

Methylation-associated PHOX2B gene silencing is a rare event in human neuroblastoma

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Abstract

Neuroblastoma (NB), an embryonic tumour originating from neural crest cells, is one of the most common solid tumours in childhood. Although NB is characterised by numerous recurrent, large-scale chromosome rearrangements, the genes targeted by these imbalances have remained elusive. We recently identified the paired-like homeobox 2B (PHOX2B, MIM 603851) gene as disease-causing in dysautonomic disorders including Congenital Central Hypoventilation Syndrome (CCHS), Hirschsprung disease (HSCR) and NB in various combinations. Most patients with NB due to a germline heterozygous PHOX2B gene mutation are familial and/or syndromic. PHOX2B, at chromosome 4p12, does not lie in a commonly rearranged locus in NB. To evaluate the role of PHOX2B in sporadic, isolated NB, we analysed 13 NB cell lines and 45 tumours for expression, mutations of coding and promoter sequences, loss of heterozygosity (LOH), or aberrant hypermethylation of PHOX2B (13 cell lines and 18 tumours). We didn't identify any mutation but LOH in about 10% of the cases and aberrant CpG dinucleotide methylation of the 500 bp PHOX2B promoter region in 4/31 tumours and cell lines (12.9%). Altogether, both germinal and somatic anomalies at the PHOX2B locus are found in NB.

MESH Keywords Cell Line, Tumor ; DNA Mutational Analysis ; methods ; Female ; Gene Frequency ; Gene Silencing ; Genetic Predisposition to Disease ; genetics ; Homeodomain Proteins ; genetics ; Humans ; Loss of Heterozygosity ; Male ; Methylation ; Neuroblastoma ; genetics ; Pedigree ; Transcription Factors ; genetics

Introduction

Neuroblastoma (NB; MIM 256700) is a tumour of the sympathetic nervous system that accounts for 10% of all cancers in children. Several lines of evidence support the involvement of genetic factors in NB, namely, rare familial cases with vertical transmission and the association of NB with other genetically determined congenital malformations of neural crest origin, such as Hirschsprung disease (HSCR; MIM 142623) and/or Congenital Central Hypoventilation Syndrome (CCHS; MIM 209880). We recently identified the paired-like homeobox 2B (PHOX2B; MIM 603851) gene as the major disease-causing gene in CCHS1 and the first gene for which germline mutations predispose to NB. 2-5 PHOX2B is a highly conserved homeotic transcription factor with two alanine tracts of nine and 20 alanines, C terminal to the homeodomain. The vast majority of mutations leading to CCHS result in an expansion of the longer alanine tract. Interestingly, patients harbouring either a missense mutation in the homeodomain or a frameshift mutation are the ones at risk of developing NB, whether or not they have CCHS. 6 However, both germline and somatic PHOX2B coding sequence mutations are rare events in sporadic, isolated NB. 7,4,8

Epigenetic abnormalities, especially alterations in DNA methylation, are involved in the development of various adult tumours. More recent studies have indicated that epigenetic aberrations may also contribute to paediatric cancer pathogenesis. In neuroblastomas, several potential tumour-suppressor genes have been found to be frequently hypermethylated and consequently down-regulated; in particular, genes of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway, CASP89 and the DCR receptors (DCR1, DCR2, DCR3 and DCR4). 10,11 DNA methylation has been shown to reduce the binding affinity of sequence-specific transcription factors while methylation-dependent gene silencing may also involve alterations in chromatin structure, mediated by methyl binding proteins. Chromosome distribution of the methyl-targeted genes are clustered and the full pattern of methylation may be generated early in tumourigenesis. 12,13 Finally, in tumours carrying a germline mutation, a second-step methylation of the DNA promoter, if present, occurs exclusively on the wild-type allele.

