**PHOX2A and PHOX2B genes are highly co-expressed in human neuroblastoma**

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**Abstract.** The detection of PHOX2B mutations in a small proportion of patients affected with either familial or sporadic neuroblastoma (NB), has arisen interest on the possible pathogenic role of this gene in the disease determination. In this light, we have carried out a quantitative expression analysis of PHOX2B and its paralogue PHOX2A on a panel of NB cell lines and NB tumour samples to identify a possible differential expression between NB cells and their normal counterpart (adrenal medulla cells). Our results revealed that both PHOX2A and PHOX2B are over-expressed in tumour samples and NB cell lines. Particularly, the expression levels of the two genes in NB cell lines show a highly significant correlation, suggesting their possible synergistic role or a coordinated expression regulation. Furthermore, PHOX2 gene over-expression in NB tumours and cell lines suggests these genes may be widely involved in NB development through either a direct mechanism of up-regulation or a failure in maintaining proper transcript levels after embryonic development.

**Introduction**

The sympathetic nervous system (SNS) is mainly composed of neurons that arise from neural crest cells, which are multipotent progenitors that migrate away from the neural tube giving rise to an impressive array of cell types. These include neurons of the sympathetic chain, neurons of glia and chromaffin cells of the adrenal medulla (AM).

Neuroblastoma (NB) is an embryonic neuroblastic tumour that develops from neural crest cells and usually occurs as a sporadic neoplasm, even if rare familial recurrence is reported (1,2). NB has also been observed in association with syndromes of neural crest (NC) growth, migration or differentiation such as Congenital Central Hypoventilation Syndrome (CCHS), Hirschsprung’s disease (HSCR) and Neurofibromatosis type 1 (NF1) (3-6), suggesting the existence of common NC-specific disease-related genes. Nevertheless, these genes have turned out unlinked to NB (7,8).

Heterozygous in frame duplications leading to polyalanine (poly-A) expansions of PHOX2B are the most frequent disease-causing mutations in CCHS, while frameshift, missense and nonsense mutations have been detected in a small subset of these patients (9). Noteworthy, about 5-10% of CCHS patients develop neural-crest derived tumours like NB, ganglioneuroblastoma and ganglioneuroma in association with frameshift and missense mutations, which are therefore regarded as predisposing to NB (6). Indeed, these PHOX2B mutations have been shown to recur in a few NB families mostly associated with other neural crest disorders like CCHS and HSCR, but not in others (10-13), supporting the hypothesis that genetic predisposition to NB is governed by more than one gene (14). Moreover, frameshift PHOX2B mutations were detected in 5 out of 237 sporadic NB samples and one out of 22 NB cell lines (15). In another study, no mutations were found in 86 sporadic NB tumour samples while one deletion was reported in 2 out of 30 NB cell lines (16). Altogether, these data confirm that PHOX2B mutations are rare not only in familial but also in sporadic NB. Very recently, a constitutional mutation of the gene was found in a patient affected with a multifocal NB and the second allele was lost in both tumours together with a specific 17q gain pattern, suggesting that loss of PHOX2B and 17q gain are early events in NB tumorigenesis (17). Interestingly, high expression of PHOX2B was reported in NB cell lines and in tumour samples whereas in a small proportion of NB
specimens aberrant methylation of PHOX2B promoter was described (16,18,19).

PHOX2A and PHOX2B are two paralogous homeo-domain transcription factors characterized by an identical homeo-domain (HD) and strictly expressed in neural crest derivatives committed to noradrenergic phenotype (20,21). Phox2b protein binds the tyrosine hydroxylase (TH) and the dopamine β hydroxylase (DBH) promoters controlling, therefore, the development of noradrenergic sympathetic neurons (22). During embryogenesis, Phox2a and Phox2b are mostly co-expressed in the same lineages but with a distinct timing (20) and, independently of each other, can induce transcription of DBH and TH in vitro (22).

