

Analysis of neuroblastoma tumour progression; loss of *PHOX2B* on 4p13 and 17q gain are early events in neuroblastoma tumourigenesis

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Received August 20, 2007; Accepted October 4, 2007

Abstract. Neuroblastomas are biologically and clinically heterogeneous tumours that most often occur sporadically in children at median age of 2 years. The *PHOX2B* gene is implicated in the development of the autonomic nervous system and has been found to be infrequently mutated in sporadic neuroblastoma tumours and in some patients with hereditary neuroblastoma. We have screened a selected series of 36 paediatric tumours with presumed genetic predisposition, 34 of them neuroblastomas, for mutations in *PHOX2B*. A constitutional heterozygous missense mutation was found in a boy who developed bilateral adrenal tumours and stage 4 disease during infancy. The second allele of the *PHOX2B* locus was lost in the tumour DNA. Histopathological evaluation of the tumours suggested growth of two primary tumours, one with diploid DNA content and the other with tetraploid DNA content, i.e. a case of neuroblastoma stage 4_M (multifocal tumour). However, array CGH (comparative genomic hybridization) data performed on both tumour masses from the patient instead supported a model where a common malignant precursor gave rise to the diploid tumour and subsequently the tetraploid tumour have progressed from the common precursor or by metastasis from

the diploid tumour with additional genetic changes. The whole genome dosage analysis showed that the remaining alleles of *PHOX2B* had been lost in both tumours together with a specific 17q gain pattern. The tetraploid tumour had these features together with additional whole chromosomal loss of chromosomes 3, 9, 14 and 15. Based on the data presented here we suggest that loss of *PHOX2B* and 17q gain are early events in neuroblastoma tumourigenesis. We also propose investigators to re-analyze the rare cases of multifocal neuroblastomas with the array CGH technique for better understanding of the origin of these tumours.

Introduction

Neuroblastoma is the most common extracranial solid tumour of childhood. It is characterized by both biologically and clinically heterogeneous tumours, ranging from spontaneously regressing growths to aggressively malignant, untreatable disease. The tumours originate from embryonal neural crest cells committed to the development of the sympathetic nervous system. Prognostic factors include patient age at diagnosis, INSS (International Neuroblastoma Staging System) stage, tumour histopathology and ploidy status of DNA content (1). Genetic markers of the disease include amplification of the *MYCN* oncogene (2), loss of a distal part of the short arm of chromosome 1 (3-6) and 17q gain (7-10), all of which are associated with a severe clinical course. Allelic loss of 11q is a common feature of aggressive neuroblastomas without 1p deletion and *MYCN* amplification (11,12). Other chromosomal regions showing potentially biologically relevant hemizygous deletions indicative of tumour suppressor gene inactivation include 3, 4, 9p, 14 and 19q (1).

A subset of neuroblastomas may be hereditary and multiple primaries are considered to be a hallmark of familial neuroblastoma (13). Linkage of hereditary neuroblastoma predisposing genes to chromosomes 16p12-13 (14) and 4p16 (15) have been found but no genes have been shown to be inactivated in these regions. Neuroblastoma may also be linked to familial predisposing conditions such as

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Abbreviations: *PHOX2B*, paired-like homeobox 2B gene; CGH, comparative genomic hybridization; INSS, international neuroblastoma staging system; ANS, autonomic nervous system; HSCR, hirschsprung's disease; CCHS, congenital central hypoventilation syndrome; LOH, loss of heterozygosity; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism

Key words: *PHOX2B*, neuroblastoma, tumour progression, multifocal tumour, neural crest cell

Table I. Primer sequences used for PCR amplification of *PHOX2B* exon regions.

Amplicon	Forward	Reverse	Length (bp)	Annealing T
Exon 1A ^a	TTTCATTATTTCCAAGTAGTGTG	CAGCCAAGTGGAAAAATGAAAT	430	58
Exon 1B ^a	TGCACATCTCCCCACTCCA	CCCTCCCTGCAATCGCTTCTAACG	488	58
Exon 2 ^a	ACCTTGGAGTCCTCACATTCT	GGGGCCCTAGGTCCTTCTCACTCG	391	58
Exon 3A ^a	CTCCGAAAATGCACAGCAATAAGA	CGCCGCCGCCGCTCCATTC	409	58
Exon 3B ^b	GCAAAGAGGCCAAGAGCACTGACC	GCGACGCCGTTTTCCCTTTATTCT	563	65

Primers were designed on the basis of the *PHOX2B* reference sequence NM_003924. Exon 1 and exon 3 were amplified by two overlapping primer pairs, A and B. ^aAmplified by use of AmpliTaqGold; ^bAmplified by use of the FailSafe system; bp, base pairs; T, temperature.

neurofibromatosis type 1 (16,17) and tumour occurrence is associated with other genetically determined congenital malformations of the autonomic nervous system (ANS), such as Hirschsprung's disease (HSCR; OMIM 142623) and congenital central hypoventilation syndrome (CCHS or Ondine's curse; OMIM 209880). CCHS is a rare but life-threatening disorder characterized by impaired control of autonomic respiration resulting in hypoventilation. HSCR, which occur in a subset of patients with CCHS, is characterized by absence of enteric ganglia along a variable length of the intestine caused by a defect in migration or differentiation of neural crest cells.

