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The Phox2B homeobox gene is mutated in sporadic neuroblastomas

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Neuroblastomas are embryonal tumours of the sympatho-adrenal lineage with a clinical course ranging from spontaneous regression to fatal progression. The Phox2B homeobox transcription factor functions in the differentiation of the sympatho-adrenal lineage. Targets of Phox2B are, for example, genes of the (nor)adrenalin synthesis route, like Dopamine Beta Hydroxylase (DBH). Congenital Central Hypoventilation Syndrome was recently found to result from Phox2B mutations and two such patients in addition developed neuroblastoma. A germline mutation in Phox2B was identified in a family with hereditary neuroblastoma. Here, we report the first analysis of Phox2B in a series of 237 sporadic neuroblastomas and 22 cell lines. Six frameshift mutations were found in exons 2 and 3; including one in cell line SK-N-SH. Two patients showed *de novo* constitutional mutations. One of them was diagnosed with Haddad syndrome. All analysed cases expressed the mutated and wild-type Phox2B alleles. Ectopic expression of TrkA, the Nerve Growth Factor receptor, strongly downregulated Phox2B and DBH expression in cell line SH-SY5Y. However, TrkA and Phox2B showed a positive correlation in a panel of 66 neuroblastoma tumours. Although Phox2B mutations are infrequent (2.3%), they implicate a role for the Phox2B pathway in oncogenesis.

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Introduction

Neuroblastoma is a childhood tumour originating from progenitor cells of the sympathetic nervous system (SNS). The tumour displays a broad spectrum of clinical behaviour, ranging from spontaneous regression to fatal progression despite intensive therapy. In general, tumours with a good prognosis are diagnosed in children less than 1 year of age. Unfavourable tumours occur in older children and are characterized by chromosomal rearrangements. Only a few genes with a causative role in neuroblastoma pathogenesis have been identified. MYCN is amplified in 20% of tumours, while amplifications of Cyclin D1, MDM2, Cdk4 and Meis1 were reported for only a few cases (Brodeur *et al.*, 1984; Schwab *et al.*, 1984; van Roy *et al.*, 1995; Spieker *et al.*, 2001a; Molenaar *et al.*, 2003). Mutated genes have not been reported to date, except for a single mutation in Cdk6 and one rearrangement of Cyclin D1 (Easton *et al.*, 1998; Molenaar *et al.*, 2003). Loss of heterozygosity of large domains of chromosome arms 1p, 4p, 11q and 14q is frequent, as well as gain of a large part of the q arm of chromosome 17.

Markers for a poor prognosis of neuroblastoma are MYCN amplification, loss of 1p36, gain of 17q and high expression of TrkB, the receptor for brain-derived neurotrophic factor (BDNF) (Brodeur *et al.*, 1984; Schwab *et al.*, 1984; Nakagawara *et al.*, 1994; Caron, 1995; Caron *et al.*, 1996). In contrast, high expression of TrkA, the receptor for nerve growth factor (NGF), is associated with a very good prognosis (Nakagawara *et al.*, 1993). Ectopic expression of TrkA in the human neuroblastoma cell line SH-SY5Y induced growth inhibition and impaired tumour growth, while ectopic TrkB expression resulted in enhanced proliferation and rapidly growing tumours (Eggert *et al.*, 2000, 2002).

Recently, it was found that the congenital central hypoventilation syndrome (CCHS) is caused by mutations in the paired homeobox transcription factor Phox2B (Amiel *et al.*, 2003; Sasaki *et al.*, 2003; Weese-Mayer *et al.*, 2003). Two CCHS patients with Phox2B mutations in these studies also had a neuroblastoma (Amiel *et al.*, 2003; Weese-Mayer *et al.*, 2003). Most Phox2B mutations in CCHS are expansions of a polyalanine tract in exon 3 (see Figure 1), but these patients did not develop neuroblastoma. The CCHS patients with neuroblastoma had a stop codon and a frameshift mutation in Phox2B, both occurring between the homeobox and the polyalanine stretch (Amiel *et al.*, 2003; Weese-Mayer *et al.*, 2003).

Phox2B controls some important differentiation steps in the sympathoadrenal lineage. The cells of this neural crest derived lineage migrate ventrally during early embryogenesis and give rise to the specialized cell types

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of the sympathetic side chain, the sympathetic paraganglia and the adrenal medulla. The human adrenal medulla is formed by primitive neuroblasts invading the adrenal cortex from the 6th week of gestation on. After migration, neuroblasts can differentiate into chromaffin cells and neuronal cells. Chromaffin cells function to produce high levels of (nor)adrenalin for secretion into the circulation. It is assumed that neuroblastomas can arise from any stage in the sympatho-adrenal differentiation route. Indeed, most neuroblastomas are marked by secretion of metabolites of the noradrenalin synthesis pathway.

