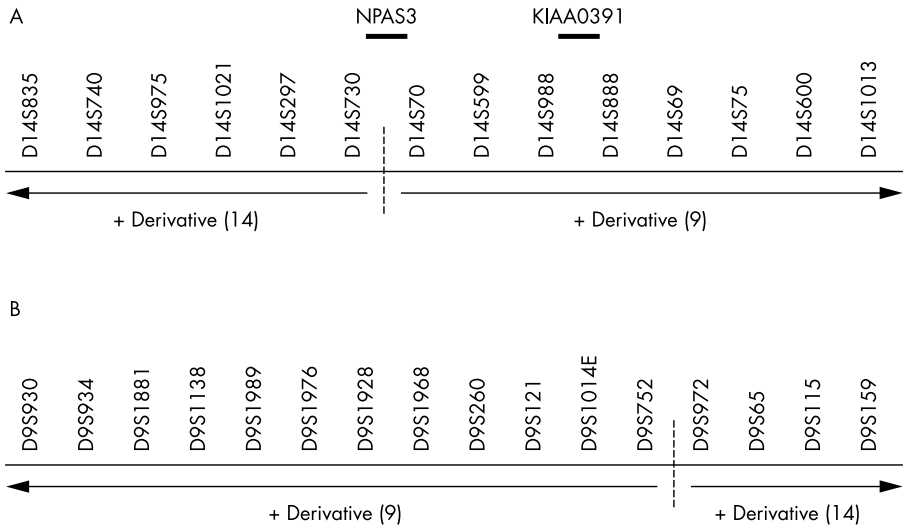

Figure 1 Breakpoint junction analysis of chromosomes 9 and 14 of the proband and mother using analysis of flow sorted chromosomes. (A) chromosome 14q13 analysis. The position of the *NPAS3* gene is shown between D14S730 and D14S70. The position of the *KIAA0391* gene is shown between D14S988 and D14S888. (B) Chromosome 9q34 analysis. Marker map distances are not drawn to scale. The plus sign indicates the presence of markers on either the derivative 9 or 14 chromosome. The dashed line indicates the translocation breakpoint junction.

(Invitrogen) and sequenced with the IRD700/800 M13 labelled primers using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences). The reactions were run on a Licor DNA sequencer Long Reader 4200.

cDNA library screening

The λ TriplEx Human Fetal Brain 5′ Stretch Plus cDNA library (20 to 25 weeks' gestation, 10 pooled white male and female) (ClonTech) was screened as outlined by the manufacturer. A PCR probe designed with Primer Premiere 3 using partial cDNA sequences obtained from database searches and RACE was used to screen the cDNA library. The primer pair (F-5′ tcttggggagcagaaggtaa 3′, R-5′ agattctgccctcagcaatg 3′) was used in standard 20 µl PCR reactions containing 1.5 mmol/l $MgCl_2$, and with the PCR cycle denaturation for three minutes, followed by 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for five minutes. One hundred ng of the PCR product was labelled with ^{32}P-α-dCTP (10 Ci/ml) using the REDIPrime kit (Amersham Biosciences). About 1.9×10^6 plaque forming units were screened in total. Hybridisation was done with the ExpressHyb solution (ClonTech) as specified by the manufacturer. Up to 35 positive plaque forming units were picked from each plate and converted to plasmids as specified by the manufacturer. The plasmids were sequenced with the IRD700/800 M13 labelled primers using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences). The reactions were run on a Licor DNA Sequencer Long reader 4200.

Genomic structure determination

Consensus sequences were assembled from partial cDNA sequences obtained from RACE, cDNA library screening, and database searches, using the GeneTool Version 1.0 software. A composite genomic sequence of the interval between the markers D14S70 and D14S730 was made by aligning the scaffolds of genomic sequence data from Celera (Celera.com) and Genbank (www.ncbi.nlm.nih.gov), using BLAST2 (www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). The genomic structure was determined by aligning the assembled cDNA sequences against the composite genomic sequence with BLAST2.