The possible involvement of non-coding mutations or promoter methylation of the PHOX2B locus in sporadic neuroblastoma has not been evaluated. In this study, we examined the level of PHOX2B expression and its methylation in NB. PHOX2B was silenced in a subset of 3/13 NB cell lines and loss of expression was associated with aberrant 50CpG dinucleotide methylation of the PHOX2B promoter.

PHOX2B promoter methylation was also detected in 2/18 tumours analysed. Treatment with the demethylating agent 5-Aza-20-deoxycytidine (5-Aza-dC) restored PHOX2B transcription in PHOX2B-negative cell lines, showing that gene silencing was due to aberrant hypermethylation. We conclude that aberrant CpG dinucleotide methylation of PHOX2B is an alternative mechanism at least as frequent as coding sequence mutations for inactivation of PHOX2B in sporadic NB.

Materials and methods

Patients

13 NB cell lines and 46 sporadic neuroblastic tumours were investigated. Patients were staged according to the International Neuroblastoma Staging System (INSS) and included seven with stage I tumour, ten stage II, seven stage III, 15 stage IV and seven stage IVS. Constitutional and tumour DNA were extracted using standard protocols. We included a patient from a two generation family with predisposition to NB due to a germline PHOX2B gene mutation (NBAF5, Table 1).²

Sequence analysis

We screened the coding sequence of the PHOX2B gene by direct DNA sequencing, as described elsewhere.¹ We studied a 533 bp sequence of the promoter region that is extremely conserved among species (97% between human and chicken at nucleotide level). The primer sequences for PHOX2B promoter region are 5'-GAAGGGGGAAAACACACAC-3' (forward) and 5'-CGTAGGCAGAGGAATTGAGG-3' (reverse). PCR Direct DNA sequencing was performed using the fluorometric method (Big Dye Terminator Cycle Sequencing kit [Applied Biosystems]).

LOH analysis

Matched constitutional and tumour DNA samples were PCR amplified using microsatellite markers of the Généthon database D4S2974 and D4S1536 flanking the PHOX2B locus. D4S1536 is 4.1Mb centromeric to PHOX2B and D4S2974 is 95 Kb telomeric to PHOX2B. Fluorescent PCR products were electrophoresed and analysed on an automatic sequencer (ABI377, Applied Biosystems, Foster City, USA).

Cell culture

Cell lines were cultured at 37°C, 5% CO₂, in Dulbecco's modified essential medium (DMEM; Invitrogen/Gibco, NY) containing 10% foetal calf serum, 292 µg/ml L-glutamine, 1% 100X MEM (non-essential amino acids medium, Invitrogen/Gibco), and 0.5% penicillin solution. Primary human neural crest cells were cultured for 3 weeks in FGF2- and EGF-containing embryonic stem cell medium with modifications available upon request.

PHOX2B expression in NB cell lines and tumours

Total RNA was isolated from cell lines and tumours by use of RNeasy (Qiagen). PHOX2B expression was first obtained from microarray on Affymetrix HG-U133 Plus 2.0 arrays. Data from 55 neuroblastic tumours were normalised using the GCRMA method. Detailed methods will be detailed elsewhere (I. Janoueix-Lerosey and O. Delattre, manuscript in preparation). In addition, RT-PCR detection of PHOX2B mRNA was performed in order to validate these data in 13 NB cell lines and eight tumours (Fig. 1a). First strand cDNA synthesis was performed on 2 µg of total RNA in a volume of 20 µl by use of [RNA kit, Applied Biosystems] and oligo(dT) primers. The specific primers used for mRNA amplification were designed within exons 2 and 3, as follows: 5'-GAGGCGCGAGTCCAGGTGTGGTTC-3' (forward) and 5'-CGACAATAGCCTTGGGCCTACCCG-3' (reverse). Expression analysis was performed in a 25-µl PCR reaction containing 1 µl of cDNA, 1 µl dNTPs (2.5 mmol/l each), 0.5 µl of each specific primers (150 ng/µl), and 0.2 µl Taq polymerase (5 U/µl; Invitrogen). PCR conditions were standard with an annealing temperature of 69°C. PCR products were loaded on a 2% agarose gel and directly visualised under UV illumination.