The paired-like homeobox 2B gene (*PHOX2B*) on chromosome 4p13, which is essential in the differentiation of the ANS (18-20), is the major gene in CCHS pathogenesis with an autosomal dominant inheritance pattern (20-22). Mutations in *PHOX2B* have been found not only in isolated CCHS patients but also in individuals with a more complex neural crest involvement including CCHS and HSCR as well as neuroblastoma (12,20-22). A low frequency of heterozygote germline alterations in *PHOX2B* have also been identified in non-syndromic sporadic neuroblastoma patients (23,24) and in some patients with familial neuroblastoma (12,22,25).

Considering the heterogenetic nature of neuroblastoma tumours, we investigated the role of *PHOX2B* mutations in a selected panel of 36 paediatric tumours with presumed genetic predisposition (associated congenital anomalies and/or early onset aggressive behavior and/or multifocal primary tumours and/or stage 4S tumours). In addition, we performed a comprehensive genetic analysis of the bilateral adrenal tumours from a patient with a *PHOX2B* constitutional missense mutation. Our data from this case suggest a model where progressive growth and additional genetic changes occurred from the first tumour after activation of the second *PHOX2B* allele through deletion of a part of chromosome 4p together with 17q gain. The constitutional *PHOX2B* missense mutation could be a predisposing factor for tumour development by preventing normal neural cell progenitor differentiation.

Materials and methods

Subjects. We have analyzed 36 patients with paediatric tumours. One of them was a Wilms' tumour and one was a

ganglioneuroma. The 34 neuroblastoma tumours were staged according to the INSS criteria (26,27); 1 was stage 1, 4 were stage 2, 8 were stage 3, 9 were stage 4, 10 were stage 4S, 1 was stage 4_M, and 1 was of unknown stage. One of the stage 4 neuroblastoma patients was also diagnosed with HSCR. Constitutional DNA was also extracted from the blood of the patient with a missense mutation in his bilateral tumours (patient 25R3) as well as from his parents and four siblings. We also screened DNA extracted from the blood of 108 anonymous healthy control individuals. DNA from blood and tumours was extracted in accordance with standard protocols. Informed consent was obtained from the patients' parents and the study was approved by a relevant ethics committee.

Mutation analysis. Primers used for amplification of DNA and for sequencing reactions were designed using the DNASTAR sequence analysis software (Lasergene, Madison, WI) on the basis of the *PHOX2B* sequence (NM_003924) and flanking genomic sequences obtained from the UCSC Genome Browser May 2004 assembly (URL: <http://genome.ucsc.edu>; Table I).

PCR amplification of fragments 1A, 1B and 2 were set up in a total volume of 25 μ l containing 100 ng of genomic DNA, 0.6 μ M of primers, 1X buffer from the supplier, 2 mM MgCl₂, 160 μ M of each dNTP and 1 U of AmpliTaqGold polymerase (Applied Biosystems, Foster City, CA, USA). Amplification of fragment 3A was set up using the same protocol but with 1.2 mM MgCl₂. These amplification reactions were run for 35 cycles at 95°C (30 sec), 58°C (30 sec), and 72°C (1 min) with an initial denaturation at 95°C for 10 min and a 7 min prolonged extension after the end of the last cycle. Amplification of the GC-rich 3B fragment was performed using the FailSafe PCR amplification system (Epicentre, Madison, WI) with 0.8 μ M of primers and PreMix J according to protocol, and run for 35 cycles at 95°C (30 sec), 65°C (30 sec), and 72°C (1 min) with an initial denaturation at 95°C for 2 min and a 10-min prolonged final extension.

Mutation analysis of the coding sequences, the exon-intron boundaries and some of the 5' and 3' untranslated flanking sequences was performed by bidirectional sequencing of purified PCR products using an ABI PRISM 3730 DNA Sequencer (Applied Biosystems) as described by Krona *et al* (28). Sequence analysis was conducted with the SeqScape sequence analysis software (Applied Biosystems).

All variations from the reference sequence were confirmed by re-sequencing of a new PCR product of the target. A total of 108 control individuals were screened for the missense mutation identified in exon 1.