Northern blot analysis

A 2.6 kb cDNA sequence encompassing exons 7 to 12 and a 1.6 kb cDNA of exon 2 of *NPAS3* were used as probes. A full length cDNA of the *GAPDH* gene was used as a control probe. Probes were labelled with 5 µl ^{32}P-α-dCTP (10 mCi/ml) using the REDIPrime labelling kit (Amersham Biosciences). The human multitissue northern blots, 12 multitissue, Blot IV and Blot II (ClonTech), were hybridised with the ExpressHyb solution (ClonTech) as specified by the manufacturer.

Protein cellular localisation studies

The open reading frame of the large NPAS3 protein isoform was cloned by standard PCR using the λ TriplEx Human Fetal Brain 5′ Stretch Plus cDNA library (pooled 10 male/female, 20 to 25 weeks' gestation) (ClonTech) and Marathon ready human fetal brain cDNA library (21-30 weeks' gestation, 10 pooled white male and female) (ClonTech) as templates. Standard PCR reactions were set up with Platinum Pfx *Taq* polymerase (Invitrogen) as specified by the manufacturer. The primer pairs (F-5′ aggcatccatcattcgactt 3′, R- 5′ cgtggtgtagaccctctgc 3′) were used with the PCR cycle denaturation for 94°C for three minutes, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 150 seconds, followed by 72°C for five minutes. The cloned open reading frame was sequenced with the IRD700/800 M13 labelled primers using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences), then subjected to site directed mutagenesis to introduce restriction sites for cloning into the EGFP-N1 vector (ClonTech). Standard PCR reactions containing about 100 ng of plasmid DNA and Platinum Pfx *Taq* polymerase were set up according to the manufacturer. The PCR cycle was denaturation at 94°C for three minutes, 30 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 90 seconds, followed by 72°C for five minutes. The primer pairs (F-5′ **agatct**atgaaccacattttgcagtccctggatg 3′, R-5′ **ggatcc**gaggaccgagtcgggaatggc 3′) introduced *Bgl*II and *Bam*HI sites to clone into the EGFP-N1 vector (ClonTech). Plasmids were sequenced with the IRDye 800 terminator sequencing kit (Amersham Biosciences) using the EGFP-N1 forward and reverse primers, in addition to internal primers within the cDNAs. The sequencing cycle was 94°C for five minutes denaturation, 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 60 seconds, for 45 cycles. The reactions were run on a Licor DNA sequencer Long reader 4200.

Table 2 Genomic structure of *NPAS3*

Exon number	Exon position	Exon size	Intron size	3′ acceptor site	5′ donor site
1	1–114	114 (64 bp 5′ UTR)	274137		AGAACG**gt**aacact
2	115–1864	1749 (1670 bp 5′ UTR)	151798	(gatttc**ag**TTTACA)	TAAAAG**gt**aagttt
3	1865–1942	77	192855	agttat**ag**GTGCAC	TTGCAG**gt**accttt
4	1943–2033	90	116393	tcctgc**ag**TCCCTG	TCACAA**gt**aagtaa
5	2034–2209	175	4221	ccctct**ag**GTGGAG	AGCCAG**gt**gggaat
6	2210–2649	439 (401 bp 3′ UTR)	54171	gtctcc**ag**TGGTTT	
7	2650–2769	119	39014	ttcctc**ag**TGGAGT	TATAAG**gt**aagccg
8	2770–2964	194	3926	ttttac**ag**GTGATT	AAATAG**gt**actttg
9	2965–3072	107	15337	ctttgt**ag**GATTAG	TGGACT**gt**aagtac
10	3073–3221	148	3414	ttcgcc**ag**TGCTGA	TCTTAG**gt**atattt
11	3222–3347	125	2153	ttccac**ag**CAATCC	TTACAG**gt**attatt
12	3348–5347	1999 (625 bp 3′ UTR)		ccacac**ag**AGGACA	

Brackets demarcate a cryptic 3′ acceptor splice site at nucleotide position 1502 of exon 2.