Phox2B is part of a regulatory network with the homologous transcription factor Phox2A, the human-achaete-scute-homologue-1 (HASH1) and the Delta-Notch pathway. HASH1 can induce expression of Phox2A and Phox2B, but Phox2B can also induce and maintain expression of HASH1 (Guillemot *et al*., 1993; Morin *et al*., 1997; Pattyn *et al*., 1999; Huber *et al*., 2002; Stanke *et al*., 2004). Phox2A and Phox2B control the expression of the genes that encode the enzymes for the synthesis of (nor)adrenalin, like DBH and Tyrosine Hydroxylase (TH) (Yang *et al*., 1998). Phox2B null mutant mice show major defects in the differentiation of the peripheral nervous system: autonomic ganglia fail to form properly and Ret and Mash1 (the murine homologue of HASH1) are not properly expressed in the anlagen of the sympathetic ganglia and the enteric nervous system (Pattyn *et al*., 1999). In addition, they do not express DBH and TH, resulting in noradrenalin deficiency and an embryonic lethal phenotype.

During the course of this study, a family with hereditary neuroblastoma was found to have a Phox2B mutation (Trochet *et al*., 2004). Here, we present the first analysis of 237 sporadic neuroblastomas and 22 neuroblastoma cell lines, and describe six cases with mutations in Phox2B. In addition, Phox2B is related to the TrkA pathway, as it was switched off by ectopic TrkA expression in a cell line, but shows a positive correlation to TrkA in a neuroblastoma tumour series.

Results

The constitutive mutations of Phox2B in two CCHS patients with neuroblastoma suggested us to analyse a possible role of this gene in neuroblastoma pathogenesis. The full coding sequences of exons 1, 2 and 3 were amplified by PCR of genomic DNA of a series of sporadic neuroblastomas and neuroblastoma cell lines. The three exons were sequenced for 237 neuroblastomas and 22 neuroblastoma cell lines. No mutations were found in exon 1. One mutation was found in exon 2 and five different mutations were found in exon 3, all resulting in frameshifts (see Table 1). Four mutations were caused by small deletions and two by small duplications. The exon 3 deletions could readily be sequenced, as the PCR reaction of this GC-rich exon shows a strong preference for the shortest allele. Also the expansions could be identified by sequencing, but required additional analysis (see below). Deletions and duplications were confirmed at least twice by independent PCR and sequence reactions.

Cell line SK-N-SH was found to carry a Phox2B allele with a deletion of 20 bp in the polyalanine encoding tract, causing a frameshift and premature stop. A 338 bp fragment of exon 3 encompassing the deletion (fragment 3C) was amplified by PCR and analysed by PAGE (Figure 1b). Both the wild-type and mutated allele were present in the cell line. The bands were excised and sequenced, confirming the presence of a wild type as well as the mutant allele.

Neuroblastoma N129 had a 38 bp deletion between the homeobox domain and the polyalanine tract, causing a 12 amino-acid deletion and a frameshift in the subsequent codons (Figure 1a and c). PCR amplification of fragment 3C showed that both the wild-type and the mutant alleles were present in the tumour, but not in the lymphocyte DNA of the patient and his parents (Figure 1b). Patient N129 was diagnosed with a stage 3 neuroblastoma at the age of 11 months and died 4 months later of therapy-related complications.

Neuroblastoma N511 had a 35 bp deletion starting at the polyalanine tract and causing a frameshift in the C-terminal end of the protein (Figure 1a). PCR amplification of fragment 3C showed that the mutated allele was present in the tumour, but not in lymphocytes of the patient. Also a wild-type fragment was present in the tumour (Figure 1b). Sequencing of these bands confirmed the presence of a wild-type and the mutated allele. Patient N511 was diagnosed with a stage 1 abdominal neuroblastoma at 10 months of age, presented with recurrent disease 3 years after diagnosis and shows no evidence of disease at age of 5 years.

Neuroblastoma N571 had a 13 bp duplication, starting just before the polyalanine encoding tract and causing a subsequent frameshift (Figure 1a and b). The mutation was not present in the DNA of normal tissue of the patient. The patient was diagnosed with an abdominal stage 3 neuroblastoma at the age of 3 years and 11 months and died of recurrent disease about a year later.

