The Phox2 pathway is differentially expressed in neuroblastoma tumors, but no mutations were found in the candidate tumor suppressor gene *PHOX2A*

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Abstract. Neuroblastoma (NB), a tumor of the sympathetic nervous system, is the most common solid tumor in childhood. By microarray expression analysis (Affymetrix HU133A) important players in the noradrenalin biosynthesis pathway (*DBH*, *DDC*, *GATA2*, *GATA3*, *PHOX2A*, *PHOX2B*, *SLC6A2 SLC18A1* and *TH*) were found to be among the top ranked genes in showing lower expression in unfavorable NB tumor types as compared to favorable ones. By quantitative PCR with TaqMan, this result was significantly verified for all transcripts ($p<0.05$, one-tailed) in a new set of 11 primary NB tumors (5 favorable vs. 6 unfavorable). *PHOX2A*, a downstream target of Phox2b, was found to be the sixth ranked gene from the microarray gene list. Since the *PHOX2A* gene is localized in a tumor suppressor candidate region at 11q, we screened this gene for mutations by DNA sequencing in 47 tumors of different stages. However, no critical changes were found that could support its role in tumor development or progression. Overall, the findings in this study either suggest that expression of this pathway could be a predictive differentiation marker of NB tumors, or our results could also imply that the noradrenalin biosynthesis pathway is involved in tumor pathogenesis.

Introduction

Neuroblastoma (NB) develops from the primitive sympathetic neuronal precursors and the tumor cells probably develop during the embryonic period. Most NB's are undifferentiated tumors and consist of immature neuroblasts. Tumors showing partial differentiation are named ganglioneuroblastoma and the most differentiated state is ganglioneuroma. NB arise in the adrenal medulla (~50%), or elsewhere along the sympathetic ganglia in the chest, abdomen or in pelvis (1). Anatomic pathophysiology of NB is closely connected to the development of the sympathetic nervous system in infants, where the origin and migration of the neuroblasts during embryogenesis correlates to the sites of the primary tumors. The localization of the tumors seems to vary with age of onset, small children, i.e. those who are diagnosed before one year, frequently have tumors in thorax and pelvis, while older children develop tumors mainly in abdominal cavity.

NB shows a wide spectrum of biological behavior and there are various genetic features observed in the tumors. The most important showing a prognostic value are *MYCN* amplification and 1p-deletion (1,2). Other chromosomal abnormalities frequently observed in NB tumors are deletions of chromosomes 11q, 14q and 3p, and gain of 17q. Recent findings show that the prevalence of 11q deletions is similar to 1p deletions and associated with stage 4 disease (3). Guo and colleagues found 11q deletions at a frequency of 43% of tumor cases, which suggest it to be the most common deletion observed in NB tumors (4). Moreover, the 11q status has also been shown to enable the identification of patients with an increased risk for relapses (3). Deletion-mapping studies have defined a crucial region at 11q23.3-q24. However, this region was initially defined by only a few tumor specimens and most cases show large hemizygous deletions from 11q14 to 11qter (4-6). The different heterozygously deleted chromosomal regions in NB tumors (e.g. 1p, 11q, 3p) have long been suggested to harbour one or several tumor suppressor genes with mutations in the remaining allele. Several tumor suppressor candidates have been proposed during the years, but so far the *PHOX2B* gene located at 4p13 is the most trustworthy candidate with identified mutations, which are believed to play a role in NB pathogenesis (7-10). Phox2b is a homeobox transcription factor of the noradrenergic biosynthesis pathway, which controls parts of the neural developmental program as well as maintenance of noradrenergic cells (11,12). Phox2b is essential to induce expression of its homologue Phox2a (13,14), which is localized near the deletion breakpoint of several 11q-deleted NB tumor specimens (i.e. 11q13.4).

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Phox2a and Phox2b are coexpressed in adult noradrenergic cells, and they are able to induce transcription of each other (12,15) and to auto-regulate their own expression (16). Together, these two highly conserved proteins control expression of downstream targets important for the noradrenergic phenotype.

In the current study, we performed a global microarray expression analysis (Affymetrix HU133A) on six primary NB tumors from a Swedish patient group. From the microarray top-ranked genes, nine transcripts belonging to the noradrenergic biosynthesis pathway were selected for further verification by real-time PCR in an expanded tumor set (n=18). The 6th ranked gene was the paired-homeobox 2a gene (*PHOX2A*). Due to its near connection to Phox2b and its location on 11q, we screened *PHOX2A* for inactivating mutations to investigate its potential role as a tumor suppressor in NB.

Materials and methods

Experimental design. Tumors were divided into two biologically based groups by the following criteria: favorable (F); NB patient with no evidence of disease (NED) with a primary tumor staged 1-3 with no *MYCN*-amplification (MNA), no 1p deletion (1pdel), and no 11q deletion (11qdel). Unfavorable (UF); NB patient with advanced stage of disease or dead of disease (DOD) with a primary tumor staged 4, or with a primary tumor staged 3 with *MYCN*-amplification and/or 1pdel and/or 11qdel (Table I).