Microsatellite analysis. Since the normal exon 1 allele with an A in position 28 was not present in the DNA extracted from patient 25R3's tumours, although it was found in heterozygous form in his constitutional DNA, we studied a number of polymorphic markers on 4p for LOH in the tumours from the patient.

Polymorphic markers on chromosome 4p were chosen from the UCSC genome browser. Amplifications were conducted with a denaturation step at 95°C for 2 min followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and ending with 7 min extension at 72°C. A volume of 10 µl Hi-Di formamide containing 1/60 GeneScan-500 ROX dye standard (Applied Biosystems), was added to 1 µl of diluted PCR product. The samples were denatured at 95°C for 5 min, followed by 10 min on ice. An ABI PRISM 3100 DNA Sequencer (Applied Biosystems) was used for capillary electrophoresis. The fragments were analyzed with GeneMapper 3.0 software (Applied Biosystems).

Fluorescence in situ hybridization. The status of chromosome 4p was further elucidated by fluorescence *in situ* hybridization (FISH) on interphase chromosome slides from one of patient 25R3's tumours. The *PHOX2B* containing BAC clone RP11-227F19 was obtained from the Wellcome Trust Sanger Institute (URL: <http://www.sanger.ac.uk>). The BAC-DNA was extracted and purified using the Qiagen, Plasmid Purification kit (Qiagen, Valencia, CA). The purified BAC-DNA was labeled with Spectrum Green (Vysis, Downers Grove, IL) and a telomeric 4q probe (Vysis) was labeled with Spectrum Red using a Nick Translation Kit (Vysis), according to protocol. A volume of 6 µl of the 4p Spectrum Green labeled BAC-DNA and 1 µl of the 4q probe were hybridized to interphase slides prepared by touch imprints from one of the patient's tumours and 6 µl of the 4p probe was also applied to metaphase chromosome control slides.

The interphase slides were pre-washed in 70% acetic acid for 30 sec, washed twice in distilled water and dehydrated in ethanol series and in acetone. All slides were then denatured in sodium hydroxide solution (1.9% NaOH in ethanol) for 6 min and dehydrated in ethanol series. The probes were denatured for 10 min at 73°C in hybridization buffer (50% formamide, 10% dextran sulphate, 2X SSC (standard saline citrate), final pH 7.0). They were then applied to the slides and hybridization was performed under a coverslip in a moist chamber at 37°C overnight. The next day, slides were washed for 5 min in 2X SSC at 73°C and stained in DAPI before visualization.

Real-time PCR expression analysis. The expression of *PHOX2B* was analysed in the tumours with *PHOX2B* DNA variations and in 11 additional neuroblastoma tumours with wild-type *PHOX2B* sequence.

cDNA from neuroblastoma tumour samples and cell lines was made using SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA), following the protocol for first

strand synthesis with 1 µg of total RNA and 0.5 µl of random hexamers (Promega, Madison, WI). Primers and probes for amplification of *PHOX2B* transcripts were derived from Applied Biosystems TaqMan® Assays-on-Demand™ Gene Expression Products and amplification of *GUSB* was used as an internal control for the integrity and relative amount of mRNA. TaqMan analysis was performed according to Applied Biosystems protocols using duplicate cDNA samples from each tumour to determine mean expression levels of *PHOX2B* normalised to the amount of *GUSB* in each sample.

GeneChip® human mapping 50K assay. The array experiments were performed at AROS Applied Biotechnology AS (ArosAB, Aarhus, Denmark) according to the Affymetrix GeneChip Mapping 50K array standard protocol (Affymetrix, Inc., Santa Clara, CA). Total genomic DNA (250 ng) obtained from neuroblastoma tissue was digested with the *Xba*I restriction enzyme. The Mapping 50K system consists of an 8-µm array designed to hybridize labelled PCR fragments from *Xba*I cleaved DNA. The array containing probes for *Xba*I cleaved DNA detects 58,960 SNPs. The *Xba*I enzyme cleaved DNA was ligated to a uniform linker and subjected to PCR amplification using a single primer. After digestion with DNaseI, the PCR products were labelled with biotin and hybridized to the microarray. The hybridized probes were washed using the Affymetrix Fluidics Station 450 and marked with streptavidin-phycoerythrin. The array was scanned using a confocal laser scanner, GeneChip Scanner 3000 (Affymetrix).

Primary data analysis was performed using the GDAS software (Affymetrix), while further statistical studies were performed using the CNAG2.0 software (Genome Laboratory, Tokyo University, <http://www.genome.umin.jp>) and additional algorithms developed at our laboratory.