Neuroblastoma N532 had a 17 bp expansion of the polyalanine tract, leading to a frameshift of the C-terminal part of the protein. PCR amplification of fragment 3C showed that a normal allele and the expanded allele were present in the tumour as well as in the lymphocytes. However, lymphocyte DNA of both parents showed normal 3C fragments (Figure 1b). The expansion is therefore a *de novo* constitutional mutation. Sequencing of the excised alleles confirmed the mutation in the tumour and the lymphocytes. The patient has a complete agangliosis of the colon (long segment Hirschsprung's disease). At the age of 8 months, the boy was diagnosed with two primary neuroblastomas of stage 2 and 3, which implies staging as 3 M. After detection of the Phox2B mutation, the patient underwent further clinical examination and was diagnosed with CCHS. The combination of CCHS and Hirschsprung disease is known as Haddad syndrome. Two years after diagnosis, the patient is in complete remission of his neuroblastoma.

Neuroblastoma N97 had an 8 bp deletion in exon 2, just upstream of the homeobox domain. PCR amplification of a fragment of exon 2 (fragment 2C) showed that the deletion was present in tumour and lymphocyte DNA of the patient, but not in lymphocyte DNA of his parents. The patient was diagnosed with a stage 2

thoracal neuroblastoma at age of 9 months. After treatment, he has remained disease free with a follow-up of 12 years. The patient was not diagnosed with further abnormalities, except for suffering already as neonate from oesophageal reflux. This symptom is associated with CCHS and may indicate a failure of local autonomic control.

In addition to these mutations, we observed 11 cases with previously reported polymorphisms of Phox2B (Amiel *et al*., 2003; Sasaki *et al*., 2003; Weese-Mayer *et al*., 2003). They were all contractions of the polyalanine encoding tract that left the reading frame intact. These polymorphisms were in all analysable cases present in the lymphocyte DNA of the patient and one of the parents.

Wild-type and mutant Phox2B alleles are coexpressed

Although the mutations in Phox2B clearly implicate this gene in neuroblastoma pathogenesis, it is not clear whether Phox2B functions as an oncogene or a tumour suppressor gene. The five exon 3 mutations start between amino acid 215 and 246 and leave the homeobox domain (a.a. 99–148) intact, while the polyalanine stretch (a.a. 241–260) is in all cases completely or largely destroyed (Figure 1a). They all cause a frameshift in the C-terminal part of the Phox2B protein, with two different reading frames (+1 frame: N532; +2 frame: N129, N511, N571 and SK-N-SH). In contrast, the exon 2 mutation causes a frameshift that completely disrupts the homeobox domain and all downstream sequences. All six mutations could therefore function to inactivate the protein, but they could as well produce an active protein with changed properties, either oncogenic by themselves or with a dominant-negative function.

Our PCR analyses of the wild type and mutated Phox2B alleles (Figure 1) clearly showed that both alleles were retained in the tumours and cell line. The tumours therefore show no loss of heterozygosity (LOH), as could be expected for a tumour suppressor gene. As a further analysis of the question whether Phox2B could be a tumour suppressor gene, we studied the Phox2B mRNA expression in three tumours with exon 3 mutations and in cell line SK-N-SH. mRNA was reverse transcribed and a fragment spanning part of exons 2 and 3 was amplified by PCR and analysed on PAGE. All three tumours and the cell line showed the wild type as well as the mutant fragment, clearly showing that both alleles were expressed in all four cases (Figure 2). This expression pattern does not support a role as classical tumour suppressor gene.

Relationship between TrkA, Phox2B and DBH expression in SK-N-SH derivatives

To further analyse the role of Phox2B and genes upstream and downstream of it, we explored cell line SK-N-SH and some of its derivatives. SH-SY5Y and SHEP are two well-studied stable subclones of SK-N-SH, with a neuronal-like and epithelial-like phenotype, respectively (Ross *et al*., 1983). Sequencing confirmed that both derivatives carry the same Phox2B mutation as SK-N-SH (data not shown). Northern blot analysis showed that SK-N-SH and SH-SY5Y have a high Phox2B expression, but the gene is completely silent in SHEP. Also DBH is expressed in SK-N-SH and SH-SY5Y, but not in SHEP (Figure 3a). As DBH is a well-documented target gene of Phox2B (Yang *et al*., 1998), these data suggest that the transcriptional activation of DBH by wild-type Phox2B is not affected by the presence and expression of a mutated Phox2B allele. These results do not support a possible dominant-negative effect of the mutated Phox2B product, at least not for DBH regulation.

