Suppression of RIZ in biologically unfavourable neuroblastomas

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Abstract. Neuroblastoma is a paediatric solid tumor characterized by recurrent genomic abnormalities of prognostic importance. One of the most commonly observed abnormalities is deletion of the short arm of chromosome 1 and reduced expression of cancer related genes in this chromosomal arm. The long isoform of the retinoblastoma protein-interacting zink finger gene (*RIZ1*) is a known tumor suppressor and a candidate neuroblastoma gene located at 1p36.2. The present study was undertaken to further assess the possible involvement of *RIZ* in neuroblastoma development. Expression of *RIZ* transcripts were quantified in a panel of neuroblastoma cell lines and tumors (33 neuroblastomas and 3 ganglioneuromas). Methylation status of promoter P1 driving *RIZ1* expression was quantified by bisulfite Pyrosequencing. Only low mean levels of promoter methylation (<10%) were observed in all samples. However, *RIZ1* and *RIZ1+2* mRNA were significantly under-expressed in biologically unfavourable tumors characterized by 1p loss ($p<0.005$) or *MYCN* amplification ($p<0.005$). Suppression of *RIZ1* is likely to contribute to the pathogenesis of biologically unfavourable neuroblastomas. In contrast to multiple other neoplasias, *RIZ1* promoter methylation is not a common event in neuroblastoma.

Introduction

Neuroblastoma is a paediatric tumor derived from precursors of the sympathetic nervous system in the adrenal gland. The clinical presentation is highly variable ranging from spontaneous regression to rapid progression and fatal outcome. Tumor stage and clinical course is strongly associated with the presence or absence of certain molecular genetic aberrations (1). Molecular features of high-risk tumors include deletions of the short arm of chromosome 1, amplification of the *MYCN* locus and activating mutations of *ALK* in chromosome 2 (2-4). Deletions of 1p occur in >70% of high-risk tumors, and several groups have mapped smallest regions of loss to 1p36 (5-11). The frequent losses of distal 1p in neuroblastomas suggest the presence of one or more neuroblastoma tumor suppressor gene loci in this region. While consistent mutations have not been demonstrated in candidate 1p genes, under-expression has been described for several cancer related genes located within as well as close to the region of loss. In addition to localized mutation, other means of allelic inactivation such as transcriptional silencing by promoter methylation or translational inhibition by micro-RNAs are anticipated (12-14).

The retinoblastoma interacting zinc finger protein gene (*RIZ*) is a known tumor suppressor located in chromosomal region 1p36.2, and a candidate neuroblastoma gene. The *RIZ* locus generates two transcripts: *RIZ1* from promoter P1 and *RIZ2* from promoter P2. The corresponding proteins are identical at the C-terminal CR domain, but differ for the SET-domain that is present in the N-terminal PR-domain of the long RIZ1 isoform but not in the short RIZ2 isoform (15). The family of SET domain proteins have important functions in chromatin mediated transcriptional regulation, which in the case of *RIZ1* involves methylation of histone H3 lysine 9 leading to transcriptional repression (16,17). Another function of RIZ is as co-activator or co-repressor of nuclear hormone receptors (18).

Several studies *in vitro* and *in vivo* support that RIZ1 but not RIZ2 could function as a tumor suppressor in cancer development (19). Loss or reduced expression of RIZ1 has been reported in tumors of the breast, ovaries, colon, and liver as well as in pheochromocytoma (20-23). RIZ1 knock-out mice frequently develop diffuse large B cell lymphomas and other tumors (17). Reconstitution of RIZ1 expression in cancer cells induced cell cycle arrest, apoptosis and suppressed growth of tumor xenograft in immuncompromized mice (21,23,24). Somatic *RIZ* mutations have also been reported, such as frameshift mutations at the C terminus in association with microsatellite instability (21,25), and missense mutations at the N-terminal PR domain in various tumors and cancer cell lines (17). Finally, hypermethylation of promoter P1 has been reported in different types of tumors (20,26-30).