Results

Clinical diagnosis of a patient with bilateral neuroblastoma tumours as stage 4_M neuroblastoma. Patient 25R3 presented at age 10 months with an enlarged abdomen in which a large right-sided tumour was detected by ultrasound (Fig. 1). Extensive work-up revealed a large right-sided primary suprarenal tumour crossing the midline and a left-sided tumour (Fig. 1A and B), histopathologically found to be a second primary tumour, also of adrenal origin (Fig. 1C-F). Furthermore, the boy had a disseminated subcutaneous tumour in his right thigh, confirmed by fine-needle biopsy. Hence, the INSS stage was assigned as 4_M (27).

A preoperative fine-needle biopsy of the right primary showed neither 1p loss of heterozygosity (LOH) nor *MYCN*-amplification (29); thus chemotherapy was not administered either pre- or postoperatively, according to the current European neuroblastoma protocol. Surgery at 12 months of age revealed two primary tumours, apparently originating from the right and left glands, respectively, that were radically extirpated (Fig. 1C-F).

At age 38 months a metastatic relapse was detected involving bone and lymphatic glands but not bone marrow. After receiving intensive chemotherapy induction (COJEC),

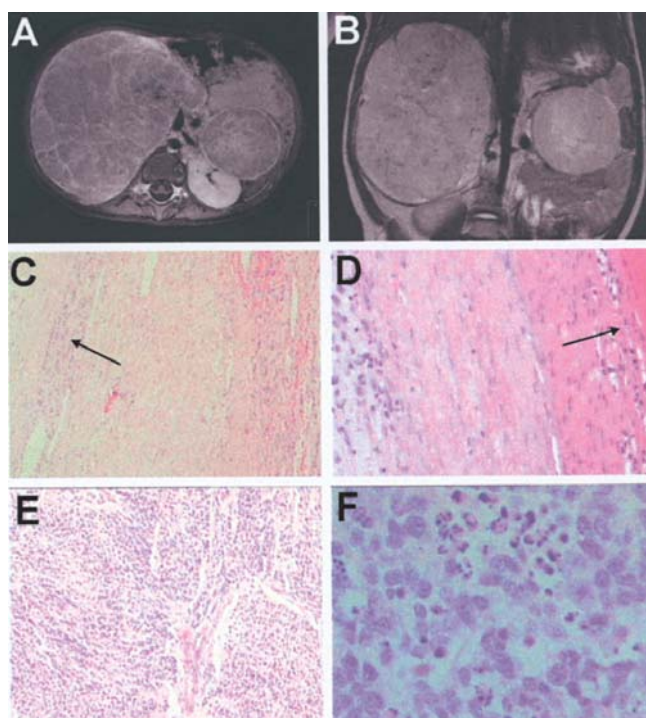


Figure 1. Clinical evaluation of patient 25R3 suggested two primary tumours (stage 4_M neuroblastoma). (A-B) Magnetic resonance tomography of the child at diagnosis revealing bilateral large tumour masses. (C-F) Tumour tissue from the extirpated tumours bilaterally showing neuroblastoma tumour tissue adjacent to normal adrenal glands, respectively. (C-D) Thin strands of light adrenal cortical cells in tumour capsules from right and left-sided neuroblastomas at arrows (H&E, x100). (E) Neuroblastoma, left adrenal, 'classical' appearance with delicate neuroendocrine vascular stroma and evident neuropil (H&E, x250). (F) Tumour area, left side, with nuclear pleomorphism (H&E, x400).

followed by high-dose chemotherapy (busulfan-melphalan) and stem cell rescue, the child was given high-dose retinoic acid in two-week pulses for six months (30). The child is now alive and well at 85 months of age, 32 months post therapy, with no signs of remaining tumour.

***PHOX2B* missense mutation in patient 25R3.** Direct DNA sequencing analysis of the coding sequences, the exon-intron boundaries and some of the 5' and 3' untranslated flanking sequences of the *PHOX2B* gene was performed in 36 patients with paediatric tumours. One of the neuroblastoma patients was also diagnosed with HSCR.

We found one missense mutation in a patient diagnosed with bilateral stage 4_M neuroblastoma. The patient had one tumour in each adrenal gland, and they were both hemizygous for a c.28A>G substitution (N10S) causing an asparagine to be replaced by a serine residue in exon 1 of *PHOX2B*. The constitutional blood of the patient was heterozygous for this mutation as was that of the patient's mother and youngest sibling, both asymptomatic. All other family members had only the normal A allele of the gene (Fig. 2). The G substitution was not present in 216 control chromosomes.