About 1×10^4-1×10^5 cells/ml of COS1 and transformed adult human skin fibroblast cell lines were grown on 22 × 22 mm coverslips at 37°C in DMEM/10% fetal bovine serum (Invitrogen) one to two days before transfection with Fugene 6 reagent (Invitrogen) as specified by the manufacturer; 3 µl of Fugene 6 reagent was used with 1 µg of EGFP plasmid construct. Transient transfection was allowed to proceed for 24 to 48 hours at 37°C. The coverslips with transfected cells were washed in PBS (room temperature) before adding 15 µl of Mounting medium containing DAPI (Vector Laboratories Incorporation). The coverslips were placed on Fisherbrand Superfrost/Plus slides (Fisher Scientific) and examined with DAPI and FITC filters of an Olympus BX50 compound microscope (with URA fluorescence). Images were captured with ImageGear 6.6.4 and the software Spot version 2.2.

RESULTS

Mapping of the translocation breakpoint junctions on chromosomes 9q34 and 14q13

In order to characterise the translocation breakpoint junctions in this family, a panel of 14 proximal chromosome 14q and 16 chromosome 9q34 markers were selected from the Whitehead Institute for Genome Research STS YAC physical maps to define the breakpoint junctions on the flow sorted aberrant chromosomes (fig 1). Both proband and mother had a breakpoint junction defined on chromosome 14 within an estimated 683 kb interval between the markers D14S730 and D14S70. On chromosome 9q34, the translocation breakpoint junction was defined between the markers D9S752 and D9S972 in both subjects, an estimated 100 kb interval (fig 1). No deletions or complex rearrangements of chromosome 9 were detected, and no genes were disrupted within this interval. The genes mapping closest, within 1 Mb of the breakpoint junction at 9q34, were three genes of unknown function (*KIAA0169* (XM_052725, *KIAA1848* (AB058751), *LOC204994* (XM_114811)), five hypothetical genes (*LOC255259* (XM_173231), *LOC169627* (XM_095823), *LOC206943* (XM_121923), *LOC169656* (XM_095844), *LOC138519* (XM_070946)), and four known genes (*LSFR2* (XM_026945), *CRAT* (NM_000755), *PPP2R4* (NM_021131), *AD-003* (NM_014064) (Ensembl (www.ensembl.org), NCBI map viewer(www.ncbi.nih.gov/cgi-bin/Entrez/hum_srch)).