Patients. A set of 56 primary NB tumors of different stages and one Wilms tumor was used in the study, six tumors were used for the microarray study and 18 tumors were used for the quantitative PCR (QPCR) expression analysis, of which five were included in both the microarray and QPCR expression analysis (Table I). A set of 47 tumors were included for DNA sequencing, of which eight samples were used in both QPCR expression and sequencing analysis and one sample was used in both microarray and sequencing analyses. The NB cell line SK-N-AS was employed as a calibrator control for the expression analysis. Tumor samples were obtained fresh-frozen at surgery from Swedish patients with diagnosed NB. The patients were staged according to the International Neuroblastoma Staging System criteria (17). MNA and 1pdel status of the tumor samples have been investigated in previous studies (18,19) using FISH analysis and PCR-based DNA polymorphism. Moreover, copy number variations of MNA, 1pdel and 11qdel have also been investigated by Affymetrix 250K SNP arrays (20).

RNA preparation and microarray analysis. Snap-frozen tumor material (30 mg) from 19 samples were homogenized by TissueLyser (Qiagen, Hilden, Germany) and total RNA was extracted using Totally RNA (Ambion, St. Austin, TX), according to the suppliers' protocols. The total RNA purity and integrity were evaluated by spectrophotometric analysis using the ND-1000 (Saveen Werner AB, Malmö, Sweden) and the Bioanalyzer 2100 (Agilent, Palo Alto, CA), respectively. All RNA samples showed an A_{260}/A_{280} nm ratio in the range of 1.9-2.1, and RNA Integrity Numbers (RIN) >7.0. A global expression analysis was performed on six tumor samples; three favorable (14E6, 12E8 and 15E3) and three unfavorable (10R2, 15R3 and 16R4) NB tumors (Table I). The total RNA was labeled and hybridized to the Affymetrix oligo array U133A at the Swegene Microarray Resource Centre (MARC) in Lund. Swegene MARC provided expression raw data and expression evaluation. Normalization, clustering analysis and statistical analysis of expression data was done in collaboration with the Bioinformatics Core Facility in Gothenburg. Raw microarray data were processed, visualized and quality controlled using packages from Bioconductor. For all probe sets on the microarray, a two-side SE-corrected t-test on the set of six patients was performed to identify candidate genes with a significant differential expression pattern between NB patients with unfavorable vs. favorable outcome.

QPCR with TaqMan. Eighteen NB tumors, of which nine were classified as favorable and nine as unfavorable were used for real-time PCR expression analysis with TaqMan microfluid cards (MFC; Table I). The RNA samples were reverse transcribed using High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA). cDNA synthesis was performed in 20 μl reactions containing 1 μg RNA; 1X RT buffer; 4 mM dNTP mix; 1X RT random primers; 50 U reverse transcriptase and 20 U RNase inhibitor. Samples were reverse transcribed under following conditions: 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 sec.

Inventoried TaqMan assays for nine genes in the Phox2 pathway, six endogenous controls and 82 other genes of interest were selected, and added to the MFC's chart. Cards holding 96 preloaded assays were ordered from Applied Biosystems. Real-time PCR was prepared by filling each reservoir with a mixture (in total 100 μl) containing 1X TaqMan mastermix and 83 ng total RNA converted to cDNA. The 384-well cards were processed and run according to manufacturer's protocol using the ABI PRISM® 7900 HT Sequence Detection System (SDS v.2.2, Applied Biosystems). Amplification reactions were run in simplex. Thermal cycling was initiated with a 2 min incubation at 50˚C, followed by a denaturation step of 10 min at 94.5˚C and then by 40 cycles of 30 sec at 97˚C and 1 min at 59.7˚C.

QPCR - Quantification and normalization. Quantification was performed by the relative quantification method (ΔΔCt method). In summary, threshold and baseline were set manually in SDS and Ct values were extracted. All samples were normalized to the geometric mean of three endogenous genes: *ADA*, *GAPDH*, *GUSB*. To adjust for discrepancy between runs, all samples were compared to a calibrator sample (SK-N-AS), which was loaded on each card. Fold changes and statistical comparison between groups (UF vs. F) was calculated using $\log_2$ (Ct) expression values from each sample. The significance of differential expression was calculated using a one-tailed t-test. The correlation of expression between transcripts in the Phox2-pathway was calculated by Pearson's correlation test within each group. The relation of expression values detected by microarray analysis vs. QPCR analysis was also calculated by Pearson's correlation. In order to quality check the data, box plots for each gene was plotted using $\log_2$ expression values and they was exploited

Table I. Clinical data of tumors used in this study.

