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

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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 noradrenegic phenotype (20,21). Phox2b protein binds the tyrosine hydroxylase (*TH*) and the dopamine ß 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 regulating the expression of pan-neuronal genes in autonomic ganglia. It has also been shown that *PHOX2B* regulates the expression of *TLX2*, a 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 in aged animals, indicating a role of *PHOX2* genes in the maintenance of noradrenergic phenotype during the entire lifespan (27), but the underlying molecular mechanisms are mostly unknown.

In the light of the large functional overlap between *PHOX2A* and *PHOX2B*, de-regulation of one of these 2 genes may contribute to promoting NB development. Therefore, we have studied the involvement of the *PHOX2* genes in NB pathogenesis by carrying out a quantitative expression analysis in a panel of NB samples from both familial and sporadic patients and NB cell lines, demonstrating an up-regulation of both genes in all tumour samples and most of the cell lines.

Materials and methods

RNA extraction and cDNA synthesis. Total RNA was isolated from NB cell lines and tumours using the RNeasy Mini Kit (Qiagen, Germany). Total RNA from micro-dissected AM chromaffin cells of a healthy donor was isolated with the PicoPure RNA Isolation Kit (ARCTURUS, USA). The concentration and quality control of all RNAs were assessed with the Agilent 2100 BioAnalyzer (Agilent, USA). The first strand cDNA was obtained by reverse transcription of 5 μg of total RNA (High Capacity cDNA Archive Kit by Applied Biosystems, USA). All procedures were carried out according to the manufacturers' instructions.

Quantitative PCR and statistical analysis. Q-expression analysis was carried out by duplex real-time RT-PCR in the ABI 7700 PCR system (Applied Biosystems) using FAM-labelled TaqMan Gene Expression Assays for each target gene (*PHOX2A* and *PHOX2B*) and a VIC-labelled assay for the *18S* r*RNA* gene as endogenous control (reference gene) according to the manufacturer's instructions. PCR amplification efficiencies and normalization with the reference gene were performed as previously described (28). For relative quantification of gene expression the comparative Ct method (www.appliedbiosystems.com; User Bulletin 2) and the Q-gene method (29) were both employed as described (28).

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 CCHS and HSCR) was amplified using the forward primer 5'-ACGGCGGCCTCAACGAGAAG-3' lying in the second exon and the reverse primer 5'-ACCC GCTCGCCCACTCG-3' 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 paralogue *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, AM-B3).

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* r*RNA* 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 ΔCt values of each gene (the difference between the Cts of the target gene and Cts of the house-

