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The von Hippel-Lindau tumor suppressor gene expression level has prognostic value in neuroblastoma

Jasmien Hoebeeck[1], Jo Vandesompele[1], Helén Nilsson[2], Katleen De Preter[1], Nadine Van Roy[1], Els De Smet[1], Nurten Yigit[1], Anne De Paepe[1], Geneviève Laureys[3], Sven Påhlman[2] and Frank Speleman[1]*

[1]*Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium*
[2]*Department of Laboratory Medicine, Molecular Medicine, Lund University, Malmö, Sweden*
[3]*Department of Pediatric Hematology and Oncology, Ghent University Hospital, Ghent, Belgium*

Deletions of the short arm of chromosome 3 are often observed in a specific subset of aggressive neuroblastomas (NBs) with loss of distal 11q and without *MYCN* amplification. The critical deleted region encompasses the locus of the von Hippel-Lindau gene (*VHL*, 3p25). Constitutional loss of function mutations in the *VHL* gene are responsible for the VHL syndrome, a dominantly inherited familial cancer syndrome predisposing to a variety of neoplasms, including pheochromocytoma. Pheochromocytomas are, like NB, derived from neural crest cells, but, unlike NB, consist of more mature chromaffin cells instead of immature neuroblasts. Further arguments for a putative role of *VHL* in NB are its function as oxygen sensitizer and the reported relation between hypoxia and dedifferentiation of NB cells, leading to a more aggressive phenotype. To test the possible involvement of *VHL* in NB, we did mRNA expression analysis and sought evidence for *VHL* gene inactivation. Although no evidence for a classic tumor suppressor role for *VHL* in NB could be obtained, a strong correlation was observed between reduced levels of *VHL* mRNA and low patient survival probability ($p = 0.013$). Furthermore, *VHL* appears to have predictive power in *NTRK1* (*TRKA*) positive tumor samples with presumed favorable prognosis, which makes it a potentially valuable marker for more accurate risk assessment in this subgroup of patients. The significance of the reduced *VHL* expression levels in relation to NB tumor biology remains unexplained, as functional analysis demonstrated no clear effect of the reduction in *VHL* mRNA expression on protein stability of its downstream target hypoxia-inducible factor α.

Key words: 3p-deletion; VHL (von Hippel-Lindau); neuroblastoma; survival; hypoxia

Neuroblastoma (NB) is the most frequent solid extracranial childhood tumor originating from immature neural crest derived sympathetic nervous system cells. NB displays a remarkable genetic heterogeneity, which underlies a highly variable clinical behavior, ranging from spontaneous regression to aggressive growth and metastasis. One of the major aims of ongoing genetic research in NB is the search for genes and signaling pathways implicated in tumor initiation and progression. This information may contribute to improved therapeutic stratification and could also lead to the development of molecular therapies. Amongst others, the identification and fine-mapping of genomic defects in the various NB subtypes might guide the search for candidate target genes. Recently, new insights were obtained into the various genetic changes that characterize the different subgroups of NB. In addition to the well-described NBs with 1p-deletion and *MYCN* amplification (MNA), a second major subgroup of aggressive NBs was recognized. These tumors exhibit neither 1p-deletion nor MNA, but present with 11q-deletions often in association with 3p-deletions.[1–5] Abnormalities of the short arm of chromosome 3 are involved in many tumor types such as carcinomas of the kidney, lung, breast, cervix, ovary, and head and neck. The most commonly deleted regions are 3p25 (encompassing the *VHL* gene), 3p21.3, 3p14.3 and 3p12. Although only limited attempts have been made to delineate a critical region of loss for 3p deletions,[6,7] evidence from comparative genomic hybridization (CGH) analysis indicates a rather distal localization of a NB tumor suppressor gene (TSG) on 3p as well as a more proximal region on 3p21.31-3p22.1, indicating that more than 1 TSG on 3p might be involved.[1,8] The distal 3p candidate region harbors the locus for the von Hippel-Lindau (*VHL*) gene predisposing to the VHL syndrome (OMIM 193300), a dominantly inherited familial cancer syndrome. Both malignant and benign neoplasms arise in affected patients, most frequently retinal, cerebellar and spinal hemangioblastoma, renal cell carcinoma, pheochromocytoma and pancreatic tumors.[9] Pheochromocytoma is a catecholamine-secreting tumor of chromaffin cells and, like NB, originates from neural crest cells. Further arguments for a possible role of *VHL* in NB development are (*i*) the expression of VHL in the neuronal cells of the fetal and adult central nervous system (CNS)[10–13] (*ii*) involvement of VHL in neuronal differentiation and in the proliferative state of CNS development[14] and (*iii*) the neuronal differentiating potential of VHL to transform NB cells into functional neuron-like cells.[15] These elements prompted us to perform an extensive investigation toward the possible role of the *VHL* gene as TSG in NB. To this purpose, we screened the *VHL* gene for genetic and epigenetic modifications and performed sensitive real-time quantification of *VHL* transcript levels in NB. Furthermore, as VHL has a very well documented role as an oxygen sensitizer by targeting the α-subunits of the hypoxia-inducible transcription factors (HIFs) for ubiquitin-dependent degradation, we evaluated HIF-1α stability in NB cells under normoxic (21% O_2) and various hypoxic (4%, 1% O_2) conditions.