No.	Case	Stage	Age	Outcome	NMA	1pdel	11qdel	Analysis	Group in expression analysis
1	18E8	1	9	NED	Neg	Neg	Neg	*QPCR*	F
2	14E6	1	13	NED	Neg	Neg	Neg	*MA, PHOX2A seq*	F
3	35R5	1	14	NED	Neg	Neg	Na	*PHOX2A seq*	
4	35R8	1	14	NED	Neg	Neg	Neg	*QPCR*	F
5	16E1	1	18	NED	Neg	Neg	Neg	*PHOX2A seq*	
6	15E6	1	22	NED	Neg	Neg	Na	*PHOX2A seq*	
7	14R4	1	32	NED	Neg	Neg	Neg	*PHOX2A seq*	
8	31R8	1	45	NED	Neg	Neg	Neg	*QPCR*	F
9	20R9	2	3	NED	Na	Na	Na	*QPCR*	F
10	33R7	2	3	NED	Neg	Neg	Na	*QPCR*	F
11	25R8	2	6	NED	Neg	Neg	Neg	*PHOX2A seq*	
12	23R4	2	9	NED	Neg	Neg	neg	*QPCR, PHOX2A seq*	F
13	35R2	2	48	NED	Neg	Neg	Neg	*PHOX2A seq*	
14	10E4	2	2	DOD	Neg	Neg	Neg	*PHOX2A seq*	
15	12E1	2	76	DOD	Pos	Neg	Pos	*PHOX2A seq*	
16	25R0	3	1	NED	Neg	Neg	Na	*QPCR*	F
17	12R4	3	2	NED	Neg	Pos	Neg	*PHOX2A seq*	
18	34R1	3	6	NED	Neg	Neg	Na	*PHOX2A seq*	
19	12E8	3	7	NED	Neg	Neg	Neg	*MA, QPCR*	F
20	15R8	3	9	NED	Neg	(Pos)	Na	*PHOX2A seq*	
21	15E3	3	12	NED	Gain	UB	Neg	*MA, QPCR, PHOX2A seq*	F
22	23R2	3	29	NED	Pos	Pos	Na	*PHOX2A seq*	
23	16R4	3	30	NED	Pos	Neg	Neg	*MA, QPCR, PHOX2A seq*	UF
24	23R0	3	43	NED	Neg	Neg	Na	*PHOX2A seq*	
25	19R6	3	8	DOD	Pos	Pos	Neg	*QPCR, PHOX2A seq*	UF
26	9R9	3	24	DOD	Neg	Pos	Pos	*QPCR, PHOX2A seq*	UF
27	13R1	3	37	DOD	Pos	Pos	Neg	*PHOX2A seq*	
28	13E6	3	48	DOD	Pos	Pos	Pos	*PHOX2A seq*	
29	10R8	3	83	DOD	Neg	(Pos)	Pos	*PHOX2A seq*	
30	10E6	4	11	NED	Pos	Pos	Neg	*PHOX2A seq*	
31	29R2	4	15	NED	Pos	Pos	Na	*QPCR*	UF
32	26R0	4	36	NED	Pos	UB	Pos	*PHOX2A seq*	
33	17R3	4	44	NED	Neg	Neg	Na	*PHOX2A seq*	
34	16E3	4	10	DOD	Pos	Pos	Neg	*PHOX2A seq*	
35	36R3	4	10	DOD	Neg	UB	Neg	*PHOX2A seq*	
36	10R2	4	15	DOD	Na	(Pos)	Neg	*MA, QPCR, PHOX2A seq*	UF
37	26R8	4	21	DOD	Pos	Pos	Na	*QPCR*	UF
38	13R0	4	27	DOD	Pos	Pos	Neg	*QPCR, PHOX2A seq*	UF
39	15R3	4	28	DOD	Neg	(Pos)	Na	*MA, QPCR*	UF
40	11R9	4	37	DOD	Neg	Neg	Pos	*PHOX2A seq*	
41	11E2	4	41	DOD	Neg	Neg	Neg	*PHOX2A seq*	
42	12R6	4	50	DOD	Pos	Pos	Neg	*PHOX2A seq*	
43	19R0	4	52	DOD	Neg	(Pos)	Na	*PHOX2A seq*	
44	34R9	4	53	DOD	Neg	Neg	Pos	*PHOX2A seq*	
45	3E2	4	58	DOD	Neg	Neg	Pos	*PHOX2A seq*	
46	17R2	4	80	DOD	Neg	Neg	Na	*PHOX2A seq*	
47	12E6	4	137	DOD	Pos	Neg	Na	*QPCR, PHOX2A seq*	UF
48	25R3	4M	10	NED	Neg	Neg	Neg	*PHOX2A seq*	
49	16E2	4S	0	NED	Neg	Neg	Neg	*PHOX2A seq*	

Table I. Continued.