The role of RIZ1 in neuroblastoma is not well understood. While *RIZ1* and *RIZ2* are highly expressed in normal neural tissues such as the adrenal medulla (31), under-expression of *RIZ* has been observed in aggressive neuroblastoma at genome-wide transcriptional profiling. Furthermore, promoter P1 methylation was found using qualitative approaches in one of the two studies where this was assessed (32,33). To further characterize the involvement of *RIZ* in neuroblastoma we quantified mRNA expression levels and promoter P1 methylation density in a series of well characterized tumors and cell

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lines and correlated the findings to various clinico-pathological factors.

Materials and methods

Cell lines. Seven established neuroblastoma cell lines were studied: SK-N-DZ, SK-N-SH, SK-N-BE(2), SK-N-FI, SK-N-AS, IMR-32, and SH-SY-5Y. Cells were grown under standard conditions (RPMI-1640 or Eagles's MEM for SH-SY-5Y, 10% fetal bovine serum, and 2 mmol/l L-glutamine) at 37˚C in the presence of 5% CO_2.

Tumor samples. The study includes a total of 36 tumors from 36 children surgically treated for peripheral neuroblastic tumor (33 with neuroblastoma and 3 with ganglioneuroma) at the Karolinska University Hospital, Stockholm, Sweden (Tables I and II). All tissues were obtained with informed consent from patients or their legal guardians, as approved by the local ethics review board. After surgical removal tumor samples were dissected and snap-frozen in liquid nitrogen and stored at -70˚C.

Detailed clinical and genetic information has been previously published for each case (34). In short, the cases represent all clinical and biological subsets of neuroblastoma, with a pattern of patient demography and tumor biology similar to that of the Swedish population. Diagnosis and staging followed the International Neuroblastoma Staging System (INSS) (35). *MYCN* amplification and 1p loss were determined as part of the standard characterization (36). In the case of pre-operative treatment this was given at the latest of 2 weeks before surgery, and radiation therapy was only given post-operatively. Four patients died of the disease (DOD) within ≤18 months after diagnosis (cases 2, 3, 5 and 10), 2 patients died of surgical complications (cases 30 and 31) while 30 patients have survived for at least 17 months with a mean of 102 months. DNA from normal adrenal medulla was purchased from Clinomix (Watervliet, NY, USA) and used as non-tumorous reference.

RNA extraction and cDNA synthesis. Total RNA was extracted from frozen tumor samples using RNeasy RNA extraction kit (Qiagen). RNA yield was quantified by spectrophotometry, and RNA quality was assessed by demonstration of distinct *28S* and *18S* bands at denaturing gel electrophoresis (1% agarose). cDNA was synthesized by reverse transcription from total RNA (2 μg) in 100 μl reactions using High-Capacity cDNA Archive kit (ABI) according to the recommendations of the manufacturer.

Real-time quantitative PCR (qRT-PCR). Gene expression of *RIZ* transcripts were quantified using TaqMan technology and an ABI PRISM 7700 Sequence Detection System. *RIZ1* (RIZ-PR) and *RIZ1+2* (RIZ-CR) were separately analysed using primers and probes that have been described in detail elsewhere (37). In addition two house-keeping genes were analysed in parallel using commercially available assays for *18S* and B2 microglobulin *B2M* (ABI assays on demand, assay Hs99999901_s1 and Hs00187842_m1). cDNA (65 ng) was amplified by PCR in TaqMan 2X Universal Master mix (final concentration 1X) under the following conditions: 50˚C for 2 min; 95˚C for 10 min; and 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Each measurement was performed in duplicates.

A standard curve for relative expression quantification was generated by parallel amplification of serially diluted cDNA from human lymphocytes. Expression values for tumor samples and cell lines were subsequently related to the standard curve and then normalized to *18S* used as endogenous control. As an independent confirmation, expression values were also normalized to *B2M* with highly similar results as compared to *18S* (data not shown). Furthermore a relative value of 1 was assigned to represent the average expression in tumors without 1p deletion.

DNA extraction and bisulfite treatment. High molecular weight DNA was extracted from tumor samples and cultured cells

Table I. Clinical and genetic details for the tumor cases studied.