The only additional alterations observed in this study were a 5'UTR-located base substitution, c.-97G>A, in a patient with stage 4S neuroblastoma; a silent substitution, c.761A>C (A254A), in the sequence coding for the carboxy-

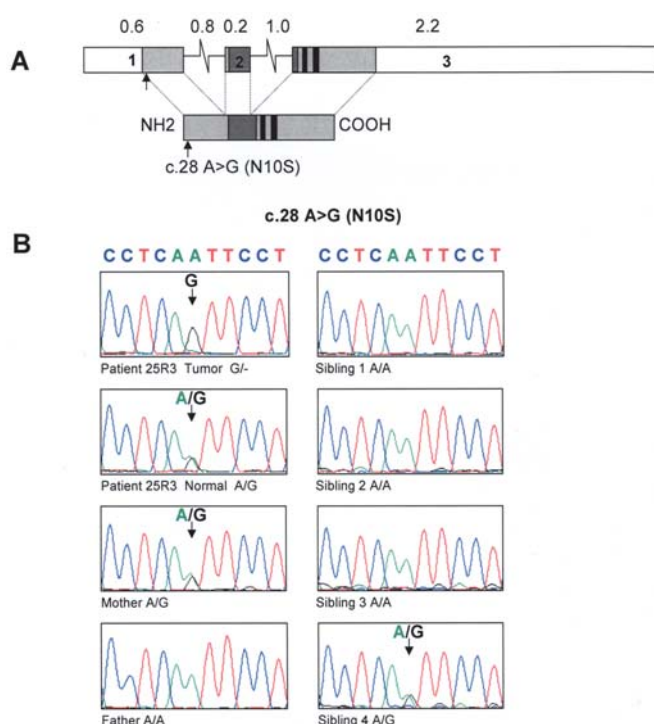


Figure 2. *PHOX2B* mutation in a patient (25R3) with bilateral neuroblastoma. (A) Genomic organization of *PHOX2B* and schematic representation of the protein. Genomic distances are indicated above the figure in kb. The coding sequence, the homeobox domain and the 9- and 20-residue polyaniline repeats are indicated by light grey, grey and black boxes, respectively. The location of the 28A>G mutation is shown (arrow). (B) Electropherograms of the exon 1 sequence, showing the hemizygous missense mutation in one of the tumour samples and the heterozygous sequence in the constitutional DNA from the patient and in DNA from the patient's mother and youngest sibling. The wild-type sequences of the patient's father and the 3 older siblings are also shown. Both of the tumour samples were hemizygous for the missense mutation but only one representative electropherogram is shown in the figure.

terminal 20-residue polyaniline tract in a patient with stage 3 neuroblastoma; and a 3'UTR-located tri-nucleotide repeat subtraction, c.958 TGC[GGC]₆GAC>TGC[GGC]₃GAC, 14 bases after the translation termination in a patient with stage 2 neuroblastoma.

Loss of second *PHOX2B* allele in both tumours. Fragment analysis of eight polymorphic markers on chromosome 4p showed that the normal *PHOX2B* allele had been lost in both tumours from the patient together with the rest of the chromosomal arm (Fig. 3). The marker D4S3357, located 1.5 Mb proximal to the *PHOX2B* locus, was retained, indicating that the breakage occurred close to the gene in both tumours. FISH analysis of one of the patient's tumours using the *PHOX2B*-containing BAC clone RP11-227F19 confirmed that the deletion 4p13-pter encompasses the *PHOX2B* locus (Fig. 4).

The mutation is in a highly conserved region of the *PHOX2B* protein sequence. In order to determine the significance of the missense mutation, N10S, we studied the evolutionary conservation of the protein by comparing the sequences of different organisms found in the public databases. Our