Isolation and characterisation of the *NPAS3* gene

In order to identify genes within the 683 kb translocation breakpoint interval on chromosome 14q13, the genomic sequence was annotated for EST clusters. These partial cDNA sequences were used to design probes for the screening of a λTriplEX 5′ stretch human fetal brain cDNA library, and perform 5′ and 3′ RACE on a Marathon Ready human fetal brain cDNA library. In this interval, two cDNAs of sizes 2.5 and 3.4 kb (GenBank accession numbers AY157302, AY157303) were isolated. These cDNAs were highly similar to the mouse neuronal PAS3 gene (*Npas3*) (Genbank accession number AF173871) on mouse chromosome 12, in a region of conserved synteny with genes on human chromosome 14. We were unable to extend beyond the 5′ of these two alternative cDNAs using RACE on a Marathon Ready human fetal brain cDNA library; however, the possibility that these are partial cDNAs cannot be excluded. The human neuronal PAS3 gene (*NPAS3*) is estimated to be about 863 kb in size with 12 exons that encode two alternative transcripts (table 2). Two pseudogenes (Genbank accession numbers AK002000, AK000865), of unknown identity and of sizes 2.2 kb and 2 kb, map within the second and fifth introns of this gene, respectively. These pseudogenes have short open reading frames and contain a 3′ poly A tail. A 901 amino acid protein encoded by the 3.4 kb cDNA has a bHLH (basic helix loop helix) dimerisation domain in the amino terminus (amino acids 31 to 72), a PAS (**P**eriod, **A**ryl hydrocarbon receptor, **S**ingle minded) domain (amino acids 117 to 183), a PAC (PAS associated carboxyl terminus) motif (amino acids 361 to 404), and a bipartite nuclear localisation signal in the carboxyl terminus (amino acids 568 to 585). This protein is about 90% identical to the mouse NPAS3 protein. The smaller 2.5 kb cDNA encodes a truncated 153 amino acid protein that contains a PAS domain (amino acids 45 to 113). Northern analysis with a 2.6 kb probe containing exons 7 to 12 showed expression of an approximately 7.5 kb transcript only in several human adult brain tissues, including cerebellum, cerebral cortex, medulla, occipital lobe, frontal lobe, temporal lobe, putamen, amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra, and thalamus (fig 2). An exon 2 probe specific to the smaller 2.5 kb alternative cDNA showed no expression in human adult tissues. However, both 2.5 and 3.4 kb cDNAs were found to be expressed in human fetal brain (20-30 weeks' gestation). Cellular localisation studies with the 901 amino acid NPAS3 protein isoform, tagged in the carboxyl terminus with enhanced green fluorescent protein, showed that this protein was localised in the nucleus of COS1 and transformed adult human skin fibroblast cell lines (fig 3).

High resolution mapping within the *NPAS3* gene

Mapping was performed with an additional 16 amplimers within the *NPAS3* gene, in order to define more precisely the breakpoint junction on chromosome 14q13. Physical mapping with these markers showed that the translocation breakpoint junction of both proband and mother was between *66M11SR* and *SDK1*, an estimated 7.9 kb interval within the third intron (fig 4). The translocation breakpoint junction affected both alternative transcripts, which resulted in the first 124 amino acids of the amino terminus of the larger 901 amino acid isoform, and the first 52 amino acids of the amino terminus of the putative smaller 153 amino acid isoform being displaced. The bHLH domain in the amino terminus of the larger protein was disrupted, preventing the protein from binding to DNA.

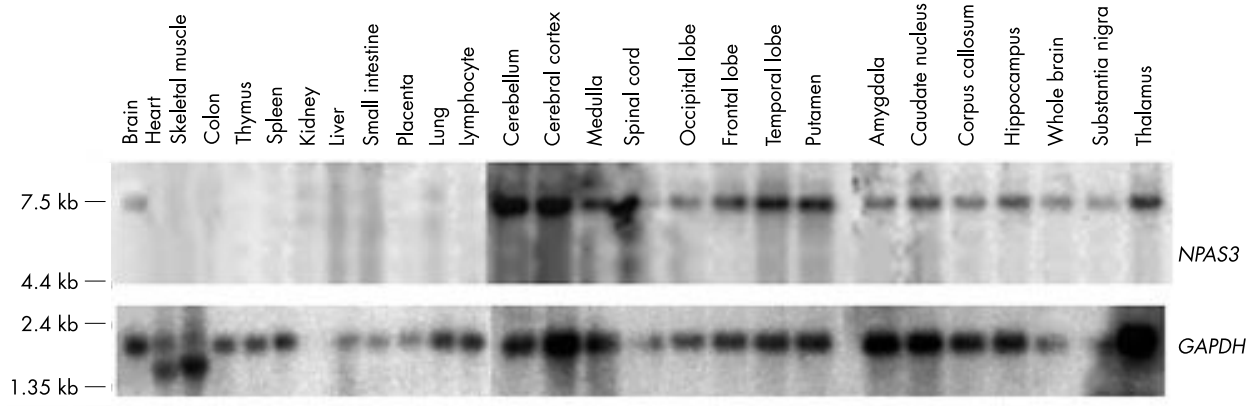

Figure 2 Northern analysis of the *NPAS3* gene showing a single approximately 7.5 kb transcript, ubiquitously expressed in the adult human brain. A 2.6 kb cDNA encompassing exons 7 to 12 of the large transcript isoform was used as a probe to hybridise on ClonTech human adult multitissue northern blots (12 adult multitissue; adult brain blot II and adult brain blot IV). A full length cDNA of the *GAPDH* gene was used as a control probe.