Material and methods

NB tumors and cell line samples and cell culture conditions

Primary NB tumor samples prior to therapy (at least 70% tumor cells) were collected at the Ghent University Hospital (Ghent, Belgium). Informed consent was obtained from the patients' relatives. The study was approved by the review board of the Ghent University Hospital, Ghent, Belgium. The patients were staged according to the International Neuroblastoma Staging System (INSS). In addition, 28 well-characterized NB cell lines were included in our study. Comparative genomic hybridization (CGH) data and multi-

Abbreviations: CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; HIF, hypoxia-inducible factor; INSS, international neuroblastoma staging system; LOH, loss of heterozygosity; MNA, *MYCN*-amplification; MSP, methylation-specific PCR; NB, neuroblastoma; TSG, tumor suppressor gene; VHL, von Hippel-Lindau.

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***Correspondence to:** Center for Medical Genetics, Ghent University Hospital MRB, De Pintelaan 185, 9000 Ghent, Belgium.
Fax: +32-9-2406549. E-mail: Franki.Speleman@UGent.be

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color fluorescence *in situ* hybridization (M-FISH) results on 16 cell lines have been published.[16] Growth at hypoxic conditions in a Hypoxia Workstation 400 (Ruskinn Technology, Leeds, UK) and conditions for Western hybridizations are described in the work of Nilsson *et al*.[17]

Real-time quantitative PCR

mRNA expression was examined by an optimized two-step real-time quantitative PCR assay.[18] cDNA was synthesized using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA). The primers were designed with PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA) and are available in the public RTPrimerDB database[19] (http://medgen.ugent.be/rtprimerdb/) (gene (RTPrimerDB-ID: *VHL* (16), *VEGF* (1216), *CD44* (88), *NTRK1* (118), *UBC* (8), *HPRT1* (5), *SDHA* (7), *GAPD* (3)). SYBR Green I amplification mixtures (15 μl) contained 2x SYBR Green I master mix (7.5 μl) (Eurogentec, Seraing, Belgium), 250 nM of each forward and reverse primer and template cDNA (20 ng total RNA equivalent). Reactions were run on an ABI PRISM 5700 Sequence Detector (Applied Biosystems). The cycling conditions were as follows: 10 min at 95°C, 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Each assay included (in duplicate) a standard curve of 4 serial dilution points of SK-N-SH, a no-template control and about 20 ng of test cDNA. The results of the Sequence Detection Software (Applied Biosystems) were exported as tab delimited files and imported into the relative quantification software qBase (Hellemans *et al*., in preparation; http://medgen.ugent.be/qbase/) for further analysis. The transcription levels were normalized by the geometric mean of 4 stably expressed reference genes (*UBC*, *HPRT1*, *SDHA*, *GAPD*).[20]