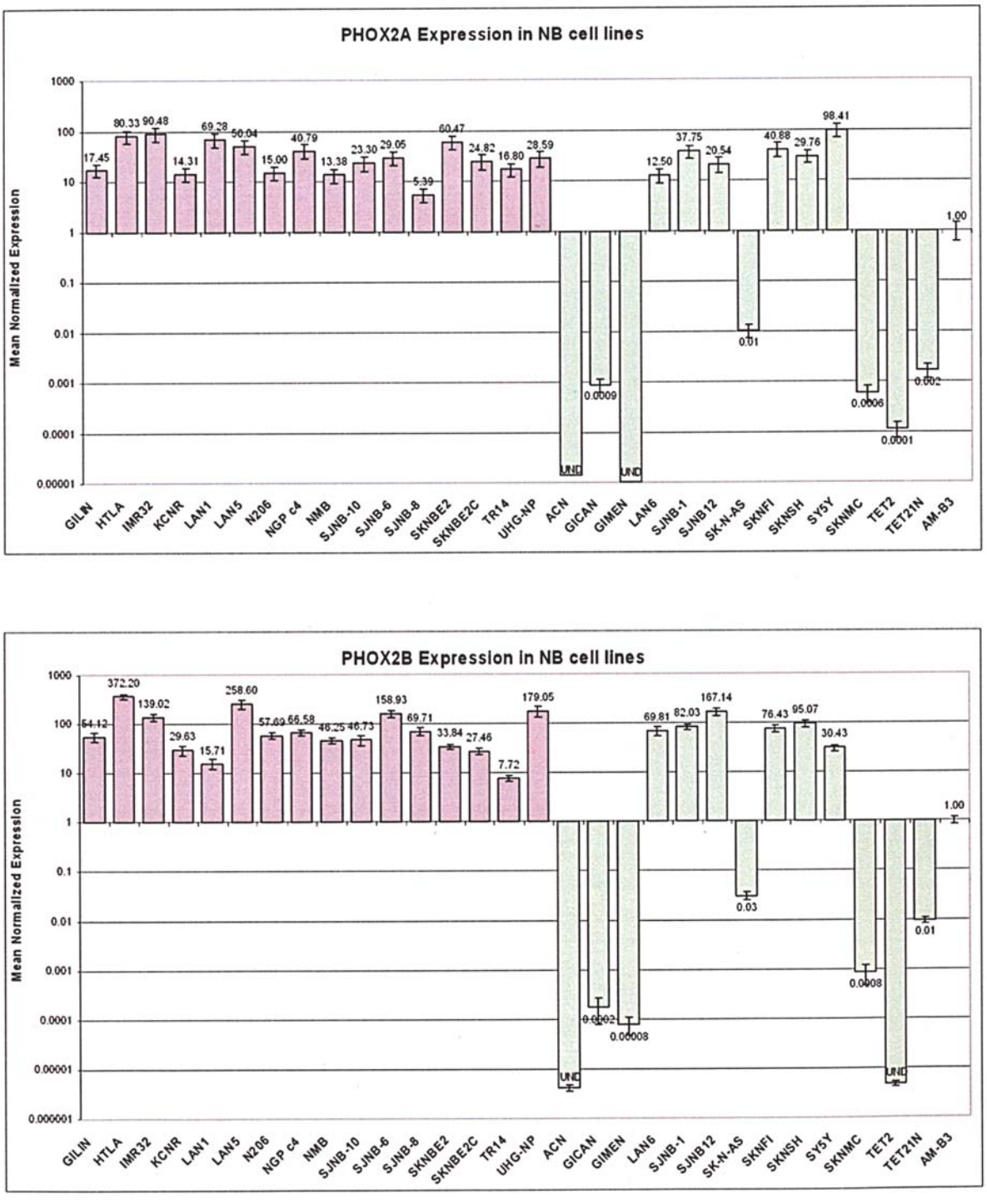

Figure 1. Relative Q expression analysis of *PHOX2A* and *PHOX2B* in NB cell lines. Q-expression analysis of *PHOX2* genes was performed in 29 NB cell lines. Bars represent the normalized *PHOX2A* and *PHOX2B* transcript levels from the Q-Gene analysis, employing sample AM-B3 (adrenal medulla from a 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.