The transcriptional activity of PHOX2B promoter in NB cells depends on its self-transactivation, a process that may allow progenitor cells to acquire independence from extrinsic signals (23). Consistent with this observation, a very recent functional study has shown that mutant PHOX2B fails to transactivate its consensus promoter sequence, suggesting that PHOX2B is critical for normal terminal differentiation of neuroblasts (16). Phox2b lies upstream of Phox2a in all the adrenergic centres whose formation depend on these two factors, with the exception of the locus coeruleus and the cranial sensory ganglia (20). Consistently, PHOX2B regulates the PHOX2A promoter in human neuroblastoma cell lines (24). PHOX2B is also required in developing ganglia for maintaining the expression of the Mammalian Achaete Scute Homologue-1 (MASH-1) (25), a bHLH transcription factor crucial for correct differentiation of enteric neurons and ganglia, by direct binding its promoter (26).

In studies on rats, PHOX2B and PHOX2A have been shown persistently expressed also after birth and during adulthood, with a significant decrease of both mRNAs only shown persistently expressed also after birth and during all the adrenergic centres whose formation depend on these (22). Phox2a and Phox2b are mostly co-expressed in the same lineages but with a distinct timing (20) and, independently of each other, can induce transcription of DBH and TH in vitro (22).

Mutation screening, PHOX2A and PHOX2B mutational screening was performed by DHPLC analysis and direct sequencing, respectively, as already described (30,31). cDNA of tumour sample NB44 (from a patient affected with NB associated with CHHS and HSCR) was amplified using the forward primer 5'-ACGGCGGCCTCAACGAAG3' lying in the second exon and the reverse primer 5'-ACCC GCTGCCCACTCG3' lying in the third exon. Isolation of the alleles was obtained by means of 'Topo TA-cloning' (Invitrogen).

Results and Discussion

To investigate the role of PHOX2B and its parologue PHOX2A in NB pathogenesis, we carried out quantitative (Q) gene expression analysis of both PHOX2A and PHOX2B in three NB families, 32 sporadic NB samples and 29 NB cell lines. To this end, we used real-time RT-PCR to compare PHOX2A and PHOX2B gene expression levels with the quantity detected in a normal tissue of common embryonic origin (AM: adrenal medulla). Chromaffin cells were obtained from a healthy subject (AM-B3, a 5-year old child) by laser micro-dissection of the biopsy specimen (32), to be used as calibrator (Fig. 1). The comparative Ct method (User Bulletin #2, 2001, www.appliedbiosystems.com) and the Q-gene method (29) were adopted for relative quantification of gene expression (Fig. 1). After normalizing the Ct values of each target gene with the reference house-keeping gene 18S rRNA values, we calibrated the normalized data with the AM-B3 sample. Twenty-two out of 29 (76%) NB cell lines revealed over-expression of PHOX2A and PHOX2B (Fig. 1). High expression levels of the PHOX2 genes were observed in 100% of MYCN amplified NB cell lines and also in 46% of MYCN single-copy NB not supporting a correlation between expression of these genes and MYCN amplification, which is mostly associated with a high proliferation rate and a more aggressive tumour behaviour.

Remarkably, the two genes show a very similar expression profile in each NB cell line analyzed (Fig. 1, panel A vs. panel B and vice-versa). To assess the degree of correlation between the expression level of PHOX2A and PHOX2B in NB cell lines we used the Pearson’s correlation coefficient (r), calculated using the ∆Ct values of each gene (the difference between the Cts of the target gene and Cts of the house-
keeping gene): the r coefficient resulted equal to 0.97 (p<10^{-4}), thus indicating a high correlation that suggests a possible synergistic role of PHOX2A and PHOX2B or a coordinated expression regulation.

Subsequently, we determined the expression levels of the two genes in NB tumour samples. As NB is a tumour characterized by a considerable histological heterogeneity, we chose tumour samples having a neuroblast cell content of at least 80% in order to minimize the contribution of stroma cells. Setting an arbitrary 2-fold cut-off, all NB tumours showed an over-expression of both PHOX2 genes, except for PHOX2A in sample NB19, with respect to the calibrator sample AM-B3 (Fig. 2).