Sequencing analysis

To screen the *VHL* gene for mutations, we performed direct sequencing of the coding region. The 3 *VHL* exons and their immediately flanking sequences were amplified by PCR as described.[21]

Methylation-specific PCR

Methylation-specific PCR (MSP) was done according to Herman *et al*.[22] with minor modifications. SssI methylase (New England Biolabs, Beverly, MA) treated and untreated normal human genomic DNA were used as a positive and negative control, respectively, after bisulphite modification.

Fluorescence in situ *hybridization*

FISH analysis was performed on interphase nuclei of NB cells as described.[23] Dual-color FISH was carried out with 2 BAC clones both covering the *VHL* gene (RP11-382A21, RP11-1016H17) combined with a probe for the centromere of chromosome 3 (pα3.5).[24] Probes were biotinylated (biotin-16-dUTP, Roche Diagnostics, Vilvoorde, Belgium) or digoxigenated (digoxygenin-11-dUTP, Roche Diagnostics) by standard nick translation. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and diamidiophenylindole for counterstaining. Microscopic analysis of images was performed using the ISIS digital imaging system (Meta Systems, Altlussheim, Germany).

Statistical analysis

Univariate survival analysis was performed with the Kaplan-Meier method and log-rank statistic using SPSS 12.0 software. Overall survival was defined from the date of diagnosis to the date of last follow-up or up to date of death. The relationship between the *VHL* mRNA expression level and genetic aberrations, including MNA, 1p-deletion, and 3p-deletion or other parameters (age at diagnosis, tumor stage) was determined using Fisher's exact test or Spearman rank correlation.

Results

Sample set and validation

For our study, 62 patients were randomly selected from pediatric oncology centers in Belgium from 1988 to 2002. To demonstrate that our patient cohort is a representative sample of NB patients, we assessed the distribution of several clinical and biological parameters and confirmed the predictive power of known markers. The median age at diagnosis is 21 months (range 3 days – 9.5 years). Median follow-up time for survivors ($n = 42$) is 60.4 months. Nineteen tumors were classified as stage I, 6 as stage II, 7 as stage III, 26 as stage IV and 4 as stage IVS, according to the INSS. Twenty-five percent of the primary tumors harbor a 1p-deletion, 18% demonstrate *MNA*, and 12% present with 3p-deletion. Univariate Kaplan-Meier survival analysis of the 62 cases confirmed that MNA, 1p-deletion, age at diagnosis >1.5 year, high-stage tumor (stages III and IV), and *NTRK1* (*TRKA*) and *CD44* mRNA expression below the median correlate significantly ($p < 0.01$) with poor survival (Table I).

Expression levels of the VHL *gene in relation to patient survival*

mRNA expression level of the *VHL* gene was determined in our cohort of NB tumor samples ($n = 62$) by real-time quantitative PCR (Table II). Patients were dichotomized as having high or low *VHL* expression based on the median of all normalized expression values. On average, the difference between high and low relative expression is 2.41 (±1.29) (ratio of 90th percentile to 10th percentile is 3.53). Kaplan-Meier survival analysis shows that *VHL* mRNA expression level below the median is significantly associated with a reduced survival probability ($p = 0.013$; Fig. 1*a*). No significant correlation between the *VHL* mRNA expression status and MNA, 3p-deletion status, and tumor stage (favorable stages I, II, IVS *versus* unfavorable stages III, IV) was found. In contrast to the primary tumor data, we did observe a correlation between 3p-deletion and reduced *VHL* mRNA expression in 28 NB cell lines (Spearman rank correlation of -0.679 ($p = 7.11 \times 10^{-5}$)).