Analysis of PHOX2B gene hypermethylation by bisulfite DNA sequencing

Genomic DNA was isolated from cell lines and primary tissues by standard procedures. Bisulfite treatment and DNA sequencing were performed as described.¹⁴ The primer sequences for PHOX2B are 5'-AAATGTAATTTATAAGATGTTT TTTTTTTG-3' (forward) and 5'-CACACTACTTAAAAATAATAA AAATTAAAT-3' (reverse). PCR conditions were standard with an annealing temperature of 58 °C. PCR products were loaded on a 2% agarose gel and directly visualised under UV illumination. PCR products were cloned in a TA cloning vector (Promega, Madison, WI) and ten individual clones were sequenced for each sample.

Treatment of NB cells with 5-Aza-dC

Cells were seeded, allowed to attach over a 24 h period, and treated for 72 h with the demethylating reagent 5-Aza-dC (Sigma) at a final concentration of 1 µM. After the treatment period the medium was removed and RNAs were extracted.

Results

Mutation and LOH at the PHOX2B locus in neuroblastoma

As previously reported, a heterozygous frameshift mutation was identified in the SK-N-SH cell line (721–740del20nt,3). No mutation, either somatic or germinal, of the PHOX2B coding sequence and promoter conserved region were identified in the other 12 NB cell lines and the 45 individuals with sporadic NB. A heterozygous missense mutation (R100L) was identified in patient NBAF5 originating from a family predisposed to NB over three generations. No second molecular event could be identified in tumoural DNA (Table 1). We detected three known synonymous base substitutions, 552C > T(S184S), 750G > A (A250A) and 870C > A (P290P). In one patient, the P290P variant was heterozygous in constitutional DNA and homozygous in tumoural DNA. LOH was confirmed in this patient and identified in four other tumours (5/46 tumours, 10.8 %) with fluorescent microsatellite markers.

PHOX2B expression in NB cell lines and tumours

PHOX2B expression levels were obtained from Affymetrix HG U133 Plus 2.0 arrays. High level of PHOX2B expression was found in 10/13 cell lines and 15/17 tumours whereas no expression could be detected in three cell lines and two tumours (Table 1). PHOX2B expression was examined by RT-PCR in 13 NB cell lines and eight tumours and two neural crest-derived tissues: adrenal gland medulla (MSR) and human trunk-level neural crest cells. These primary, non-transformed cells were derived from a human embryo at 28 days of development with a normal karyotype. Loss of expression was confirmed by the absence of PHOX2B mRNA transcripts in 3/13 cell lines (SK-N-SH, SK-N-BE and GIMEN) whereas PHOX2B cDNAs were visualised in all other cell lines and non-cancerous adrenal (MSR) and neural crest cells (NC) (Fig. 1a). PHOX2B was silenced in three tumours: one ganglioneuroma (NBAF21), one ganglioneuroblastoma (NBAF38) and one NB (NB10) (Fig. 1a).

Methylation and silencing of PHOX2B in NB cell lines and tumours

Promoter-associated CpG islands of PHOX2b were analysed by methyl sequencing in a panel of 13 cell lines, 18 tumours and two neural crest-derived tissues: adrenal gland medulla (MSR) and human trunk-level neural crest cells. All 12 CpG dinucleotides were 100% methylated (10/10 clones) in 2/13 cell lines (SK-N-BE and GIMEN) and in 2/18 tumours (NBAF21 and NB10 (Fig 1b)). As sequencing of the PCR products showed that cytosines outside the CpG sites were converted to thymine, an incomplete bisulfite conversion could be ruled out.

Restoration of PHOX2B expression in negative cell lines by 5-Aza-dC

5-Aza-dC, a methyltransferase inhibitor, was used to investigate whether PHOX2B expression could be restored cell lines for which PHOX2B was not expressed. PHOX2B mRNA expression levels, analysed by RT-PCR, were restored in SK-N-BE and GIMEN cell lines, while no re-expression was observed for SKN- SH.