For both protein isoforms, the PAS domains that are required for dimerisation were disrupted. The PAC motif and bipartite nuclear localisation signal within the carboxyl terminus of the larger protein isoform remained intact. Interestingly, the proband was found to have deletions of three markers (I3DK, D14S49, I4DK) that mapped within the second intron of *NPAS3*, suggesting an estimated 94 kb maximum microdeletion within this intron (fig 4). An analysis of this deleted genomic sequence showed several possible transcription factor binding sites. None of these additional microrearrangements was found in the mother.

The chromosome 14q13 region outside *NPAS3* was also screened for additional rearrangements in the mother and proband. In the proband, a microdeletion with an estimated maximum size of 22 kb within the third intron of the *KIAA0391* gene (Genbank accession number NM_014672) was found. Specifically, markers D14S1014 and K2DK were deleted. This rearrangement was absent in the mother. *KIAA0391* has an unknown function, unknown identity, and maps about slightly over 1 Mb distal to *NPAS3*. Furthermore, the deleted interval contains several possible transcription factor binding sites.

DISCUSSION

The proband and mother have a translocation breakpoint junction in the third intron of the *NPAS3* gene which disrupts the coding potential of both alternative transcripts. Studies with FISH have confirmed a break within this gene.[10] The *NPAS3* gene belongs to the bHLH-PAS (**b**asic **H**elix **L**oop **H**elix, **P**eriod, **A**ryl hydrocarbon receptor, **S**ingle minded) superfamily of transcription factors, that are involved in a wide range of functions including circadian ossicilations (*Npas2, Per, Clock*), neurogenesis (*Sim*), toxin metabolism (*Arnt*), hypoxia (*Hifia*), and tracheal development (*Trh*).[11–14] A small protein isoform that may be encoded by the small transcript isoform of *NPAS3* was identified. This small protein isoform may form heterodimers via its PAS domain with the larger NPAS3 protein isoform or other proteins containing a PAS domain, to regulate activity. This mode of regulation is seen with the PITX2D protein isoform[15] and other bHLH-PAS transcription