TABLE I – UNIVARIATE KAPLAN-MEIER SURVIVAL ESTIMATES

Parameters	No. of patients	OS (months)	SE	p*
Tumor stage				
1, 2, 4S	29 (0)[1]			<0.0001
3, 4	33 (20)	75.68	17.84	
Age at diagnosis (year)				
<1.5	39 (4)	179.77	12.07	<0.0001
>1.5	23 (16)	52.2	10.23	
MYCN gene				
Single copy	51 (10)	160.28	13.07	<0.0001
Amplification	11 (10)	16.91	3.33	
Chromosome 1p				
Normal	43 (8)	127.63	10.26	<0.0001
Deletion	13 (9)	51.55	15.27	
Not known	6 (3)	–	–	
Chromosome 3p				
Normal	43 (12)	117.04	10.39	0.92
Deletion	6 (2)	98.36	20.35	
Not known	13 (6)	–	–	
NTRK1 mRNA expression				
<Median	31 (15)	73.28	11.14	0.0025
>Median	31 (5)	169.41	14.55	
CD44 mRNA expression				
<Median	31 (15)	79.61	11.91	0.0036
>Median	31 (5)	168.33	15.14	
VHL mRNA expression				
<Median	31 (14)	93.89	12.86	0.013
>Median	31 (6)	160.51	16.12	

OS, overall survival; SE, standard error of the mean.
*Log rank statistic.–[1]Numbers in parentheses indicate the no. of patients deceased.

TABLE II – PATIENT TUMOR SAMPLE INFORMATION

Patient	Stage	Age at diagnosis (months)	Status[a]	OS (months)	*VHL* expression[b]	SE
1	I	6.54	1	159.39	*	–
2	IV	12.66	1	59.15	0.057	0.004
3	IV	66.71	2	31.04	0.130	0.007
4	IV	2.04	1	58.32	0.136	0.014
5	IV	113.79	2	27.06	0.175	0.009
6	I	1.51	1	60.59	0.394	0.021
7	I	65.03	1	79.33	0.481	0.033
8	IV	25.35	2	9.86	0.498	0.046
9	IV	9.99	1	60.76	0.503	0.050
10	IV	57.47	2	24.79	0.544	0.043
11	III	22.39	2	17.82	0.545	0.035
12	IV	54.64	1	73.05	0.577	0.038
13	IVS	2.93	1	79.63	0.600	0.081
14	IV	56.65	2	21.47	0.605	0.038
15	II	5.10	1	43.56	0.629	0.032
16	IV	29.49	2	5.49	0.637	0.071
17	IV	27.65	2	31.76	0.661	0.055
18	IV	46.45	2	8.81	0.689	0.048
19	IV	19.59	2	6.54	0.699	0.076
20	I	5.39	1	60.23	0.714	0.078
21	III	20.12	2	21.01	0.768	0.082
22	I	7.99	1	40.24	0.774	0.103
23	III	62.50	1	63.52	0.785	0.066
24	IV	100.67	2	19.23	0.790	0.060
25	IVS	8.48	1	39.19	0.826	0.078
26	I	3.55	1	35.84	0.849	0.080
27	IV	49.91	2	18.71	0.852	0.070
28	IV	11.74	2	13.55	0.858	0.074
29	I	12.82	1	9.37	0.889	0.075
30	I	23.21	1	127.40	0.983	0.063
31	IV	3.12	1	35.57	0.991	0.107
32	I	2.96	1	53.92	1.009	0.170
33	IV	34.03	2	27.95	1.017	0.059
34	IV	5.03	1	57.96	1.036	0.077
35	I	2.96	1	81.63	1.056	0.076
36	II	0.10	1	39.35	1.085	0.085
37	I	1.28	1	31.69	1.107	0.036
38	IV	26.43	1	43.04	1.165	0.148
39	I	14.10	1	95.34	1.180	0.056
40	IV	1.25	1	28.70	1.191	0.160
41	I	12.53	1	37.94	1.199	0.159
42	I	1.87	1	67.23	1.210	0.079
43	IV	63.55	2	85.81	1.232	0.094
44	III	7.50	1	107.57	1.261	0.153
45	I	1.45	1	32.68	1.276	0.221
46	IVS	2.30	1	149.49	1.280	0.116
47	IV	43.82	1	43.56	1.293	0.063
48	I	5.69	1	73.22	1.298	0.085
49	II	18.35	1	88.11	1.333	0.068
50	III	7.43	1	53.62	1.360	0.136
51	III	17.33	2	7.13	1.431	0.077
52	IV	3.25	2	71.24	1.471	0.078
53	1	0.92	1	74.04	1.547	0.105
54	III	0.30	2	6.84	1.648	0.152
55	IVS	3.68	1	42.64	1.669	0.167
56	IV	31.50	2	20.91	1.711	0.135
57	I	17.49	1	158.40	1.935	0.125
58	II	10.88	1	143.41	1.951	0.129
59	II	10.39	1	133.58	1.971	0.266
60	II	15.52	1	36.43	2.415	0.152
61	IV	1.71	1	204.36	2.422	0.122
62	I	0.20	1	67.17	3.555	0.397