No.	Case	Stage	Age	Outcome	NMA	1pdel	11qdel	Analysis	Group in expression analysis
50	12E5	4S	1	NED	Neg	Neg	Neg	*PHOX2A seq*	
51	13E7	4S	2	NED	Na	Na	Na	*PHOX2A seq*	
52	13R3	4S	3	NED	Neg	Neg	Na	*PHOX2A seq*	
53	10R4	4S	6	NED	Neg	Neg	Na	*PHOX2A seq*	
54	21R6	4S	10	NED	Neg	Neg	Na	*PHOX2A seq*	
55	12R7	4S	25	NED	Neg	Neg	Na	*PHOX2A seq*	
56	11E5	4S	0	DOD	Neg	Neg	Neg	*PHOX2A seq*	
57	19R3	Wilms	0	Na	Neg	Pos	Na	*PHOX2A seq*	

Stage, clinical stage of NB; Wilms, Wilms tumor; Age, months at diagnosis; NED, no evidence of disease; DOD, dead of disease; Na, not available; 1p-del, 1p-deletion; Pos, positive; Neg, negative; UB, unbalanced; gain, *MYCN* <4 times the ploidy; (Pos), Ambigous results; MA, micro array; QPCR, quanatitative PCR and PHOX2A seq, sequencing analysis of *PHOX2A*.

to identify outliers that could disturb the analysis and interpretation of the data.

DNA sequencing - sample preparation. DNA was enriched from 47 fresh-frozen tumor samples by the DNeasy kit from Qiagen (Table I). Throughout the PCR and sequencing steps, CEPH standard DNA (CEPH 1347-02, Applied Biosystems) was used as a positive control and water as a negative control.

DNA sequencing - primer design and PCR amplification. The genomic DNA sequence and the mRNA sequence (Accession number: NM_005169) of the *PHOX2A* gene were obtained from UCSC Genome Bioinformatics webpage (http://genome.ucsc.edu; Fig. 1). The primers, covering the promoter region (4 fragments) and exon 2, were designed using ExonPrimer (http://ihg.gsf.de/ihg/ExonPrimer.html), or PrimerExpress (Applied Biosystems). Primers covering exon 1 and exon 3 were adapted from Nakano *et al* (21). Primer sequences are available on request. The PCR reactions were set up using the automated workstation Biomek® FX (Beckman Coulter, www.beckmancoulter.com) in 384-well reaction plates. Touch Down-PCR (TD-PCR) was carried out in 10 μl reactions containing 1X PCR buffer; 20 mM dNTP mix, 0.25 U Hot Star TaqPlus DNA polymerase (Qiagen), 5-10 μM primer (forward and reverse) and 25-50 ng genomic DNA. GC-rich fragments were run with 1X Q-solution (Qiagen) in the master mix. PCR amplifications for exon 1 and exon 3 were run according to Nakano *et al* (21) and thermal profiles for the other five fragments are available on request. TD-PCR products were cleansed using magnetic beads (AMPure, Agencourt, Bioscience Corporation, Beverly, MA) in the automated workstation Biomek NX (Beckman Coulter) and eluted in dH_2O.

DNA sequencing - sequence PCR and data analysis. Sequence PCR, using BigDye® Terminator v 3.1 Cycle Sequence kit (Applied Biosystems), was carried out in 10 μl reactions containing 6 μl 1:3 diluted template (TD-PCR product), 0.25-2 μl BDT, 1X BDT buffer; and 1.6 μM primer (forward or reverse) and run according to standard procedures. The

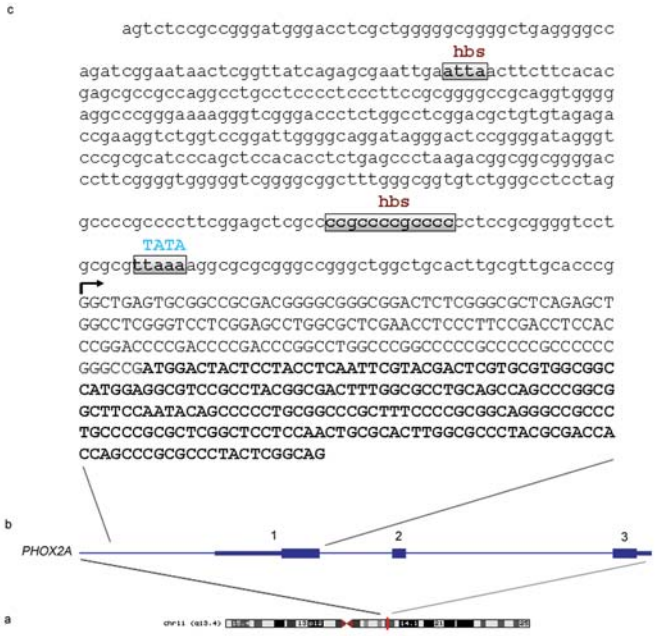

Figure 1. Schematic representation of the *PHOX2A* gene. (a) *PHOX2A* is located at chromosome band 11q13.4. (b) The gene contains three exons; exon 1 [389 base pairs (bp)], exon 2 (188 bp) and exon3 (1121 bp). (c) Regulatory sites in the promoter region of *PHOX2A* are the Homeobox Binding Sites (hbs) marked in red, TATA-box (blue) and the transcription start site marked by an arrow. The translated sequence is shown in bold.

thermal profile for GC-rich fragments (exon 1 and 3) was modified to an initial denaturation step at 94˚C for 3 min, followed by 50 cycles of 96˚C for 30 sec, 50˚C for 10 sec and 60˚C for 3 min. Sequence-PCR was performed using the same primers as in the previous TD-PCR amplifications, except for two fragments (one in the promoter and one in exon 2) in which special sequencing primers were used. The sequence-PCR products were cleansed using magnetic beads (CleanSeq, Agencourt) in the automated workstation Biomek NX (Beckman Coulter) and eluted in High Dye Formamide. The sequence reaction products were loaded on a 3730 DNA Analyzer (Applied Biosystems) and the results were analysed

Table II. Transcripts analyzed by QPCR with TaqMan.