SH-SY5Y has been extensively used to study the effects of ectopic expression of TrkA and TrkB. We

Table 1 Summary of Phox2B mutations

Patient	*Age (months)*	*Stage*	*Outcome*	*Mutation*[a]	*Status*
SK-N-SH	—	—	—	721–740del20nt	Cell line
N129	11	3	died of complications	633–670del38nt	Tumour specific
N511	10	1	NED	721–755del35nt	Tumour specific
N571	47	3	died of disease	702–714dup13nt	Tumour specific
N532	8	2+3 (3 M)	NED	721–737dup17nt	Constitutional de novo
N97	9	2	NED	284–291del8nt	Constitutional de novo

[a]Nucleotide (nt) numbering refers to the CDS of Phox2B consisting of 942 nt, homeodomain: nt294–nt444, 20 alanine tract: nt721–nt780
NED = no evidence of disease

Figure 1 Phox2B is mutated in five neuroblastoma tumours and a cell line. (**a**) The organization of the Phox2B gene and the positions of the mutations. Nucleotide positions 630–759 and 241–361 refer to the coding sequence. Deletions are indicated by dots, duplications are in bold. The homeodomain is in blue, the polyalanine encoding stretch is in green. (**b**) PCR products of exon 2 fragment 2C and exon 3 fragment 3C were analysed on PAGE. The alleles in SK-N-SH are shown next to two normal controls (**c**). The mutant alleles of N129 and N511 are only present in the tumour (1T) and not in lymphocyte DNA of the patient (1L) and parents (2L and 3L). The mutant alleles of N97 and N532 are present in the tumour (1T) and lymphocyte DNA of the patients (1L), but not in lymphocyte DNA of the parents (2L and 3L). The mutant alleles in N571 are present in two tumour samples (1T1 and 1T3) and not in normal tissue of the patient (1N). Note the presence of heteroduplex bands of mutant and wild-type alleles in the upper part of the gel. (**c**) An electropherogram of the Phox2B sequence of tumour N129 showing the 38 bp deletion

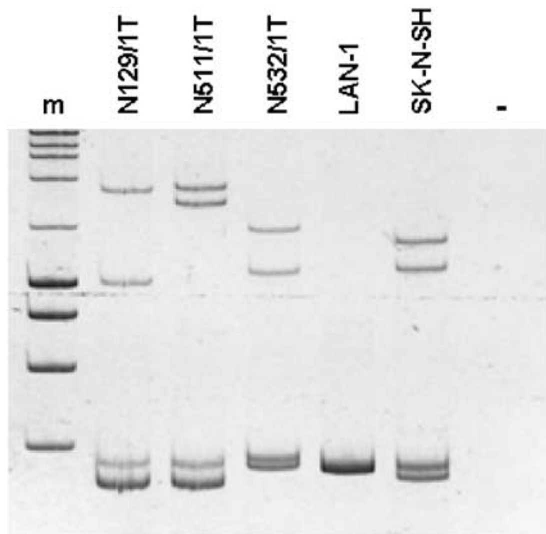

Figure 2 Three neuroblastomas and cell line SK-N-SH express both the wild-type and mutated Phox2B alleles. Oligo dT-primed cDNA of tumours N129, N511 and N532 and cell lines LAN-1 (control) and SK-N-SH were amplified by PCR. The fragment includes part of exons 2 and 3 spanning the deletions and expansion. Except for the control sample, wild type and mutant cDNA products are always observed together

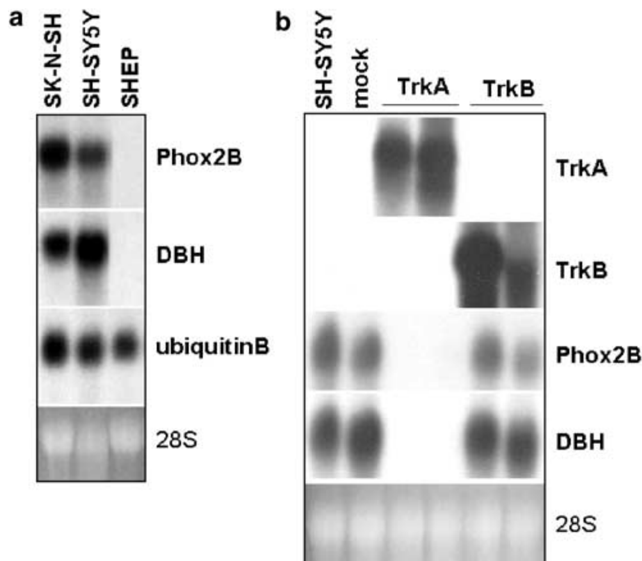

Figure 3 Phox2B and DBH expression in neuroblastoma cell line SK-N-SH and derivatives. (**a**) A Northern blot with total RNA from SK-N-SH, its neuronal derivative SH-SY5Y and its epithelial derivative SHEP was hybridized with Phox2B and DBH probes. UbiquitinB hybridization and 28S serve as loading controls. (**b**) Phox2B and DBH expression are downregulated by TrkA, but not by TrkB. A Northern blot with total RNA from SH-SY5Y, SH-SY5Y transduced with an empty vector (mock), two clones transduced with a TrkA expression construct (TrkA) and two clones transduced with a TrkB expression construct (TrkB) was hybridized with probes for the indicated genes