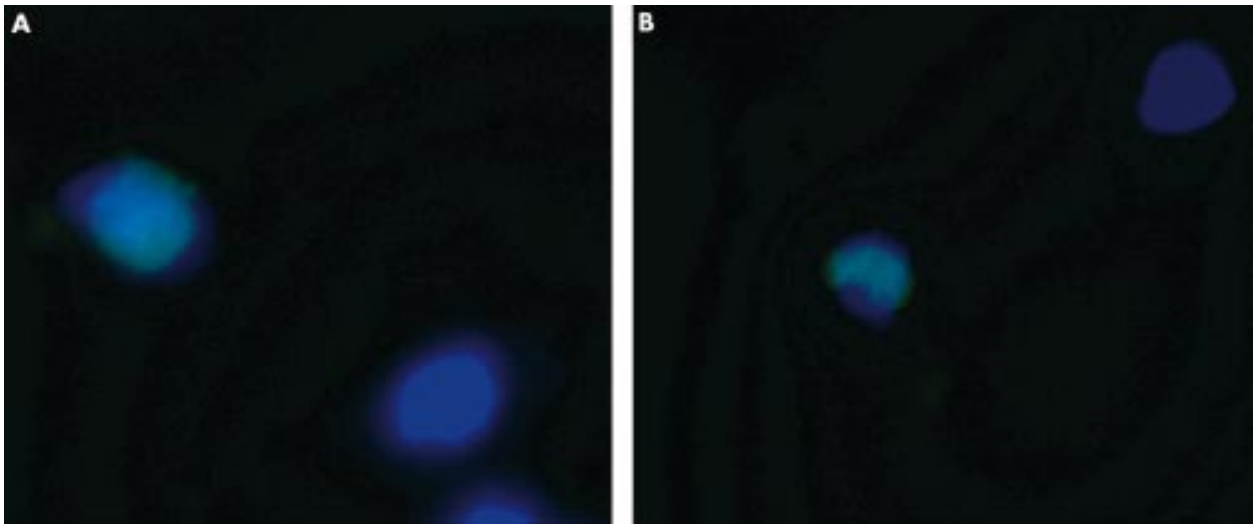

Figure 3 Cellular localisation of the large NPAS3 protein isoform. Transient transfections of the NPAS3-EGFP-N1 construct were done in (A) COS1 and (B) transformed adult human skin fibroblast cell lines. DAPI and FITC filters were used to visualise the signals. Images obtained from each filter were merged with the software Spot version 2.2. Blue signals represent the DAPI stained nucleus; green signals represent the EGFP tagged protein.