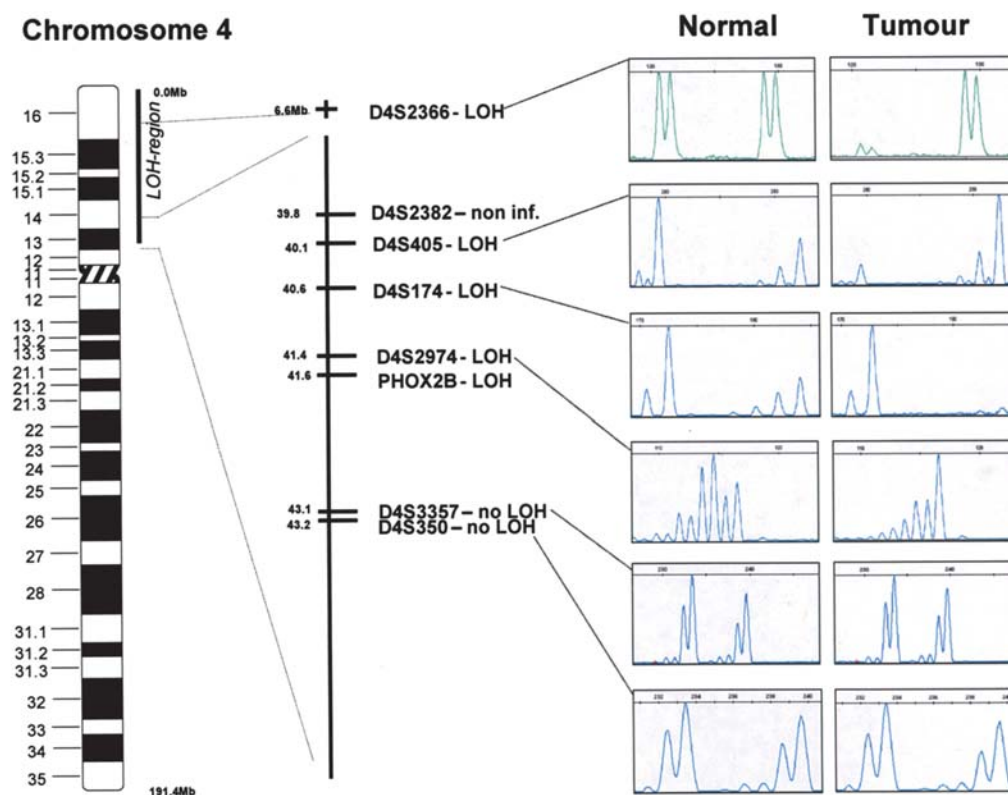


Figure 3. Loss of heterozygosity in the terminal part of chromosome 4 in both tumours of patient 25R3. Fragment analysis of polymorphic sites in DNA from the tumour and normal tissue (blood) of the patient reveals that all informative markers distal to *PHOX2B* (D4S2366, D4S405, D4S174 and D4S2974) have lost one allele in the tumour DNA, whereas the proximal markers D4S3357 and D4S350 remain heterozygous. The megabase (Mb) distance of the markers from the telomeric end of the chromosome is indicated. LOH, loss of heterozygosity; non inf, non-informative. The results were concordant in both of the patient's tumours but only one representative panel is shown for each locus.

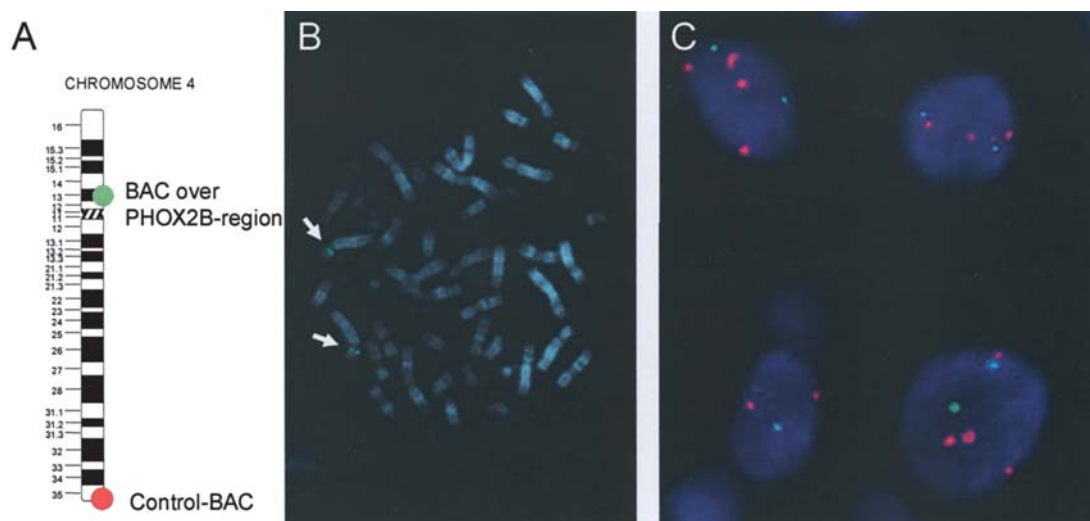
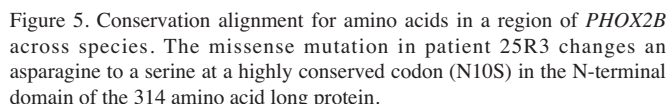