Discussion

This study of a series of 13 cell lines and 46 neuroblastic tumours aimed to determine the role of PHOX2B in sporadic NB. PHOX2B, not known so far for being either a tumour-suppressor gene or a proto-oncogene, is the first predisposing gene identified in NB. Most mutations occur in familial and syndromic NB cases. A heterozygous germline mutation was found in about 20% of published pedigrees and reached 50% when HSCR is associated with NB either in the index case or relatives.^{2,5,7,4} However, germline PHOX2B mutations are rare in sporadic isolated NB (1/215 cases in the series reported by Van Limpt and 2/86 in the series reported by McConville).^{3,8} Somatic PHOX2B mutations have also been described in NB.³ In the series of 45 sporadic NB cases we report, no PHOX2B gene mutations were identified either in the coding sequence or the promoter region. LOH at the PHOX2B locus could be detected in 5/46 cases.

This ratio of about 10% has to be balanced with the known high rate of chromosomal rearrangement in NB. It is worth noting that LOH of the short arm of chromosome 4 is found in roughly 20% of NB, but PHOX2B is centromeric to the smallest region of overlap (SRO).^{15,16}

We subsequently asked the question of epigenetic events at the PHOX2B locus and identified clonal aberrant CpG island methylation of the promoter in 2/13 NB cell lines and 2/18 tumours (ganglioneuroma in one case and NB stage II in one case). The demethylating drug 5-Aza-dC reinduced PHOX2B expression in NB cell lines, suggesting that PHOX2B methylation correlates with gene silencing. Moreover, it is well established that DNA demethylating agents as 5-Aza-dC induce adrenergic differentiation in NB cell lines¹⁷ and have an antiproliferative effect in mouse NB models.¹⁸ Hypermethylation and downregulation of potential tumour-suppressor genes such as genes involved in cell-cycle control or apoptosis is often associated with a poor outcome in NB.^{9,19,20}

We first considered PHOX2B as a potential tumour-suppressor gene since, i) at least some mutations identified in syndromic or familial NB cases (i.e. missense mutations of the homeodomain) are likely loss-of-function mutations, ii) a 'second hit' model has been

proposed in NB21 and, iii) some constitutional LOH at chromosome 4p encompass the PHOX2B locus.⁴ However, in patients with germinal PHOX2B mutation, neither a second mutation nor LOH or aberrant promoter hypermethylation in the tumours have been identified as reported earlier.²

PHOX2B has been shown to promote differentiation by controlling G1-S transition during cell cycle of sympathetic neuroblast precursors²² and is an essential regulator of normal autonomic nervous system development.²³ Mice with a homozygous inactivation of *Phox2b* fail in proper differentiation of the sympathetic nervous system. A gain-of-function or a dominant negative effect of PHOX2B frameshift mutations is not, however, ruled out. When tested, mutant transcripts were present and stable.² While mutant proteins localised to the nucleus, we observed some ability to bind DNA for two of the three PHOX2B frameshift mutations tested in vitro, although transactivation of the dopamine beta-hydroxylase promoter was always severely impaired.²⁴ Moreover, Bachetti and coworkers reported an increased transactivation of the PHOX2A promoter for proteins resulting from frameshift mutations when compared to the wild type protein.²⁵ PHOX2B is expressed not only in neural crest cells but also in mature sympathetic tissue of adrenal gland (Fig. 1a and 1). Methylation-associated repression of PHOX2B could result in a differentiation block of sympathetic neuroblasts.