Differently from NB cell lines, no statistically significant linear correlation between the amounts of the two PHOX2 transcripts could be highlighted in NB samples, although almost all tumour samples clearly show high expression levels of both genes. The high heterogeneity shown by tumour specimens in terms of PHOX2 genes expression profiles is consistent with a high variability of the genetic

Figure 1. Relative Q expression analysis of PHOX2A and PHOX2B in NB cell lines. Q-expression analysis of PHOX2 genes was performed in 29 NB cell lines. Bars represent the normalized PHOX2A and PHOX2B transcript levels from the Q-Gene analysis, employing sample AM-B3 (adrenal medulla from a healthy child donor) as calibrator. Samples in violet are from NB cell lines with amplification of MYCN oncogene; samples in light green are from NB cell lines with MYCN single copy. Standard deviations are indicated. UND: undetectable.
background and is likely to represent the effects of the PHOX2 genes de-regulation in NB. Considering the general over-expression of PHOX2A and PHOX2B in all tumours analyzed and their preferential expression in noradrenergic cell types (22), the variable, even low (≤20%), percentage of stroma cells present in the NB biopsies makes this quantification likely to be affected by an underestimate of the PHOX2 gene expression level.

During development, the expression of PHOX2B is triggered by bone morphogenetic proteins (BMP-2, -4, -7), but may become independent from these stimuli through the establishment of a positive feedback. Indeed, 65% of the activity of the PHOX2B promoter seems to depend on an auto-regulatory loop in which the transcription factor PHOX2B binds and transactivates its own promoter (23). The over-expression of PHOX2B we have found in NB may amplify this physiological feedback mechanism. Moreover, the observed concomitant over-expression of PHOX2A may be due to the direct binding of PHOX2B to PHOX2A promoter, already described (24).
**PHOX2A** and **PHOX2B** play a role in a complex regulatory network, although their cross-talk and interacting pathways are still to be clarified. Particularly, **PHOX2B** acts upstream of **PHOX2A** and activates the expression of **MASH1**, **dHAND**, and **GATA3**, which are supposed to induce a cell switch toward **DBH** expression on the basis of gain and loss of function studies (33). **DBH** can also be directly induced by **PHOX2A** and **PHOX2B** (34) as partially confirmed by ChIP (Chromatin Immuno Precipitation) assays (35). Moreover, both **PHOX2** genes are involved in the **DELTA-NOTCH** pathway for chromaffin lineage differentiation (18,36,37). In particular, **MSX1**, a homeobox gene involved in embryonic neural crest development, was recently demonstrated to strongly induce the **DELTA-NOTCH** pathway genes and to be down-regulated by **PHOX2B** (38). Hence, a de-regulation of the **PHOX2** genes expression might affect these pathways that are essential for the normal differentiation program of the SNS.

On the basis of the possible interchangeable mechanisms of action of **PHOX2A** and **PHOX2B** through binding the same target promoters, we decided to carry out a mutation screening of both genes on our familial and sporadic cases. Similarly to the negative results already obtained from **PHOX2B** analysis in NB families (12), no mutations in the **PHOX2A** coding sequence were identified. In addition, we also screened 16 matched samples of sporadic NB without finding **PHOX2A** and **PHOX2B** mutations, except for a syndromic patient presenting CCHS and HSCR in association with NB. In this patient carrying the heterozygous c.930 insG frameshift mutation of the **PHOX2B** gene (39) we demonstrated the expression of both wild-type and mutated allele in the tumour sample cDNA (Fig. 3, NB44). The

![Figure 3. Sequence analysis of the PHOX2B gene in tumour sample NB44. The upper box represents the nucleotide sequence of cDNA from the tumour sample of a patient affected with NB associated with CCHS and HSCR, demonstrating PHOX2B biallelic expression. Sequences of the single alleles, obtained after PCR cloning, are provided underneath.](image-url)
observed biallelic expression, also reported for different PHOX2B frameshift mutations (15), suggests a dominant role of this PHOX2B mutation in tumour development.

Although no mutation was observed for PHOX2A and only few were reported for PHOX2B in human NB, their over-expression in tumours and NB cell lines indicates that the PHOX2 genes may be involved in NB development through either a direct mechanism of up-regulation or a failure in maintaining proper transcript levels of these genes after embryonic development.

Although in vitro forced over-expression of either wild-type or mutant PHOX2B in SK-N-AS cell line decreased growth rate and proliferation and promoted apoptosis (16), the consequences of the endogenous concomitant PHOX2 gene over-expression as observed in biotic tumour samples from NB patients and in the majority of NB cell lines remains unclear. Additional functional studies need to be carried out to clarify the molecular details underlying the involvement of PHOX2 genes in the above-mentioned pathways and their impact on the tumour phenotype.

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