previously described clones of SH-SY5Y which were virally transduced with TrkA and TrkB expression vectors (Eggert *et al*., 2000, 2002). The TrkA and TrkB receptors expressed in the clones were activated even in the absence of exogenously added ligands, probably due to dimerization of the over-expressed receptors (Eggert *et al*., 2000). We have analysed the mRNA expression profiles of TrkA and TrkB expressing clones by oligonucleotide arrays (Schulte *et al*., 2004). The profiles of the clones expressing TrkA showed a strongly reduced signal for Phox2B. RNA of three SH-SY5Y clones transduced with TrkA, two TrkB-transduced clones, SH-SY5Y wild-type and a control clone transduced with an empty vector were analysed by Northern blot. TrkA and TrkB were highly expressed in the clones transduced with the respective genes, but not in the wild-type cells and control clones (Figure 3; shown for two TrkA clones). Indeed, Phox2B was strongly silenced in the TrkA-expressing clones, but not in the wild type and control cells and the two TrkB-expressing clones. A regulatory connection between TrkA and Phox2B has never been reported before. Also DBH expression was completely abrogated in all SY5Y clones with TrkA expression (Figure 3b). DBH expression remained unaffected in the SY5Y-TrkB clones. Phox2B is therefore downregulated by TrkA, but not by TrkB, and connects the NGF-TrkA pathway with the noradrenalin synthesis route. The mutated Phox2B is unable to interfere with the regulation of DBH by wild-type Phox2B.

TrkA and Phox2B expression are positively correlated in neuroblastoma tumours

To analyse whether the relationships observed in SK-N-SH and its derivatives are representative for neuroblastomas *in vivo*, we analysed TrkA, Phox2B and DBH expression in a series of 66 neuroblastomas, representative for the normal neuroblastoma spectrum (see Materials and methods). Surprisingly, the real-time rtPCR data showed a positive correlation between expression of TrkA and Phox2B (Figure 4a). As expected, also Phox2B and DBH were positively correlated (Figure 4b). All correlations were significant: between TrkA and Phox2B (Pearson correlation coefficient $r=0.6$; Wilcoxon rank-sum test $P<0.001$), between Phox2B and DBH ($r=0.52$; $P<0.001$) and between TrkA and DBH (Figure 4c; $r=0.74$; $P<0.001$). Although the *in vitro* and *in vivo* results both indicate a relationship between TrkA and Phox2B, the relationship in SH-SY5Y is opposite to the apparent relation in the tumour series. Of note, TrkA is constitutively active in SK-SY5Y, while it is unknown whether TrkA was activated by its ligand NGF at the moment of surgical excision of the tumours.

Discussion

Here, we present the first analysis of a series of sporadic neuroblastoma for Phox2B mutations. Six different mutations were found in a series of 237 tumours and 22 cell lines (2.3%). The five exon 3 mutations cluster in or close to the polyalanine tract and induce frameshifts, while the exon 2 mutation induces a frameshift that