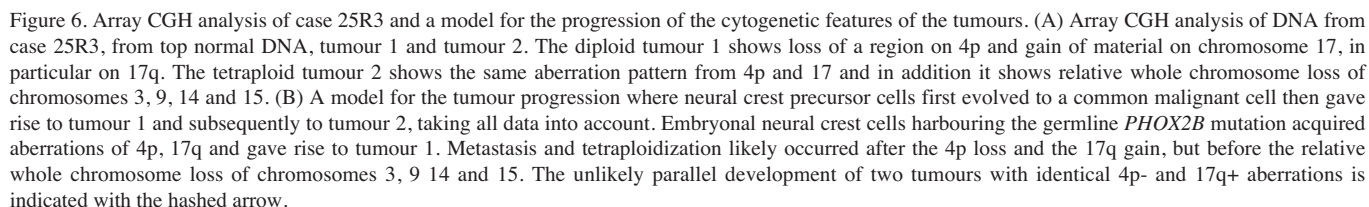
Figure 4. Confirmation of 4p deletion at the *PHOX2B* locus in patient 25R3 by FISH analysis. (A) Representation of chromosome 4 displaying the location of BAC RP11-227F19 and the telomeric 4q control probe. (B) Metaphase chromosomes in a normal cell showing the location of BAC RP11-227F19 on chromosome 4p. (C) Interphase neuroblastoma cells from one of the patient's tumours. There is a mixed population of diploid and tetraploid cells, both showing loss of the 4p signal on one or two chromosomal homologues, respectively.

findings indicated that the amino acid position N10S and the N-terminal part of the protein are very well conserved (Fig. 5).

PHOX2B sequence variations do not reduce mRNA levels. Quantitative expression analysis was performed to determine if the identified *PHOX2B* sequence variations had an effect



Array CGH shows that both tumours have a common origin. Microarray analyses using Affymetrix 50k arrays were performed on normal DNA and DNA from both tumour masses from patient 25R3 and the data were analysed using CNAG software (Fig. 6A). Both tumours showed loss of material on chromosome region 4p. A detailed analysis of



last lost markers and first retained markers indicate that the region of loss is from 4pter (base pair 0) to 4p13 (at 41.8 Mb from pter; data not shown). The *PHOX2B* is located 41.4 Mb from 4pter and it is thus just, but well, within the region of 4p-loss in the tumours. Both tumour 1 and 2 have the same 4p-loss and 4p-breakpoint as far as the array resolution show. The intensity of the loss indicates that the loss involves half of the original material, i.e. from 2 to 1 in a diploid setting and from 4 to 2 in a tetraploid setting. Both tumours also share an identical chromosome 17 gain with more material of the 17q region (Fig. 6A). In addition tumour 2 only has relative whole chromosome loss of chromosomes 3, 9, 14 and 15. The levels of intensity for these chromosomes are compatible with a loss of one chromosome in a tetraploid tumour, i.e. leading to a loss from 4 to 3 chromosomes.

Discussion

It is conceivable that autonomic nervous system disorders such as CCHS and HSCR share a common molecular pathogenesis with tumours of neural crest origin, with defects in one or more genes that control differentiation and/or migration of cells committed to development of the sympathetic nervous system. The *PHOX2B* gene is involved early in the embryologic origin of the ANS, both through activation of neuronal differentiation and repression of expression of neurogenesis inhibitors (31).

PHOX2B encodes a highly conserved homeobox transcription factor of 314 amino acids with two short and stable polyalanine repeats of 9 and 20 residues, respectively (32). The length of both polyalanine tracts is conserved in mice and humans. However, heterozygous expansions within the carboxy-terminal 20-residue polyalanine tract are common in CCHS, probably resulting from non-homologous recombination (20,21). In contrast, neuroblastoma patients with or without associated congenital abnormalities have only been found to have either heterozygous frame shift, nonsense or missense *PHOX2B* mutations (12,20-25).

Only one mutation with a predictable effect on the protein sequence of *PHOX2B* was observed in our study. This missense mutation was found in the bilateral tumours of a patient diagnosed with stage 4_M neuroblastoma. The constitutional DNA of the patient was heterozygous for the mutation and the tumours were hemizygous (Fig. 2B). It is difficult to determine the effect of an asparagine to a serine substitution on the protein function, since they are both polar amino acids with neutral side chains. However, *PHOX2B* is an extremely well conserved gene with only a single amino acid differing between the human and mouse encoded proteins; i.e. glycine to alanine at position 292, and with substantial conservation of the 5'-upstream region (32). The region in the vicinity of the detected missense mutation is also highly conserved (Fig. 5). Although the allele remaining in the tumour DNA was also present in heterozygous form in the patient's mother and youngest sibling, it was not found in any of 108 screened control individuals (i.e. 216 alleles). The occurrence of the mutation in the healthy mother and one of the patient's four siblings could be explained by the occurrence of spontaneous regression in neuroblastoma or by the short developmental window where embryological neural

crest cells may be sensitive for loss of *PHOX2B* function causing incomplete penetrance of the gene.