Gene	Description	Location	Accession no.	Assay no.	Microarray		QPCR			
							Ver (6F + 6UF)		Total (8F + 9UF)	
					Rank	Fold change	Fold change	P-value	Fold change	P-value
DBH	GOI	9q34	NM_000787	Hs01089840_m1	18	10.8	15.0	0.004	11.8	0.0002
DDC	GOI	7p11	NM_000790	Hs00168031_m1	16	3.4	23.8	0.016	15.9	0.0024
GATA2	GOI	3q21.3	NM_032638	Hs00231119_m1	19	8.1	4.2	0.026	6.0	0.0014
GATA3	GOI	10p15	NM_001002295	Hs00231122_m1	50	5.8	6.2	0.002	5.7	0.0004
PHOX2A	GOI	11q13.4	NM_005169	Hs00605931_mH	6	4.2	26.3	0.022	11.0	0.0149
PHOX2B	GOI	4p12	NM_003924	Hs00243679_m1	178	3.9	17.6	0.004	8.5	0.0047
SLC18A1	GOI	8p21.3	NM_003053	Hs00161839_m1	3	6.8	25.0	0.013	24.1	0.0010
SLC6A2	GOI	16q12.2	NM_001043	Hs00426573_m1	7	4.1	540.3	0.001	201.1	0.0002
TH	GOI	11p15.5	NM_199292	Hs00165941_m1	31	4.9	10.6	0.013	7.6	0.0023
GUSB	End. control	7q11.21	NM_000181	Hs99999908_m1						
ADA	End. control	20q13.12	NM_000022	Hs00163553_m1						
GAPDH	End. control	12p13.31	NM_002046	Hs02786624_g1						

GOI, Gene of interest; End. control, endogenous control; location, chromosomal location; Assay no, TaqMan assay number according to Applied Biosystems (www@appliedbiosystems.com); rank, rank in gene-list sorted by statistical significance; Fold change, expression fold between biological groups (UF vs. F); P-value, one-tailed t-test assuming unequal variance; Ver (Verification), quantitative PCR results from the new tumor set (6 F vs. 6 UF) not used in the micro array study; total, quantitative PCR results from all tumor samples (8 F vs. 9 UF).

using the software programs Sequencing Analysis v. 5.2 and SeqScape v.2.5 (Applied Biosystems).

Results

Microarray analysis. The global microarray expression analysis (Affymetrix HU133A) containing ~33,000 genes, was performed on three favorable and three unfavorable NB tumors. Genes showing lower expression in unfavorable compared to favorable tumors with a fold change over two were ranked according to statistical significance (Table II), resulting in a list of 1084 probe-sets. Out of the 50 top-ranked genes found to show lower expression in unfavorable tumors, eight genes belonged to the noradrenergic biosynthesis pathway. The 178th ranked transcript was the paired-like homeobox 2b gene (*PHOX2B*), which has been found to be mutated in a few hereditary and sporadic NB cases (7-10).

QPCR verification. Nine genes belonging to the noradrenergic biosynthesis pathway were selected for further verification by real-time RT-PCR; *PHOX2A*, *PHOX2B*, *GATA2*, *GATA3*, *DDC*, *DBH*, *SLC6A2*, *SLC18A1* and *TH*. Six endogenous controls showing uniform expression on the microarray were also selected. Out of these, two transcripts (PEX7 and GK) showed expression values beneath the detection limit and they were therefore excluded from the analysis. In order to check for the stability of the remaining four endogenous controls, M-values were calculated according to Vandesompele and colleagues (22). The control gene 18S which showed the highest M-value (M=2,59) was not uniformly expressed within the sample group and it was therefore excluded from the analysis (data not shown). Thus, the relative expression of the genes were quantified and normalized to the geometric mean of *ADA*, *GAPDH* and *GUSB*. The distribution of expression values ($\log_2$ values) for each transcript was visualized using box plots and identified case 25R0 (belonging to the favorable group) as an outlier for all transcripts (data not shown). This sample was therefore removed from the study, resulting in an analysis set of eight favorable versus nine unfavorable tumors (n=17).

All nine transcripts showed significant lower expression ($p<0.05$) in unfavorable vs. favorable NB tumors, both in the verification group and in the total analysis (Table II; Fig. 2). Gene expression values of all transcripts also correlated significantly within groups, except for *GATA2* and *GATA3* which only partially correlated to the others (data not shown). The correlation between microarray data and QPCR data was significant (Pearson's correlation = 0.6; Fig. 3).