Interestingly, NB10 patient had HSCR. We found aberrant homozygous PHOX2B promoter methylation on the tumour sample DNA while neither a mutation nor a deletion could be identified on either germinal or tumour DNA. Interestingly, PHOX2B was not methylated on lymphocyte DNA. We could speculate that PHOX2B methylation occurred during embryonic development and is responsible for both HSCR and NB development. As malignant transformation of cells could happen at different stages of tissue maturation, aberrant methylation may contribute to the diversity that characterises NB and other genes of the RET-PHOX2B pathway could be implicated. Interestingly, aberrations in the p53/MDM2/p14 (ARF) pathway have recently been described in the two NB cell lines where PHOX2B was found methylated²⁶; a p53 mutation in SKNBE(2)C and aberrant p14 (ARF) methylation in GIMEN.

Promoter methylation is not only implicated in silencing tumour-suppressor genes but also in regulation of developmental pathways during embryogenesis.²⁷ Abnormal methylation in NB could result from abnormalities in this process in a self-renewing multipotent stem cell becoming the malignant progenitor of this neural crest cancer. Within a single tumour, cell phenotypes are characteristic of embryonic structures, particularly neuroblasts, Schwann cells and melanocytes. Cellular heterogeneity and maturation stage correlate with clinical stage and prognosis of the disease.

However, down-regulation of PHOX2B was not always associated with hypermethylation. In SK-N-SH, the mutation is heterozygous and no PHOX2B expression could be detected. This result is discordant with the one reported by van Limpt and coworkers who observed the expression of both wild-type and mutant cDNAs in this cell line. However, they observed a PHOX2B gene silencing in the SHEP cell line, a stable subclone of SK-N-SH and also carrying the frameshift mutation. One could speculate on aberrant hypermethylation outside of the promoter region studied. However, it is worth noting that no expression of H-ASH1 could be detected either in the SK-N-SH cell line or in NBAF38 tumour (data not shown). This could also contribute to PHOX2B down-regulation. Finally, one can speculate on the effects of other mechanisms such as micro RNAs Fig. 2.

Genetic heterogeneity in predisposition to neuroblastic tumours is likely. The high recurrence of loss of heterozygosity for chromosomes 1p36 and 11q23 supported the existence of putative tumour-suppressor genes within these regions. However, these loci do not segregate with NB in most familial cases. On the other hand, linkage analysis has focused attention on 16p12-13 and 4p16.^{28,16} Several studies have clearly demonstrated that PHOX2B is a major predisposing gene in syndromic NB cases (i.e. associated with other autonomic dysfunction).^{6,3,29} Aberrant methylation of the PHOX2B promoter seems to be an alternative mechanism as frequent as mutation in sporadic NB cases and argues that loss-of-function by haploinsufficiency is the NB-predisposing mechanism.

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Footnotes:

This is an imperfectly formatted author manuscript. Please be indulgent about italics and reference formatting; some special characters converted poorly as well.

Conflict of interest statement None declared.

References:

1. Amiel J , Laudier B , Attie-Bitach T Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. *Nat Genet.* 2003; 33: (4) 459- 61
2. Trochet D , Bourdeaut F , Janoueix-Lerosey I Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. *Am J Hum Genet.* 2004; 74: (4) 761- 4
3. van Limpt V , Schramm A , van Lakeman A The *Phox2B* homeobox gene is mutated in sporadic neuroblastomas. *Oncogene.* 2004; 23: (57) 9280- 8
4. Perri P , Bachetti T , Longo L PHOX2B mutations and genetic predisposition to neuroblastoma. *Oncogene.* 2005; 24: (18) 3050- 3