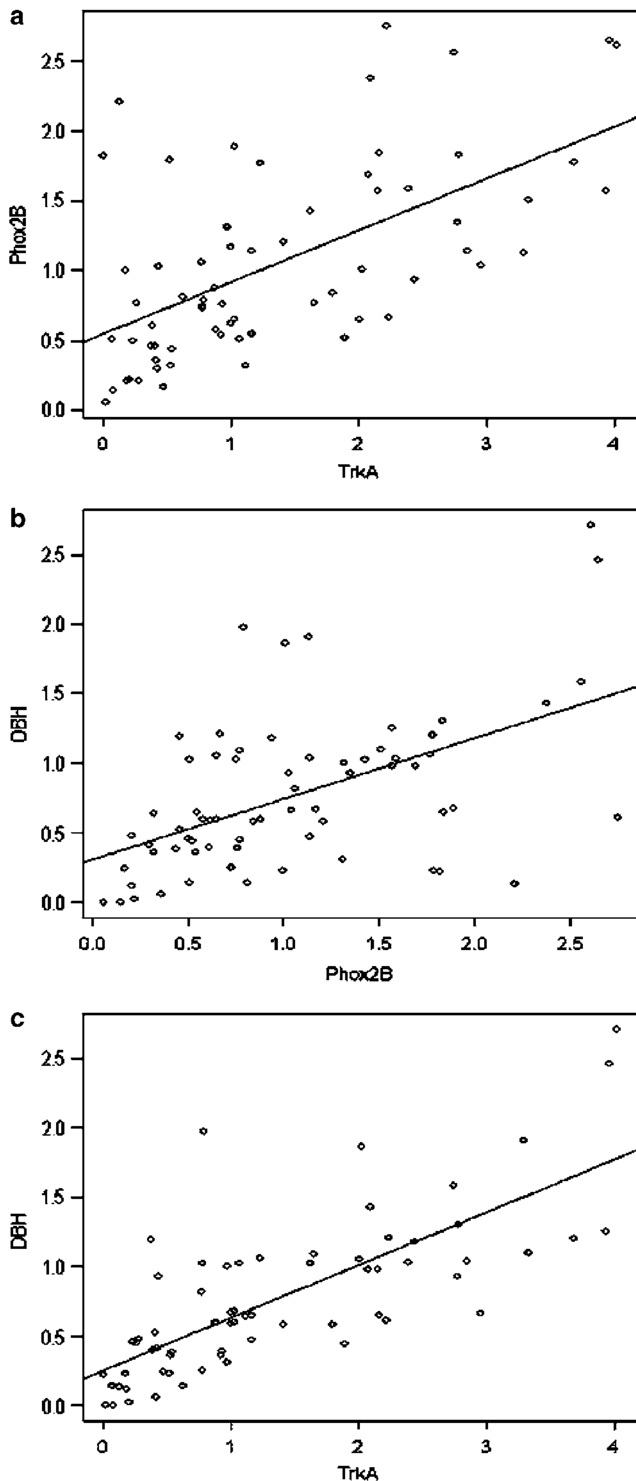

Figure 4 Real-time rtPCR analysis of TrkA, Phox2B and DBH expression in a series of 66 neuroblastomas. (**a**–**c**) Scatter plots of normalized gene expression for the indicated genes. Trendlines indicate the degree of positive correlation. All correlations were significant ($P<0.001$, Wilcoxon rank-sum test)

disrupts the homeobox and all downstream sequences. The mutations induce shifts towards both the $+1$ or $+2$ frames. They thereby differ from the large majority of the Phox2B mutations in CCHS, which are expansions of the C-terminal polyalanine tract that do not induce frameshifts (Amiel *et al*., 2003; Sasaki *et al*., 2003; Weese-Mayer *et al*., 2003). These in-frame expansions are never associated with neuroblastoma. Only two constitutive Phox2B mutations in CCHS patients with neuroblastoma have been described. One had a stop codon immediately after the homeobox domain (Weese-Mayer *et al*., 2003), and the other one had a frameshift in the C-terminal end of the protein due to an insertion, leaving the homeobox intact and destroying the polyalanine stretch (Amiel *et al*., 2003). Neuroblastoma-associated mutations therefore seem to share the destruction of a significant part of the normal amino-acid sequence.

The mutations raise the question whether Phox2B should be considered as a tumour suppressor gene or oncogene. Phox2B could be argued to have a differentiation-stimulating function, as it activates genes of the (nor)adrenalin synthesis route, which characterize terminally differentiated neuroblasts. In addition, Phox2B maps to the short arm of chromosome 4, which we and others found to show LOH in about 20% of neuroblastomas (Caron *et al*., 1996; Perri *et al*., 2002). It maps at about 37 Mb proximal of the SRO defined in one study (Perri *et al*., 2002), but most 4p deletions are large and include the Phox2B region, opening the possibility that Phox2B could act as a tumour suppressor gene. If Phox2B had a tumour suppressor function, the mutations would either cause inactive proteins, or may have a dominant-negative effect and prevent the functioning of the wild-type allele. The dominant negative model is not supported by the observation that SK-N-SH and SH-SY5Y cells have a normal expression of the Phox2B-target gene DBH, while they express both the mutant as well as the wild-type Phox2B allele. The mutant allele evidently cannot prevent DBH expression. The fact that in all tumours with Phox2B mutations, the wild type allele was present and, as far as analysed, also expressed also seems to contradict a tumour suppressor function, but does not exclude tumorigenesis by haploinsufficiency of Phox2B. Alternatively, the mutations that we observed in neuroblastoma could be activating and change Phox2B into an oncogene. The frameshifts always destroy the polyalanine tract, which may be targeted by an unknown protein that forms an inactive complex with Phox2B. Polyalanine tracts are found in many transcription factors, including a series of homeobox proteins (Han and Manley, 1993; Lanz *et al*., 1995; Lavoie *et al*., 2003). However, the mutation upfront of the homeobox in patient N97 suggests that, if this were an activating mutation, the activated protein would not even need the DNA-binding homeodomain. All together, the available data are as yet inconclusive for the mechanism by which Phox2B mutations contribute to neuroblastoma pathogenesis. It is even possible that the different mutations are oncogenic by different mechanisms.