PHOX2A mutation analysis. From the microarray gene list the 6th ranked gene was found to be the paired-like homeobox 2a (*PHOX2A*). A mutation screening of all coding regions as well as the regulatory sites of the *PHOX2A* gene was performed by PCR-based sequencing analysis (Fig. 1). All sequence fragments of *PHOX2A* were quality checked and aligned to the reference sequence (Accession number: NM_005169 and 365 bp promoter sequence derived from the UCSC genome browser). Sequences were run both in forward and reverse direction and covered 100% of the coding regions in 90% of the samples. The remaining 10% showed an 80-99% coverage. In the promoter and the 5' UTR region there were coverage of 100% of the sequences in 83% of the samples

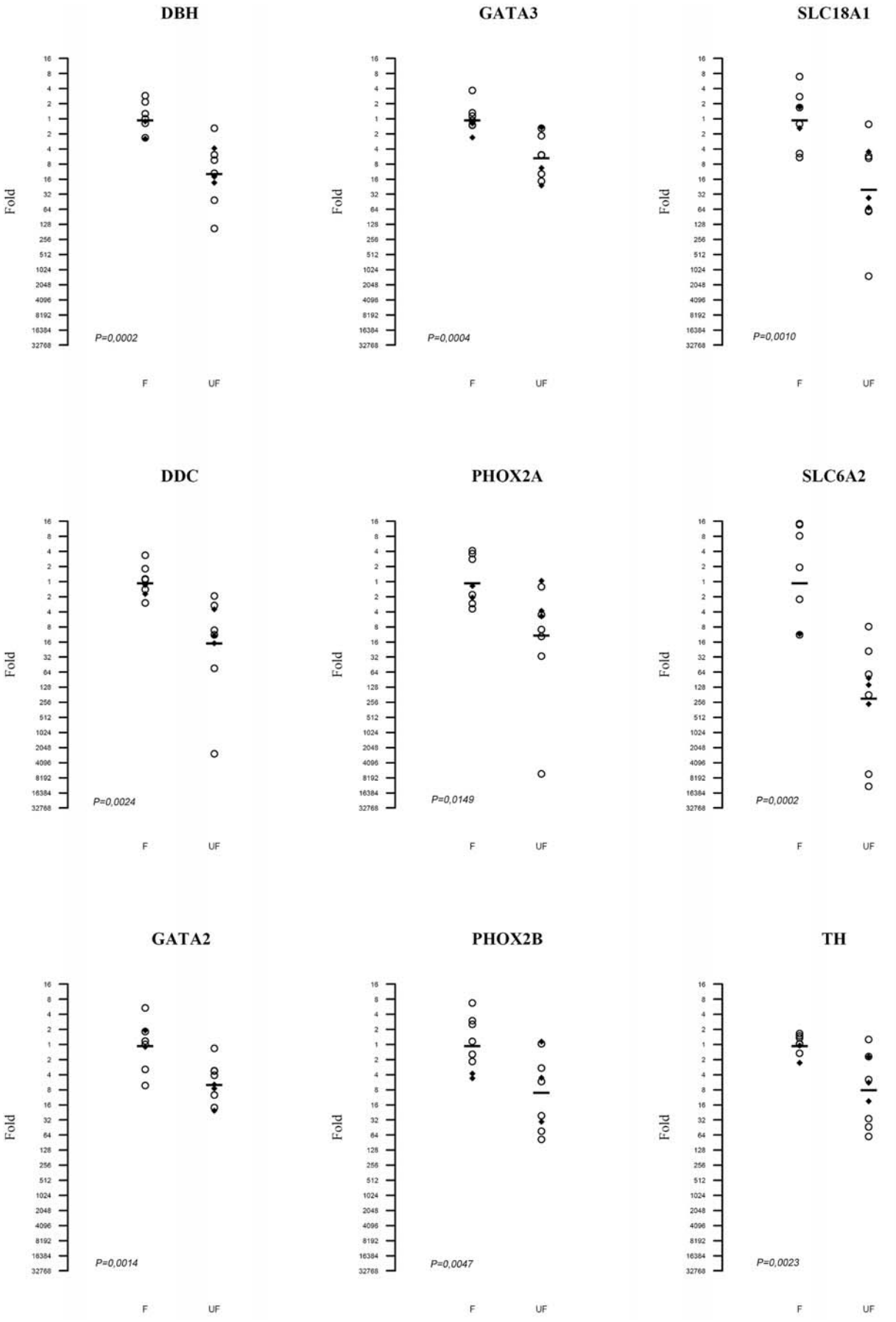

Figure 2. Scatter plots of the real-time PCR data from nine genes in the noradrenergic biosynthesis pathway. Expression values are presented as fold values from the mean of the favorable group (F). Expression values were normalized to the mean of three endogenous controls; ADA, GAPDH and GUSB. Filled square, cases used in the microarray analysis as well as in the real-time PCR study; open circle, cases used only in the real-time PCR analysis. Group: F, favourable tumor types; UF, unfavourable tumor types. P-values are calculated on the total data set (see Table II) by a two sample t-test on $\log_2$ (i.e. Ct) expression values, assuming unequal variance.