[a]1, alive; 2, dead.–[b]Expression values rescaled to median expression.
*No detectable *VHL* expression.

More interestingly, there appears to be a negative relation between *VHL* mRNA expression in the primary tumor and age at diagnosis (Fisher's exact test: $p = 0.0079$ and Spearman rank correlation of -0.353 ($p = 0.0049$)). To determine whether *VHL* mRNA expression contributes to survival within specific subgroups, we performed stratified subgroup analysis (Fig. 1*b*). Within the high *NTRK1* expressing tumors with presumed favorable prognosis, *VHL* mRNA expression lower than the median expression was associated with lower overall survival probability ($p = 0.058$).

Mutation analysis and methylation status of the VHL *gene in NB*

Despite a thorough investigation, no evidence for either genetic or epigenetic modification of the *VHL* gene could be found in our tumor series. Mutation analysis was carried out by direct sequencing of the coding region of the *VHL* gene in 28 cell lines and 62 primary NB, of which at least 6 tumors displayed a 3p-deletion by CGH, FISH and/or loss of heterozygosity analysis. In 1 patient, a rare base pair change was detected in both tumor and constitutional DNA. This base pair change, originally reported as a mutation, concerns a rare variant (with a frequency of about 0.5%) of the *VHL* gene.[25]

Subsequently, we sought for evidence of *VHL* gene promoter hypermethylation in 28 NB cell lines and our cohort of primary tumors using MSP. In all cell lines and tumors a positive signal was obtained with primers specific for unmethylated DNA, whereas none of these samples showed a positive signal with primers specific for methylated DNA (data not shown), thus providing no evidence for *VHL* gene silencing through methylation.

VHL *functional analysis*

Given the observed reduced *VHL* expression in unfavorable NBs, functional analysis was performed in order to asses the functional consequence of this reduced expression. One of the most well-known functions of VHL is its role in angiogenesis by regulating the stability of hypoxia-inducible factors such as HIF-1α. These proteins are transcription factors that regulate the production of mRNAs implicated in angiogenesis, such as the vascular endothelial growth factor (VEGF). HIF-1α protein stability and VHL protein levels were investigated, using Western blot analysis, in 5 NB cell lines, of which 2 present with low *VHL* mRNA expression and 3p-deletion (SK-N-AS, SJNB-6) and 3 NB cell lines with mRNA expression higher than the median with intact 3p regions (IMR-32, SK-N-SH, LA-N-5) (Fig. 2). All investigated cells displayed approximately similar amounts of VHL protein (Fig. 2*a*). No differences could be detected for HIF-1α between the different cell lines, neither under normoxic (21% O_2) nor under hypoxic (4%, 1% O_2) conditions. None of the cells expressed detectable HIF-1α protein under normoxic conditions and reanalysis of the 2 cell lines with 3p-deletions and prolonged filter exposure confirmed that basal HIF-1α levels were low also in these cell lines (Fig. 2*c*). These results indicate that there is no clear effect of reduced *VHL* mRNA expression on HIF-1α protein stability. Furthermore, no significant correlation between the *VHL* mRNA expression status and *VEGF* mRNA expression levels was found, although hypoxia induced VEGF expression, as previously reported.[26]