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

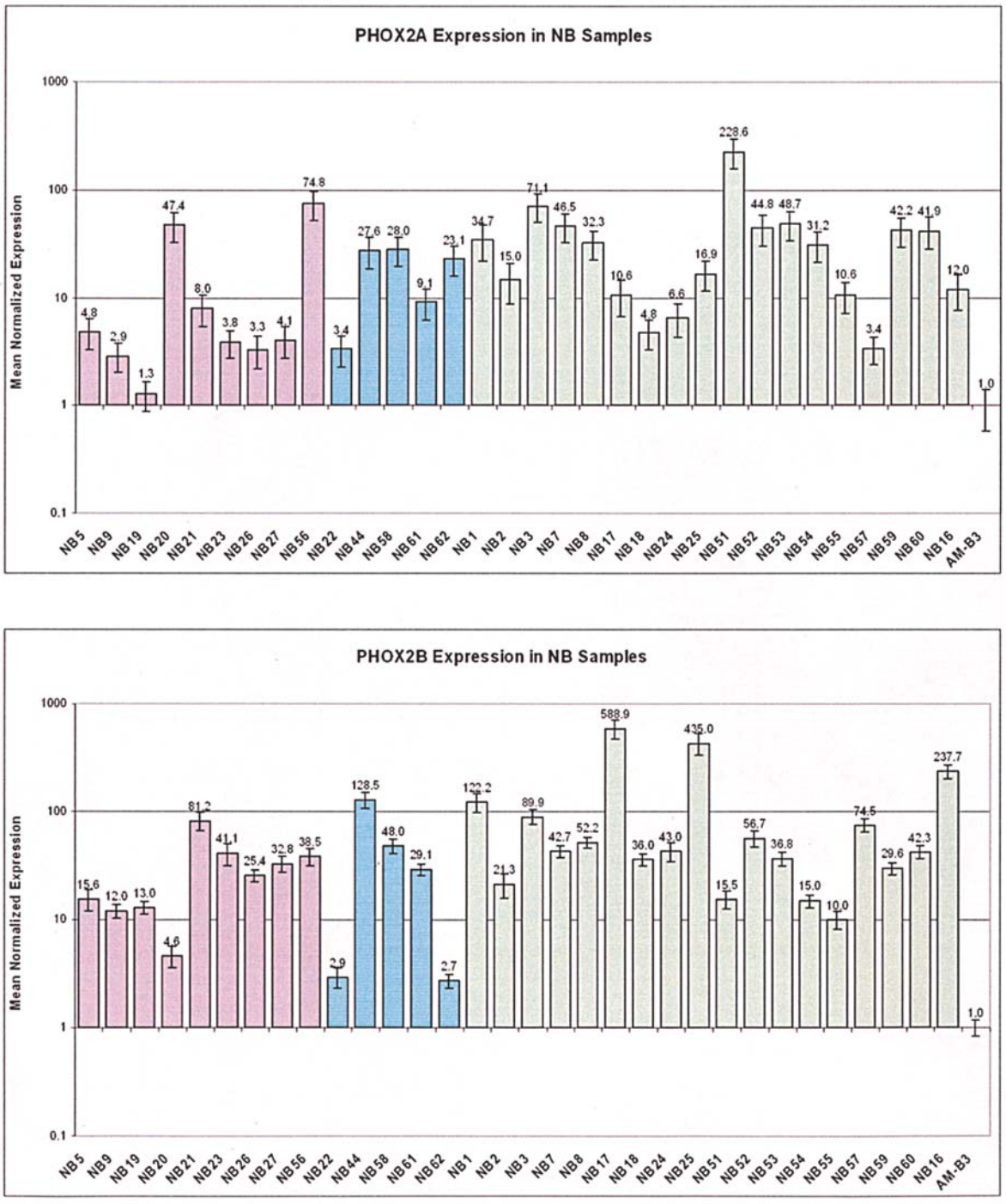

Figure 2. Relative Q expression analysis of *PHOX2A* and *PHOX2B* in NB tumours. Q-expression analysis of *PHOX2* genes was performed in 32 NB samples with an estimated percentage of neuroblasts above 80% in order to minimize the content of non-malignant cell. Tumour samples were from patients affected with either localized or disseminated disease. Sample AM-B3, adrenal medulla from a healthy child donor, was employed as calibrator. Samples in violet are from NB tumours with amplification of *MYCN* oncogene (*MYCN* ≥5 copies); samples in light blue are from tumours with *MYCN* gain (≥2 *MYCN* ≤4 copies); samples in light green are from tumours with *MYCN* single copy. 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).