- 5. Mosse YP , Laudenslager M , Khazi D Germline PHOX2B mutation in hereditary neuroblastoma. *Am J Hum Genet.* 2004; 75: (4) 727- 30
- 6. Trochet D , O'Brien LM , Gozal D PHOX2B genotype allows for prediction of tumor risk in congenital central hypoventilation syndrome. *Am J Hum Genet.* 2005; 76: (3) 421- 6
- 7. van Limpt V , Chan A , Schramm A Phox2B mutations and the Delta-Notch pathway in neuroblastoma. *Cancer Lett.* 2005; 228: (1-2) 59- 63
- 8. McConville C , Reid S , Baskcomb L PHOX2B analysis in non-syndromic neuroblastoma cases shows novel mutations and genotype-phenotype associations. *Am J Med Genet A.* 2006; 140: (12) 1297- 301
- 9. Teitz T , Wei T , Valentine MB Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nat Med.* 2000; 6: (5) 529- 35
- 10. Abe M , Ohira M , Kaneda A CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. *Cancer Res.* 2005; 65: (3) 828- 34
- 11. Banelli B , Di Vinci A , Gelvi I DNA methylation in neuroblastic tumors. *Cancer Lett.* 2005; 228: (1-2) 37- 41
- 12. Esteller M , Fraga MF , Guo M DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. *Hum Mol Genet.* 2001; 10: (26) 3001- 7
- 13. Ushijima T , Okochi-Takada E Aberrant methylations in cancer cells: where do they come from?. *Cancer Sci.* 2005; 96: (4) 206- 11
- 14. Herman JG , Graff JR , Myohanen S Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A.* 1996; 93: (18) 9821- 6
- 15. Caron H , van Sluis P , de Kraker J Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *N Engl J Med.* 1996; 334: (4) 225- 30
- 16. Perri P , Longo L , Cusano R Weak linkage at 4p16 to predisposition for human neuroblastoma. *Oncogene.* 2002; 21: (54) 8356- 60
- 17. Okuse K , Mizuno N , Matsuoka I Induction of cholinergic and adrenergic differentiation in N-18 cells by differentiation agents and DNA demethylating agents. *Brain Res.* 1993; 626 : (1-2) 225- 33
- 18. Bartolucci S , Estenez M , Longo A 5-Aza-2'-deoxycytidine as inducer of differentiation and growth inhibition in mouse neuroblastoma cells. *Cell Differ Dev.* 1989; 27: (1) 47- 55
- 19. van Noesel MM , van Bezouw S , Voute PA Clustering of hypermethylated genes in neuroblastoma. *Genes Chromosomes Cancer.* 2003; 38: (3) 226- 33
- 20. Yang Q , Zage P , Kagan D Association of epigenetic inactivation of RASSF1A with poor outcome in human neuroblastoma. *Clin Cancer Res.* 2004; 10: (24) 8493- 500
- 21. Knudson AG Jr , Meadows AT Developmental genetics of neuroblastoma. *J Natl Cancer Inst.* 1976; 57: (3) 675- 82
- 22. Dubreuil V , Hirsch MR , Pattyn A The Phox2b transcription factor coordinately regulates neuronal cell cycle exit and identity. *Development.* 2000; 127: (23) 5191- 201
- 23. Pattyn A , Morin X , Cremer H The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. *Nature.* 1999; 399: (6734) 366- 70
- 24. Trochet D , Hong SJ , Lim JK Molecular consequences of PHOX2B missense, frameshift and alanine expansion mutations leading to autonomic dysfunction. *Hum Mol Genet.* 2005; 14: (23) 3697- 708
- 25. Bachetti T , Borghini S , Ravazzolo R An in vitro approach to test the possible role of candidate factors in the transcriptional regulation of the RET proto-oncogene. *Gene Expr.* 2005; 12: (3) 137- 49
- 26. Carr J , Bell E , Pearson AD Increased frequency of aberrations in the p53/MDM2/p14(ARF) pathway in neuroblastoma cell lines established at relapse. *Cancer Res.* 2006; 66: (4) 2138- 45
- 27. Theise ND , Krause DS Toward a new paradigm of cell plasticity. *Leukemia.* 2002; 16: (4) 542- 8
- 28. Maris JM , Weiss MJ , Mosse Y Evidence for a hereditary neuroblastoma predisposition locus at chromosome 16p12-13. *Cancer Res.* 2002; 62: (22) 6651- 8
- 29. Maris JM The biologic basis for neuroblastoma heterogeneity and risk stratification. *Curr Opin Pediatr.* 2005; 17: (1) 7- 13