Discussion

In our study, we demonstrated that reduced *VHL* mRNA expression level is a poor prognostic indicator for NB. For this purpose, a representative set of 62 primary tumors were tested using a sensitive real-time PCR assay.[18] No correlation was found with known prognostic markers such as *MYCN* status or tumor stage, whereas an inverse negative correlation was found with age. Interestingly, stratified survival analysis to assess the prognostic role of *VHL* levels in NB subgroups revealed reduced survival for patients whose tumor is characterized by high *NTRK1* and low *VHL* expression levels. In view of the fact that these tumors are generally considered favorable, *VHL* expression levels may help in the identification of a subgroup of patients with reduced survival probability. The sensitivity, specificity, and the positive and negative predictive value of *VHL* mRNA expression as prognostic marker are comparable to the established marker *NTRK1*. Stratification of patients with respect to both *VHL* and *NTRK1* mRNA expression appears to be more specific and has a higher negative predictive value than evaluating each marker alone (Table III). As this subgroup analysis is inherently based on

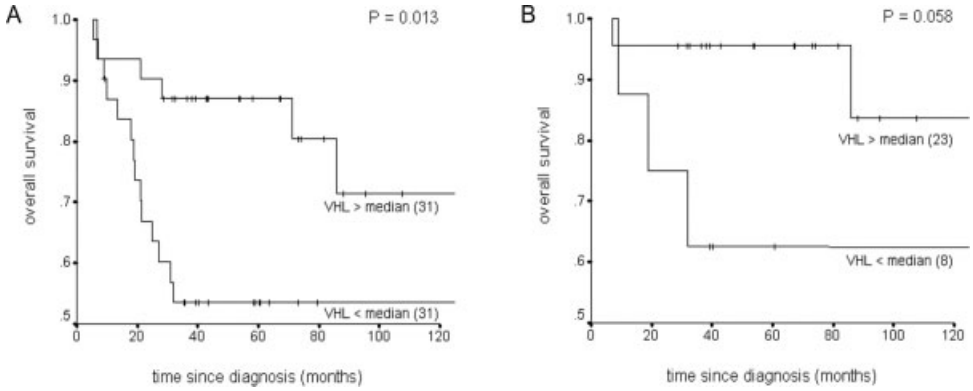

FIGURE 1 – (*a*) Kaplan-Meier survival curve for patients with NB. Patients were dichotomized with respect to the median *VHL* gene expression. The survival rates of patients with *VHL* expression higher than the median expression were significantly higher than those with a *VHL* expression lower than the median expression. (*b*) Stratified survival analysis for 31 high *NTRK1* expressing tumors subdivided by *VHL* expression below/above median level. Within the group of high *NTRK1* expressing tumors with presumed high survival probability, low *VHL* expression was associated with a reduced survival probability.