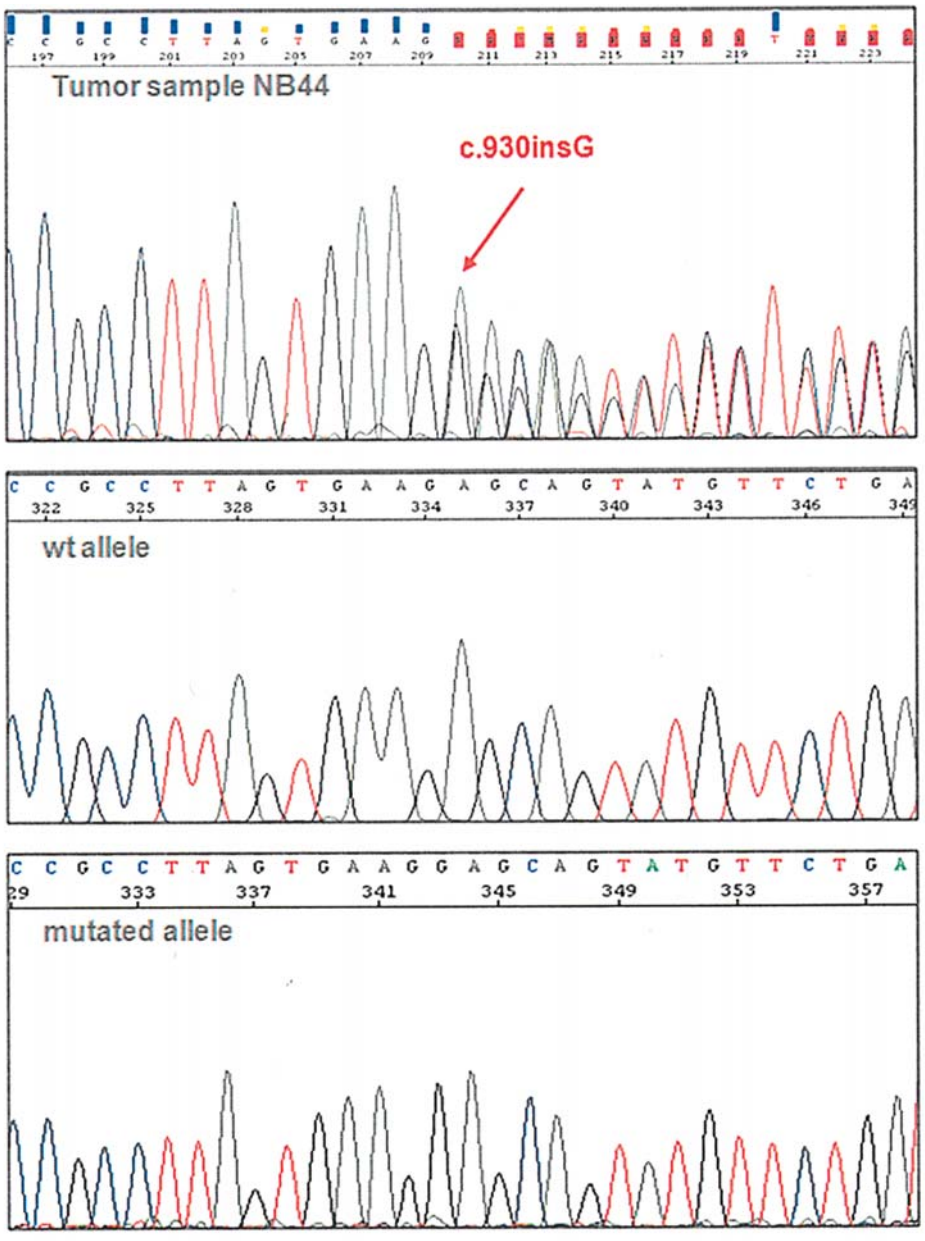

Figure 3. Sequence analysis of the *PHOX2B* gene in tumour sample NB44. The upper box represents the nucleotide sequence of cDNA from the tumour sample of a patient affected with NB associated with CCHS and HSCR, demonstrating *PHOX2B* biallelic expression. Sequences of the single alleles, obtained after PCR cloning, are provided underneath.

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

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 bioptic 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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