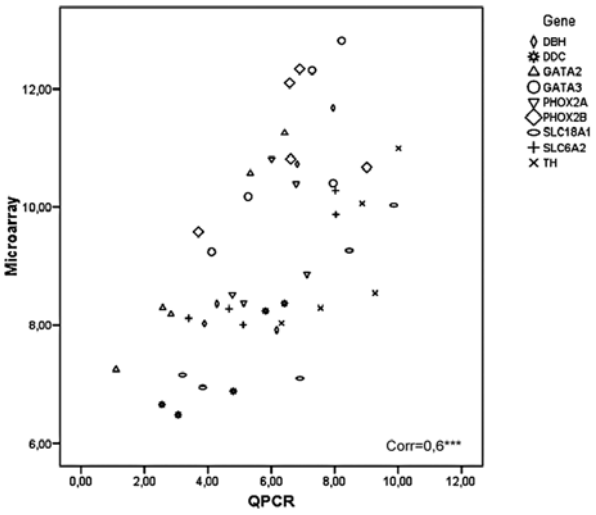

Figure 3. Correlation scatter plot between microarray and QPCR $\log_2$ expression values. Pearson's correlation = 0.6.

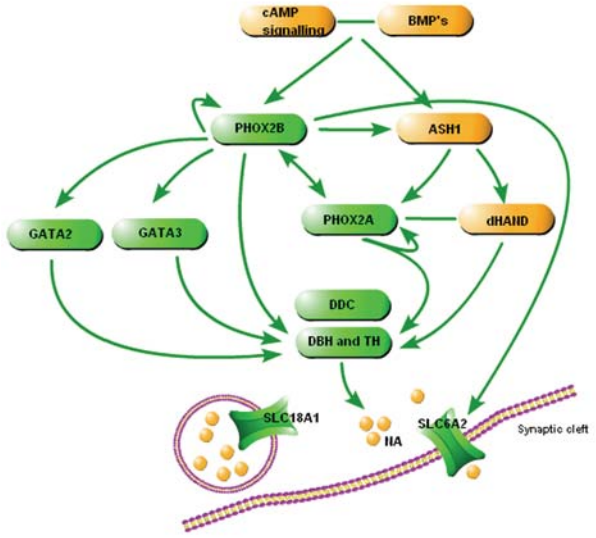

Figure 4. A simplified schematic representation of the noradrenalin biosynthesis pathway. Bone morphogenetic proteins (BMP's) induce expression of paired-like homeobox 2b (Phox2b) and ASCL1. cAMP signalling works synergistically with BMP's in promoting expression of these genes. ASCL1 and Phox2b are needed to induce expression of Phox2a. The two, highly conserved Phox2-proteins are involved in the expression of dopamine ß hydroxylase (DBH) and tyrosine hydroxylase (TH), which together with dopamine decarboxylase (DDC) are involved in the final catalysis of dopamine to noradrenaline (NA). SLC6A2 participates in the re-uptake of neurotransmitter from and to the synaptic cleft, and SLC18A1 participates in the transport of monoamines across the vesicle membranes. Genes marked in green showed significantly lower expression in unfavorable tumor types in the present study.

and a 62-98% coverage in the remaining 17%. However, all important regulatory sites [two homeobox binding sites (hbs) and TATA-box, see Fig. 1] were covered in all samples in both directions. The sequencing of the *PHOX2A* gene revealed only one silent base-pair substitution in exon 1 (156 C>T, Leu52Leu), which was found in two cases (15E6, 25S8) and in the CEPH control.