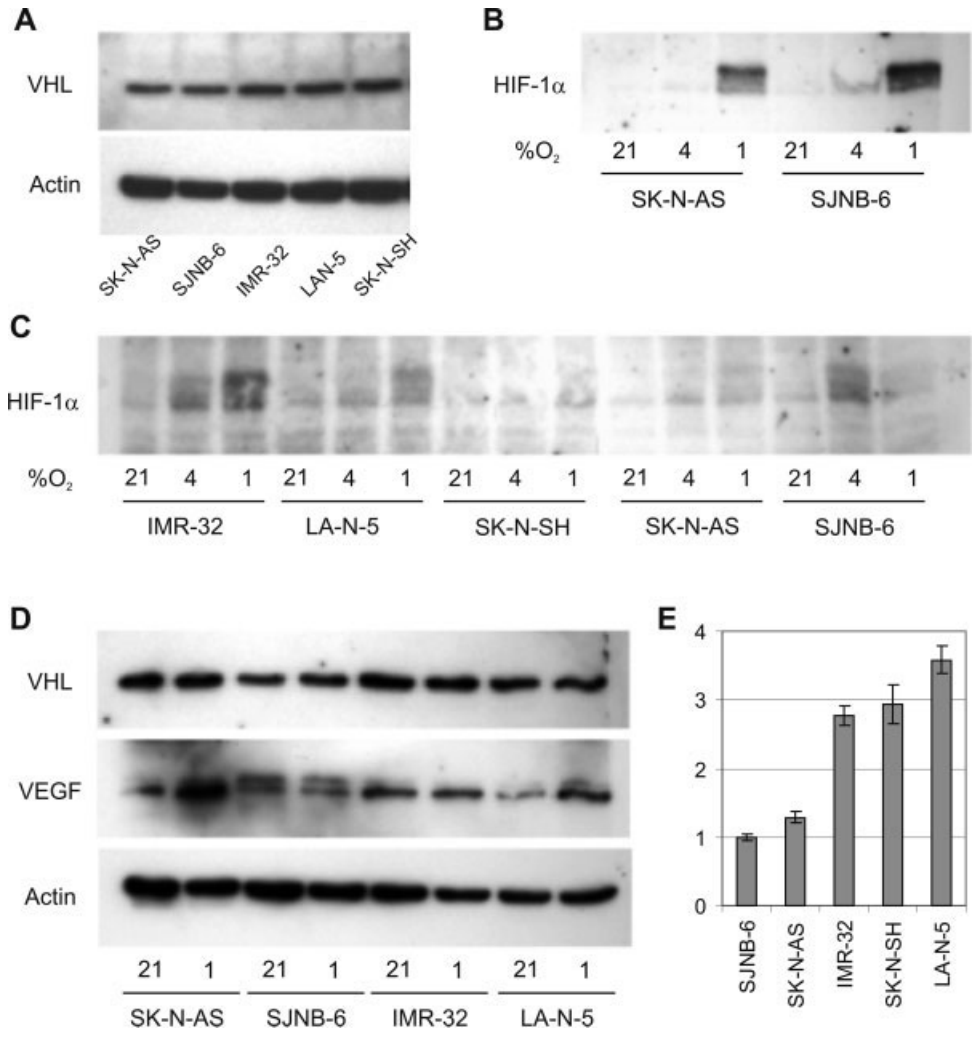

FIGURE 2 – Western blot analyses of VHL, HIF-1α and VEGF protein levels in NB cell lines. (*a*) VHL expression in 3p-deleted (SK-N-AS and SJNB-6) and 3p-undeleted NB cell lines. (*b*) and (*c*) HIF-1α protein levels in NB cells grown at normoxic (21% O_2) and hypoxic (4 and 1% O_2, respectively) conditions. In (*c*) cell lysates from the 3p-deleted cell lines were reanalyzed and exposed longer to confirm low HIF-1α expression levels in normoxic cells. (*d*) Hypoxia-induced VEGF expression (note the actin loading control). (*e*) Normalized relative *VHL* mRNA expression (minimum level rescaled to 1) (±standard error of the mean).

relatively small number of patients, care must be taken and further study of a larger cohort of patients is needed to confirm these results. Currently, therapeutic risk-group assignment is based on INSS stage, age at diagnosis, *MYCN* copy number and Shimada tumor histopathology. However, additional factors have been reported to have prognostic value. By analyzing additional prognostic markers such as *VHL* and *NTRK1* expression, it should become possible to determine whether a tumor is truly aggressive and does require extensive treatment, thereby refining current treatment stratifications.