Fig. 1

(a) PHOX2B mRNA in NB cell lines. PHOX2B mRNA is detected in 10/13 cell lines as in human adrenal gland medulla (MSR) and non-transformed human neural crest cells (NC). No expression could be observed in three NB cell lines; SKNSH, SKNBE and GIMEN. (b) Bisulfite sequencing analysis of the methylation status of the 12 CpG from the start site. One microgram of tumour DNA was denatured by sodium hydroxide and modified by sodium bisulfite treatment, which converts unmethylated cytosines to uracil. Bisulfite treated DNA was amplified using specific primers (added in methods). PHOX2B promoter is 100% methylated in SKNBE and GIMEN but not in other cell lines. All the 12 CpG dinucleotide are methylated in two primary tumours NBAF21 and NB10. All ten clones showed identical methylation in each sample. • methylated cytosine ○ non-methylated cytosine.

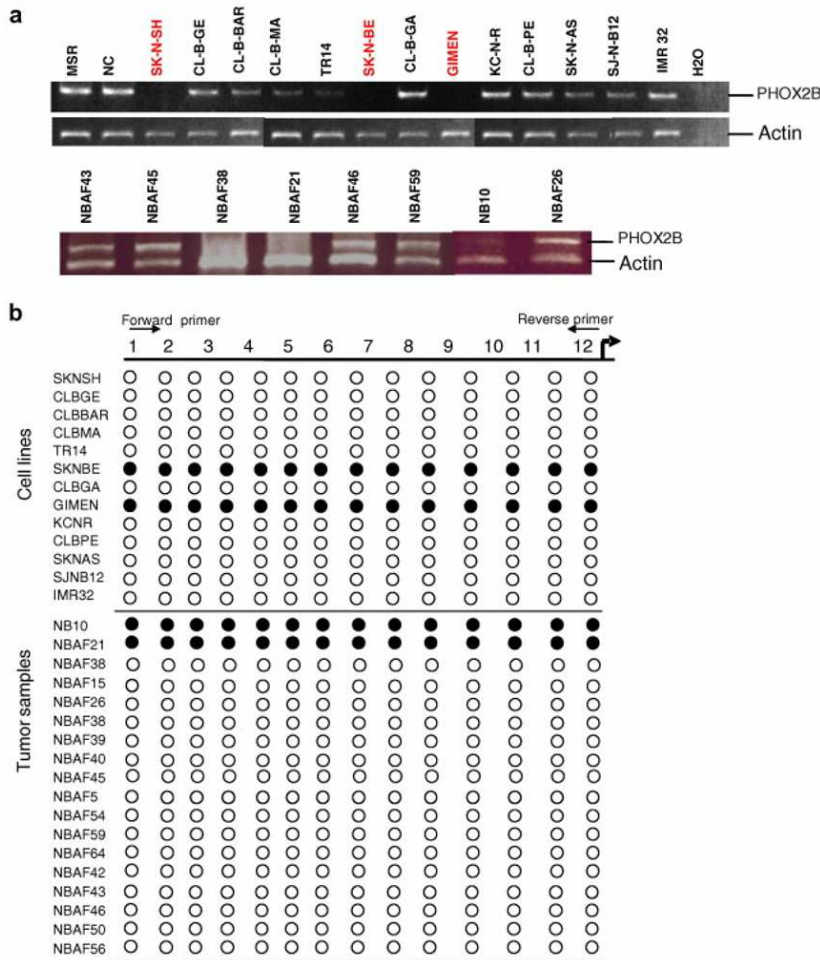


Fig. 2

(a) RT-PCR: Demethylation of the PHOX2B promoter CpG islands restores expression in PHOX2B-negative NB cell lines, SKNBE and GIMEN. SKNBE, GIMEN and SKNSH were treated (+) or not treated (-) with 1 μmol/l 5'-aza-2'-deoxycytidine for three days. (b) Methyl sequencing results of PHOX2B promoter before (1) and after treatment (2) by 5-azacytidine.