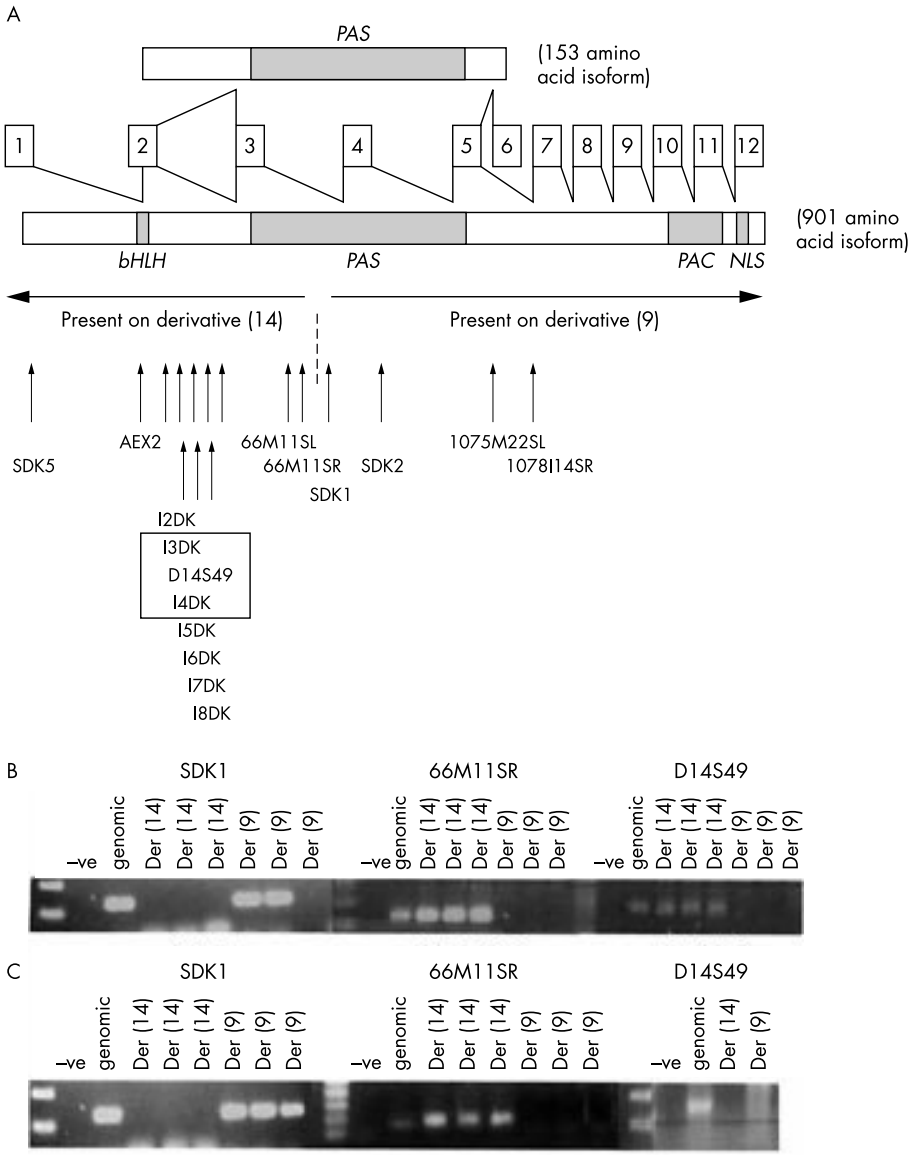

Figure 4 Identification of rearrangement within the *NPAS3* gene. (A) Schematic of the *NPAS3* gene showing positions of markers used in mapping. The stippled line indicates the translocation breakpoint junction. The functional domains or motifs of the NPAS3 protein isoforms are bHLH (basic helix loop helix), PAS (period, aryl hydrocarbon receptor, single minded), PAC (PAS associated carboxy terminus), NLS (bipartite nuclear localisation signal). Markers placed in a box are those found deleted in the proband. (B) Flow sorted chromosome analysis of mother with selected markers within the *NPAS3* gene. (C) Flow sorted chromosome analysis of proband with selected markers within the *NPAS3* gene. Mapping was performed on three separate flow sorted der(14) or der(9) chromosomes or a pool of three der(14) or der(9) chromosomes.

factors.[11–14] The NPAS3 protein contains a bHLH domain that is known to bind to DNA, and in this study is found to be localised in the nucleus, suggesting that it may be a transcription factor. Expression of both transcript isoforms was also found in the developing human fetal brain (20 to 30 weeks' gestation). The murine *Npas3* gene is extensively expressed in the developing central nervous system,[16] but its role is yet to be defined. Expression of the murine *Npas3* gene specifically in the developing nervous system during early embryogenesis suggests that haploinsufficiency could result in central nervous system anomalies causing mental impairment. So far, only the *SIM2* gene of the bHLH-PAS family of transcription factors, mapping on human chromosome 21, has been suggested to be associated with behavioural problems, as seen in Down syndrome patients.[17] No other members of this transcription factor family have yet been associated with schizophrenia, suggesting that *NPAS3* is the first gene of this superfamily to be associated with this disease. The fact that this gene was expressed in 13 adult brain tissues including the hippocampus, thalamus, and cortex supports a possible role in development and/or function of these structures, which may play a role in schizophrenia.[18]

The PAS domain of both protein isoforms, required for dimerisation, is disrupted in both proband and mother. Such a disruption is expected to destroy the function of the small NPAS3 protein isoform. In the case of the large protein isoform, the translocation breakpoint junction leaves the bHLH domain intact in the amino terminus, and the PAC and bipartite nuclear localisation motifs intact in the carboxyl terminus. With such disruption of the *NPAS3* gene, the NPAS3 large protein isoform is likely to be non-functional, compatible with a contribution of haploinsufficiency to schizophrenia in mother and proband. Interestingly, the proband was found to have a translocation breakpoint junction in conjunction with a 94 kb microdeletion within *NPAS3* and a 22 kb microdeletion within *KIAA0391*. This finding is not unexpected, since familial translocation chromosomes acquire additional rearrangements owing to unequal recombination during meiosis. The

possibility of minor rearrangements in other genes cannot be excluded. The function of the *KIAA0391* gene is currently unknown and is not similar to other genes with known functions. These deleted intervals contain potential transcription factor binding sites. These deletions separate from, or in conjunction with, a genetic background inherited from the father, may explain the more severe phenotype observed in the proband.