The observation that constitutive ectopic TrkA expression in SH-SY5Y can largely silence Phox2B expression is potentially very interesting, but has to be interpreted with respect to the paradoxical observation

that TrkA and Phox2B expression is positively correlated in a series of 66 neuroblastoma tumours. The positive correlation in the tumour series does not imply that one of the genes induces (indirectly) the expression of the other. However, the effect of TrkA on Phox2B in SH-SY5Y reveals a regulatory connection, in which TrkA is upstream of Phox2B. A major difference between the physiological status of the SH-SY5Y clones *in vitro* and the tumour series *in vivo* is that the first have a constitutively active TrkA protein, probably due to the high expression and spontaneous dimerization of TrkA, while the tumour cells need NGF stimulation for TrkA activation and signalling. As we do not know whether NGF, which can be produced by Schwann cells in or around tumour lesions, was present in the tumours, it is currently impossible to determine the level of TrkA activity in the tumours and asses whether it can potentially downregulate Phox2B *in vivo*. Interestingly, ectopic expression of TrkB does not have any effect on Phox2B. TrkA and TrkB are associated with strongly different phenotypes. TrkA expression is associated with a favourable prognosis, while TrkB expression associates with a poor prognosis (Nakagawara *et al*., 1993, 1994). The question whether a possible relationship between TrkA and Phox2B plays a role in tumour behaviour *in vivo* requires further analysis.

The frequency of Phox2B mutations in neuroblastoma is relatively low. Also the two patients with *de novo* constitutive mutations have only one and two primary tumours, indicating that Phox2B mutations need additional defects to give rise to full blown tumours. Phox2B is evidently involved in control of development and mutations may only contribute to cancer during a short time-frame in neuroblast differentiation. Phox2B functions in a developmental pathway including HASH1, Phox2A and RET and is by these genes connected to a regulatory network including the Delta-Notch pathway and a range of proneural genes. This study shows that Phox2B mutations can contribute to neuroblastoma pathogenesis and indicates that the regulatory network around Phox2B plays an important role in neuroblast differentiation.

Materials and methods

Sample collection and DNA isolation

After informed consent was given, tumour tissues from neuroblastoma patients treated at the Emma Kinderziekenhuis/Academic Medical Center and other pediatric oncology centers in the Netherlands were obtained. A consecutive series of 237 tumour samples was used for sequencing of Phox2B. The large majority of the samples were pretreatment biopsies. The stage distribution of the tumours was as following: stage 1: 15 tumours; stage 2: 36 tumours; stage 3: 28 tumours; stage 4: 86 tumours; stage 4S: 17 tumours and 55 tumours of unknown stage. Peripheral blood samples of patients and their parents were also collected. Histological analysis was performed and high molecular weight DNA was prepared as described before (Mullenbach *et al*., 1989) and used for sequencing. The 66 neuroblastomas used for expression analysis were from the German neuroblastoma study NB95 and NB97. Their clinical staging was: Stage 1: 20 tumours; stage 2: 16 tumours; stage 3: eight tumours; stage 4: 14 tumours; stage 4S: eight tumours.

Cell lines

The TrkA and TrkB-transduced SH-SY5Y cells were described previously (Eggert *et al*., 2000). Cell lines were cultured as previously described (Boon *et al*., 2001; Spieker *et al*., 2001b). Cell lines sequenced for Phox2B mutations were: SK-N-FI, LAN-2, SK-N-AS, LAN-6, SJ-NB-1, SJ-NB-12, SK-N-SH, UHGNP, AMC106, IMR32, KCNR, TR14, N206, SJ-NB-8, SK-N-BE, SJ-NB-6, LAN-1, NMB, NGP, SJ-NB-10, LAN-5 and NB69wt. In addition, we sequenced the SK-N-SH derivatives SHEP, SH-SY5Y and SH-SY5Y-TrkA.