TABLE III – TEST EVALUATION FOR INDIVIDUAL AND COMBINED ANALYSIS OF THE PROGNOSTIC MARKERS *NTRK1* AND *VHL* mRNA EXPRESSION LEVELS

Expression level	Number of patients		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
	Alive	Dead				
NTRK1> median	26	5	75	62	48	83
NTRK1 <median	16	15				
VHL> median	25	6	70	60	45	80
VHL <median	17	14				
NTRK1> median *VHL*> median	21	2	60	81	38	91
NTRK1> median *VHL*< median	5	3				

At present, none of the available data from genome wide expression studies in NB has identified *VHL* as a putative prognostic marker. Re-evaluation of the publicly available microarray data published by Berwanger and colleagues did not reveal a prognostic value of *VHL* expression levels.[27] This could be attributed to the lower sensitivity of microarray mRNA measurements to detect the 2–3-fold differences that we measure using our high-resolution qPCR assay.

The predictive power of *VHL* mRNA expression levels prompted us to investigate the possible role of *VHL* as a classic TSG. In NB cell lines carrying a 3p-deletion, mutation analysis was performed but no sequence variations or deletions were found in the coding sequence of the remaining allele. Also, methylation analysis provided no evidence for epigenetic inactivation of the second allele. However, by using MSP for only 1 primer pair we cannot exclude the presence of methylated CpG dinucleotides in the promoter regions that were not covered by the primers. In analogy with recent reports in which loss of only 1 allele of a tumor suppressor might contribute to the tumorigenic process, the reduced expression of the *VHL* gene suggests that *VHL* could act in such a haploinsufficient manner. However, differences at the *VHL* mRNA transcript level between 3p-deleted and nondeleted cell lines did not apparently correlate with differences in protein levels, nor did we find evidence for increased HIF-1α stability. The lack of correlation could however be due to the fact that the measurement of mRNA expression levels by real-time PCR is far more sensitive than measuring differences in protein levels. Furthermore, multiple stably expressing reference genes were used for accurate normalization of the mRNA expression data compared to only 1 reference gene for protein analysis.

Although our study suggests that the *VHL* expression level may serve as a prognostic marker, even in high *NTRK1* expressing tumors with presumed favorable outcome, the biological significance of this finding remains unexplained. As the analysis of oxygen-sensing signaling in NB cell lines did not reveal a reduced VHL protein level in low expressing (3p-deleted) cells, other explanations must be sought for the possible contribution of lower *VHL* mRNA levels to tumor formation. Besides its most documented role, *i.e.* targeting the subunits of the HIFs for ubiquitin-dependent degradation, VHL has been implicated in diverse cellular processes. Another function is the involvement of VHL in neuronal differentiation and in the proliferative state of central nervous system development.[14] Moreover, overexpression of exogenous *VHL* in NB SH-SY5Y cells with the *VHL* gene led to the induction of neurite-like processes with varicosities and expression of the neuronal markers neuropeptide Y and neurofilament 200, whereas inhibition of VHL reduced such neuronal properties. Thus, it appears that VHL has a neuronal differentiation potential to transform NB cells into functional neuron-like cells.[15]

Furthermore, VHL may have targets other than HIF for proteasomal degradation. Indeed, a number of other VHL-binding partners have been identified, including potential substrates for ubiquitin-dependent degradation. VHL can interact with protein kinase C, a family of genes encoding isoforms of which some are implicated in the regulation of neuronal differentiation.[28] Other functions of VHL comprise cell cycle control and transcriptional regulation. Interestingly, the transcription of tyrosine hydroxylase, which encodes the rate-limiting enzyme in catecholamine biosynthesis, seems to be regulated by VHL. In analogy with NB, the role of VHL in pheochromocytoma also remains enigmatic. Pheochromocytoma-predisposing mutant VHL proteins seem to retain the ability to downregulate the hypoxia-inducible factors, suggesting that HIF is not relevant for pheochromocytoma tumorigenesis. Therefore, it is important to understand the possible tumor suppressor function(s) of the *VHL* gene, unrelated to HIF destabilization, in order to clearly understand and test the functional significance of reduced *VHL* levels in NB.

In conclusion, the *VHL* mRNA level is a promising marker to predict patient survival, especially in the subgroup of patients with presumed favorable high *NTRK1* expressing tumors. Further analysis to elucidate a mechanism of action of *VHL* in NB is warranted.

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