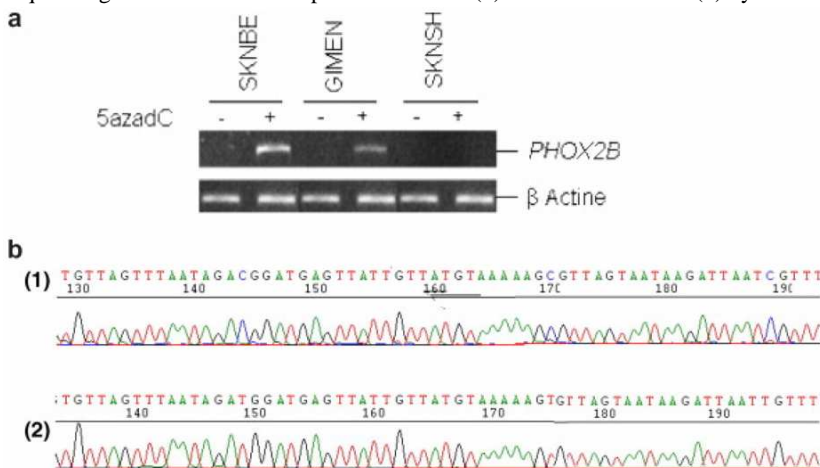


Table 1

Presentation of a series of 13 NB cell lines and 18 tumours for which PHOX2B expression data were analysed on Affymetrix HG U133 Plus 2.0 arrays (Affy Exp) and/or RT-PCR. Mutations, LOH and methylation analyses are presented as well as prognostic factors (stage, loss of 1p36, N-MYC amplification; A, Amplified; NA, Non Amplified)

		PHOX2B								
		Affy Exp	RT-PCR	Mutation	LOH	Methylation	Stage INSS	Loss of 1p36	N-MYC	
Cell lines	CLB-BAR	3679	+	-	-	-	4	+	A	
	CLB-GA	6956	+	-	-	-	4	+	NA	
	CLB-GE	5725	+	-	-	-	4	+	A	
	CLB-MA	7820	+	-	-	-	4	+	A	
	CLB-PE	387	+	-	-	-	2	-	A	
	GIMEN	6	-	-	-	+	4	+	NA	
	IMR32	8839	+	-	-	-	2	+	A	
	KCNR	5181	+	-	-	-	4	+	A	
	SJNB12	11010	+	-	-	-	3A	+	NA	
	SKNAS	2169	+	-	-	-	4	+	NA	
	SKNSH	23	-	721-740del20nt	-	-	4	-	NA	
	SKNBE	6	-	-	-	+	4	+	A	
	TR14	2534	+	-	-	-	3	+	A	
	Tumours	NBAF15	6775	ND	-	-	-	2B	-	NA
NBAF21		136	-	-	-	+	3	-	NA	
NBAF26		8259	+	-	-	-	4S	-	NA	
NBAF38		23	-	-	+	-	2A	-	NA	
NBAF39		6293	ND	-	-	-	2B	-	NA	
NBAF40		7462	ND	-	+	-	4S	-	NA	
NBAF42		7557	ND	-	-	-	4	-	NA	
NBAF43		3415	+	-	-	-	2B	-	NA	
NBAF45		7089	+	-	-	-	1	-	NA	
NBAF46		1394	+	-	-	-	2A	-	NA	
NBAF5		1053	ND	299G > T(R100L)	-	-	1	-	NA	
NBAF50		7811	ND	-	-	-	2B	-	NA	
NBAF54		5166	ND	-	+	-	4S	-	NA	
NBAF56		12059	ND	-	-	-	4S	+	NA	
NBAF59	10706	+	-	+	-	2A	-	NA		
NBAF61	10684	ND	-	+	-	4S	-	NA		
NBAF64	5349	ND	-	-	-	4	+	NA		
NB10	ND	-	-	-	+	2B	-	NA		