Discussion

NB is one of the most frequent types of cancers among young children and the most common among infants. The tumor originates from an immature cell in the sympathetic ganglia, a branch of the autonomic peripheral nervous system characterized by the production of the neurotransmitter noradrenalin. Early actors in the noradrenalin biosynthesis pathway are bone morphogenetic proteins (BMPs), produced by the developing aorta (Fig. 4). One of the many important functions of these proteins is to induce expression of the basic Helix-loop-helix transcription factor achaete-scute complex-like 1 (ASCL1), which is essential for development of peripheral sympathetic and parasympathetic neurons (23,24). BMPs also induce expression of Phox2b, a homeobox transcription factor shown to be mutated in primary NB tumors (7-10). Although independently expressed, in sympathetic ganglia it seems like Phox2b is necessary for maintained expression of ASCL1 (12,15). Recent findings suggest that cAMP signaling works synergistically with BMPs in promoting expression of these genes (25) (Fig. 4). ASCL1 and Phox2b are essential to induce expression of the Phox2b homologue Phox2a (13,14). Both Phox2a and Phox2b are involved in the expression of dopamine ß hydroxylase (DBH), which catalyze the conversion of dopamine to noradrenalin and tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of noradrenalin (26). The catecholeamine synthesis is also dependent on the dopa decarboxylase (DDC) enzyme that converts L-DOPA into dopamine. The genes encoding these three enzymes has previously been identified as the top-ranked classifiers in studies on NB (27-29). The function of the two Phox proteins differs in some aspects; while Phox2b is required for generation and maintenance of all noradrenergic cells (11,12), Phox2a seems to be more necessary for the brain noradrenergic centers (locus corelueus), (16,30). Overexpression of Phox2a and Phox2b *in vivo* engenders additional noradrenergic neurons (31), while mice lacking *PHOX2B* die before birth with abnormal and reduced chromaffin cells in the adrenal medulla (32). Other down-stream targets of ASCL1 and Phox2b, are the GATA binding protein 2/3 (GATA2 and GATA3) and dHAND, transcription factors involved in the noradrenergic pathway (12) (Fig. 4). Both GATA2 and GATA3 have previously been shown to discriminate between groups of NB tumors (28,29). Phox2b probably also controls expression of solute carrier family 6 (neurotransmitter transporter, noradrenalin, member 2; SLC6A2), which participates in the re-uptake of neuro-transmitter from the synaptic cleft (16). Another important catecholeamine transporter in noradrenaline-producing chromaffin cells or neurons is SLC18A1 (also called VMAT1; vesicular monoamine transporter 1). The differential expression levels of its transcript have been discussed in previous studies concerning neuroblastoma (33), phaeo-chromocytoma (34,35), as well as in gastrointestinal endocrine tumors (36). In a recent study, Revet and colleagues showed a connection of the Phox2-pathway to the Delta-Notch pathway via the homeobox transcription factor MSX1 (37). They found the MSX1 transcript to be down-regulated after induction of Phox2b expression and thus the role of MSX1 seems to be repression of neural differentiation. However, in

the current study we could not find any evidence for MSX1 up-regulation in unfavorable cases.

The evolutionary conserved Phox2 proteins (Phox2a and Phox2b) are the upstream key mediators of the noradrenalin biosynthesis and mutations in genes encoding them have been linked to several different syndromes. *PHOX2B* is a primary disease locus in congenital central hypoventilation syndrome (CCHS), a rare syndrome characterized by impaired autonomous breathing (38). Mutations in *PHOX2B* have also been found in patients with a complex combination of neural crest abnormalities, as well as in Hirschsprung disease (HSCR) which is characterized by absence of enteric ganglia. Several patients with CCHS and HSCR also suffer from NB (9,38,39). Moreover, both hereditary and sporadic cases of NB have been found to show mutations in the *PHOX2B* gene, although at low frequencies (7,8,10). Mutations in *PHOX2A* have been found in patients with congenital fibrosis of the extraocular muscles (CFEOM), a disorder that might result from maldevelopment of these nerve nuclei. In a study made by Nakano and colleagues, two splice-site mutations and one missense mutation were found (21). The fact that *PHOX2A* was the 6th most significant gene from the microarray gene list, that it is localized within the region often deleted in chromosome 11q and that it is directly regulated by Phox2b encouraged us to screen the coding and promoter region of *PHOX2A* by DNA sequencing. As can be visualized in Fig. 1, the promoter region contains several regulatory sites, including the Phox2b binding site, where mutations might affect the induction of *PHOX2A* expression (13). However, no alterations were found in the regulatory or coding parts of *PHOX2A* in this study.

The current study is based upon a gene list of differentially expressed genes from a microarray expression study (Affymetrix HU133A). Out of the genes found to show lower expression in unfavorable NB tumors, nine noradrenalin biosynthesis pathway actors (Fig. 4) were among the most significant transcripts (Table II). These results could be significantly verified in a second set of tumors by real-time PCR with TaqMan (Fig. 2). The interpretation of the lower expression of this pathway in unfavorable tumors can be discussed. This result could simply reflect the differentiation state in which high-risk tumors are more immature, also suggested by others (29). In that case, the present findings suggest the Phox2 pathway expression status to be an excellent marker for determination of the differentiation grade of NB tumors. On the other hand, the result could also reflect the importance of the deregulation of this pathway in tumor development. This suggestion is supported by the fact that *PHOX2B* is mutated in hereditary and in a few sporadic NB cases (7-10). Thus, one could speculate that a mutation in the *PHOX2B* gene is one of several ways to suppress this pathway in high-risk tumors.

In conclusion, a significant difference in expression of the noradrenalin biosynthesis pathway was found between high- and low-risk NB tumor types. This finding has been indicated by earlier studies (27-29), but the current study is the first to verify these results by a more robust technique in a separate patient material. In the current study, we screened the *PHOX2A* gene for mutations in primary sporadic NB tumors. However, no evidence for involvement in tumor development could be found. One could speculate that the presence of global epigenetic differences between favorable and unfavorable NB tumors might be responsible for modulating expression of this critical pathway and others. The study of such epigenetic patterns on a wide genome scale is presently ongoing at our laboratory.

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