Sequence analysis

Phox2B exons 1, 2 and 3 were amplified with primer pairs 3328F + 3329R, 3330F + 3331R and 3333F + 3336R described in Table 2, giving products of 385, 346 and 711 bp, respectively. The PCR reactions were carried out using 0.15 μl Hotstar*Taq* DNA polymerase (Qiagen) in a total volume of 25 μl containing 50 ng genomic DNA, 20 ng of each primer (or 25 ng for exon 3 primers), 1 mM $MgCl_2$, 5 μl solution Q (Qiagen), 0.2 mM dNTPs, and 2.5 μl 10 × Hotstar PCR buffer. The amplification was performed with an initial denaturation at 96°C for 10′ followed by 35 cycles of 45″ at 96°C, 1′ at 58°C (54°C for exon 2), and 90″ at 72°C. Final extension was at 72°C for 7′. Owing to its high GC content, sequencing of exon 3 was performed in the presence of 1 M betaine and reactions were started with an initial denaturation step for 10′ at 96°C. The PCR products were visualized on agarose gels. Sequence reactions were performed with the ABI PRISM™Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Nieuwerkerk, The Netherlands) in a final reaction volume of 20 μl. For exons 1 and 2, the same primers employed for initial PCR were used. For exon 3 primers 3332F, 3334R, 3366F and 3336R were used. PCR products were sequenced in both forward and reverse directions on an ABI 3730 (Applied Biosystems) automated sequencer. Sequences were base-called and assembled with the Staden-package (Bonfield *et al*., 1998), using sequence AF117979 as a reference. All sequence assemblies and polymorphisms were manually reviewed to insure accuracy of variant identification.

Analysis of Phox2B fragment 3C and 2C

The Phox2B exon 3 region coding for the polyalanine repeat was amplified with primer pair 3366F + 3334R. The PCR

Table 2 Oligo nucleotides used for sequencing

Oligo	*Sequence*	*Location*
3328F	*tgagctgtgcacatctcc*	Exon1 5′UTR
3329R	*atatacgggcggaaaggc*	Intron1
3330F	*tcctcacattctagctcc*	Intron1
3331R	*cttcgaatttcaccagcc*	Intron2
3572F	*tctcctctgtcatactctagttcctt*	Exon2
3573R	*gaccctttccagctctttgag*	Exon2
3443F	*gagtccaggtgtggttcc*	Exon2/Exon3
3332F	*acttgggccaccctaacc*	Intron2
3333F	*gccgaagtagaacttggg*	Intron2
3366F	*agtctgactcttccagg*	Exon3
3334R	*caagcgaatccgggatgg*	Exon3
3336R	*cgacgacaatagccttgg*	Exon3 3′UTR

products (338 bp for the wt allele) were analysed on 8% PAGE. For heterozygous individuals, the separated bands were isolated, dissolved in H_2O and used for re-PCR. This enabled separated sequencing of both alleles.

An 127 bp fragment overspanning the 8 bp deletion of patient N97 was amplified using primers 3572F + 3573R. Both alleles were isolated from PAGE and sequenced separately.

Expression profiling of SH-SY5Y clones

Affymetrix HGU95Av2 oligonucleotide microarrays and GeneChip Microarray Suite (MAS) 5.0. software (Affymetrix) were used to compare gene expression profiles (Sasaki *et al*., 2003).

Northern blot analysis and RT–PCR

RNA isolation and Northern blot analysis was performed as described before (Limpt *et al*., 2003). DNA probes were generated from EST clones of the IMAGE consortium; TrkA (bg110843, insert 850 bp), TrkB (AW770836, insert 1300 bp) and Phox2B (AI266171, insert 1449 bp). The DBH probe was described previously (Weese-Mayer *et al*., 2003). Oligo dT-primed cDNA was synthesized at 58°C from 3 μg total RNA with ThermoScript TM according to the manufacturers protocol (Invitrogen, Life Technologies, The Netherlands). A volume of 2 μl cDNA was used for PCR amplification with primer pair 3443F + 3334R. PCR products (wt Phox2B band of 440 bp) were analysed by 12% PAGE. For the real-time rtPCR analysis of the series of 66 neuroblastomas, we used 'Assays on demand' (Applied Biosystems). They were performed for TrkA (Assay ID Hs00176787_m1), Phox2B (Assay ID Hs00243679_m1) and DBH (Assay ID Hs00168025_m1). Expression values were normalized by geometric averaging of four housekeeping genes (SDH, GAPDH, UBC and HPRT) as proposed in Vandesompele *et al*. (2002). Correlation of gene expression was evaluated by calculating Pearson's correlation coefficients (*r*) and by applying Wilcoxon rank-sum testing using programs within the 'stats'-package of 'R' (www.r-project.org).

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