Microsatellite analysis determined that the normal allele of the gene had been lost in both of the tumours along with the distal part of chromosome 4p (Fig. 3). This was confirmed by FISH analysis (Fig. 4) and whole genome dosage analysis which also revealed that the breakpoints on chromosome 4 were identical in both tumour samples (Fig. 6). Furthermore, both tumours had identical 17q gains and the tetraploid tumour had additional whole chromosomal loss of chromosomes 3, 9, 14 and 15 supporting a model where progressive growth of the tetraploid tumour occurred from the diploid tumour after additional genetic changes. Apparently, 17q gain and loss of the distal part of chromosome 4p, including the *PHOX2B* locus and possibly other tumour suppressor genes, were early events in the development of this tumour, consistent with the importance of *PHOX2B* in embryonic ANS development. This report indicates that tetraploidization and chromosomal instability causing subsequent loss of important genes on chromosomes 3, 9, 14 and 15 occur later and may confer properties necessary for invasion or metastatic growth.

Furthermore, although the two alterations found in the carboxyterminal 20-residue polyalanine tract and in the 3'-UTR region of the gene, respectively are not previously reported in dbSNP, they did not have an impact on expression or stability of the transcript and thus probably represent polymorphisms. The effect on regulation of gene expression from the 5'-UTR located variation (c.-97G>A), found in a patient with stage 4S neuroblastoma can not be determined since only DNA was available from this patient.

To our knowledge, LOH at the *PHOX2B* locus has not previously been reported in any patient with CCHS or in any neuroblastoma with *PHOX2B* mutation of one allele. One explanation for this can be that LOH analysis has not been performed in some studies. This could also be due to lack of blood or matched tumour samples to look for deletion of wild-type allele in the few identified germline mutation carriers.

Recently, Raabe *et al* reported on a study of *PHOX2B* mutations in a selected panel of patients with presumed genetic predisposition similar to our own. Three of 47 individuals in their study showed a germline *PHOX2B* mutation (12). This is consistent with our results demonstrating that *PHOX2B* mutations are rare even in selected neuroblastoma patients. Our study also supports the previous findings that neuroblastoma tumours with mutations in *PHOX2B* do not have in-frame expansions of the polyalanine tract (12,20-23,33,34). They seem to acquire missense mutations or protein truncating mutations through some other mechanism than the non-homologous recombination thought to be responsible for the expansion mutations generally found in patients with ANS disorders who lack these tumours. However, the repeat expansions could have a dominant negative effect causing similar effect on the cell through loss of function of the protein product. Even though *PHOX2B* mutations are not common in sporadic neuroblastoma tumours, patients with ANS disorders and missense or nonsense mutations in *PHOX2B* might also have an increased risk of developing tumours and should be monitored for the occurrence of neuroblastoma.

The tumours of patient 25R3 caught our attention since it was clinically diagnosed as a rare multifocal neuroblastoma case, 4_M (27). The patient also displayed a rare germline *PHOX2B* mutation in a highly conserved codon of one allele and loss of the other allele due to a loss of a large 41.7 Mb chromosome region on 4p. Thus, this is a perfect example of Knudson's two-hit hypothesis for inactivation of a tumour suppressor (35). In contrast, molecular data, in particular array CGH data argues for a tumour progression model and that the patient has developed only one primary tumour. In this model, the first tumour developed from *PHOX2B* mutated proliferating undifferentiated neural crest precursor cells after additional chromosomal aberrations with 17q gain and 4p loss and the large tumour 2 is due to either a metastasis to the second adrenal gland from the established first tumour or a parallel tumour development from a common malignant precursor (Fig. 6B). The fact that argues for a sequential or parallel tumour development from a common malignant precursor and not independent multifocal primaries from the neural crest precursors is that both tumours in case 25R3 contain specific chromosomal breaks that have been involved in producing the 4p loss and the 17 gain region, and it seem implausible that these events have happened in two different tumour origin events. Tumour 2 have later evolved additional chromosomal changes - tetraploidization and loss of additional whole chromosomes. The molecular facts thus strongly suggest that this rare case in fact results from a single malignant cellular origin, albeit being clinically multifocal. This case was studied with an array of different complementary molecular techniques, and these data may suggest that other authors should perform a similar scrutiny of multifocal cases in particular in the era of high-density array based studies.

Acknowledgements

We gratefully acknowledge the Göteborg Genomics core facility for providing access to the ABI PRISM 3730 DNA Sequencer. This work was supported by grants from the Swedish Cancer Society, the Children's Cancer Foundation, the IngaBritt and Arne Lundberg Research Foundation, the King Gustav V Jubilee Clinic Cancer Research Foundation, the Assar Gabrielsson Foundation, the Wilhelm and Martina Lundgren Research Foundation, the Royal Physiographic Society in Lund, and the Sahlgrenska University Hospital Foundation. T.M. occupies a cancer researcher position funded by the Swedish Cancer Foundation. None of the authors had competing interests.

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