Most studies by linkage and linkage disequilibrium have reported only weak evidence for a locus for schizophrenia on the proximal region of human chromosome 14q.[6] However, with the use of genome screens using non-parametric linkage analyses, two groups have suggested significant linkage to the markers D14S79 (p=0.01) and D14S306 (p=0.005) at 14q13,[19 20] suggestive of a susceptibility locus at 14q13. Recently, using the identity by descent datasets of 30 affected sibs in 21 pedigrees from the previous study by Blouin *et al*,[20] and a modified multipoint non-parametric linkage algorithm,[21] evidence for the sharing of two alleles at 8p21 in conjunction with one allele on chromosome 14 between D14S1280 and D14S306 was found among affected sib pairs with the schizophrenia spectrum.[7] These data suggested that a susceptibility locus at 14q13 acted in conjunction with other schizophrenia loci, such as at 8p21, in the patients examined. The *NPAS3* gene resides within this interval, and therefore may be a susceptibility gene with a contributory role in the cause of schizophrenia. Proximal chromosome 14q deletion cases reported to date do not describe any symptoms of mental illness.[9 22–25] This may be because these reported patients died neonatally or in early childhood or were too young to manifest such traits. Furthermore, the deletion intervals may not extend into the *NPAS3* gene.

Of the diseases localised to date to chromosome 14q13, only Fahr disease (idiopathic basal ganglia calcification) has been associated with neuropsychiatric problems.[26] The age of onset for idiopathic basal ganglia calcification is 30 to 60 years and manifests in patients with dysarthria, extrapyramidal signs, and ataxia. The central nervous system undergoes calcification in areas such as the globus pallidus, putamen, caudate nucleus, dentate, thalamus, cerebral white matter, and cerebellum, which is postulated to result in progressive dystonia, parkinsonism, and neuropsychiatric manifestations such as schizophrenia or schizophreniform psychosis. Of the patients who showed significant linkage of disease to chromosome 14q13, only one had schizophreniform psychosis and the Fahr disease spectrum.[26] Our subjects have no features in common with the Fahr disease patients showing linkage to chromosome 14q13, except for the schizophrenia spectrum. We were unable to discern whether calcification of the central nervous system was prevalent in our subjects.

On chromosome 9q34, the breakpoint junction was defined within 100 kb. No genes were disrupted within this interval. Although chromosome 9q is speculated to contain susceptibility gene(s) for schizophrenia,[5] none of the reported genes within the breakpoint junction interval at 9q34 in our subjects appeared to be likely candidates for schizophrenia. The possibility of position effects exerted on the expression of neighbouring genes at the breakpoint junction cannot be excluded. There was evidence for a weak association of the *NMDAR1* gene (NM_000832) at chromosome 9q34 with schizophrenia in a South African Bantu speaking tribe.[27] The NMDAR1 receptor functions in the glutaminergic pathway and was shown, in mice carrying a hypomorphic allele, to result in schizophrenia-like behaviour that could be treated with haloperidol.[28] However, examination of sequences within a 1 Mb interval of the 9q34 breakpoint junction showed that the *NMDAR1* gene was not positioned within this interval. There is one case report of a 28 year old man with mental retardation, schizophrenia, short stature, short webbed neck, dysmorphic face, and mild anomalies of the fingers, who had a del(9)(q32q34.1).[29] The deletion interval in this patient is not yet characterised and the locus for schizophrenia in this patient is as yet unidentified.

In summary, we have found the *NPAS3* gene to be disrupted in a two generation family with the schizophrenia spectrum. The *NPAS3* gene is proposed to be a susceptibility gene with a contribution to the mental illness observed in this family. Other possibilities cannot be excluded, such as mental illness as a non-specific consequence of mental delay, the translocation breakpoint junction being the result of chance, or the susceptibility locus close to but not at the breakpoint junction. In order to confirm the role of *NPAS3* as a schizophrenia susceptibility gene, association studies in larger case-control samples and mutation analysis are warranted.

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