Parameter	Total	Neuro-blastoma	Ganglio-neuroma
Cases studied			
Patients	36	33	3
Tumors	36	33	3
Sex			
Female	16	15	1
Male	19	17	2
Age at diagnosis			
Range (months)	0-145	0-136	59-145
High-risk therapy			
Yes	10	10	-
No	26	23	3
Stage			
Stage 4	9	9	-
Stage 1-3	21	21	-
Stage 4S	3	3	-
Loss in 1p			
Loss	10	10	-
No loss	25	22	3
MYCN			
Amplified	10	10	-
No amplification	26	23	3
Survival			
DOD (≤18 months)	4	4	-
DOC	2	2	-
Alive (≥17 months)	30	27	3

DOD, dead of disease; DOC, dead of surgical complications.

Table II. Results from *RIZ* promoter P1 methylation and gene-expression studies.

Case no.	Loss of 1p	Other high-risk features	Tumor type	P1 methylation %			*RIZ1* mRNA/		*RIZ1+2* mRNA/	
				MetI	Min.	Max.	*18S*	No 1p loss	*18S*	No 1p loss
Tumors										
2	Loss	*MYCN*, 4, DOD	NB	5.7	3.2	11.4	1.3	0.2	5.6	0.05
3	Loss	*MYCN*, 4, DOD	NB	3.0	0.8	7.2	7.6	0.9	106.1	0.9
4	Loss	*MYCN*, 4	NB	3.9	3.1	4.3	2.1	0.3	27.6	0.2
5	Loss	*MYCN*, 4, DOD	NB	5.0	4.1	6.3	1.8	0.2	27.2	0.2
6	Loss	*MYCN*	NB	5.2	4.3	6.4	7.7	0.9	82.3	0.7
7	Loss	*MYCN*, 4	NB	-	-	-	1.3	0.2	27.9	0.2
8	Loss	*MYCN*, 4	NB	5.0	3.6	6.0	2.4	0.3	11.1	0.1
9	Loss	*MYCN*	NB	2.1	1.7	3.6	3.6	0.4	25.6	0.2
10	No	*MYCN*, 4, DOD	NB	1.4	0.0	4.8	2.4	0.3	32.2	0.3
11	No	*MYCN*	NB	1.4	0.9	1.6	-	-	-	-
12	No	No	NB	5.2	2.2	13.9	12.4	1.5	300.0	2.5
13	No	No	NB	6.1	2.8	12.8	8.8	1.1	120.3	1.0
14	No	No	NB	8.9	5.2	11.2	13.6	1.6	66.7	0.5
15	No	No	NB	2.9	2.2	3.3	4.0	0.5	87.3	0.7
16	No	No	NB	2.9	0.0	5.9	8.1	1.0	150.4	1.2
17	No	No	NB	2.8	1.2	9.8	12.7	1.5	127.4	1.0
18	No	No	NB	2.6	1.1	6.1	9.7	1.2	92.0	0.8
19	No	No	Gang.	1.3	0.0	4.4	8.2	1.0	84.0	0.7
20	No	No	Gang.	2.3	1.2	4.2	3.4	0.4	44.0	0.4
21	No	No	NB	2.6	1.5	4.2	9.4	1.1	95.1	0.8
22	No	4	NB	3.6	2.4	7.6	11.2	1.4	142.7	1.2
23	No	No	NB	6.6	4.4	15.0	3.1	0.4	178.0	1.5
24	No	No	NB	4.2	0.0	21.5	5.0	0.6	72.0	0.6
25	No	No	NB	2.0	0.0	3.9	11.7	1.4	125.9	1.0
26	No	No	NB	6.0	1.7	17.1	10.5	1.3	95.2	0.8
27	No	No	Gang.	4.5	2.7	7.5	1.6	0.2	82.1	0.7
28	Loss	No	NB	4.3	3.2	6.0	6.3	0.8	40.6	0.3
29	No	No	NB	1.0	0.0	2.2	11.4	1.4	213.7	1.8
30	No	No	NB	7.0	3.2	18.3	14.8	1.8	289.8	2.4
31	-	No	NB	2.3	0.0	4.5	3.0	0.4	40.0	0.3
32	No	No	NB	0.8	0.7	1.0	-	-	-	-
34	No	No	NB	1.2	0.9	1.5	-	-	-	-
35	No	4	NB	0.7	0.5	0.9	-	-	-	-
38	No	No	NB	1.4	0.0	2.6	-	-	-	-
39	No	No	NB	6.2	3.9	9.5	-	-	-	-
40	Loss	No	NB	7.2	9.7	12.0	-	-	-	-
Cell lines										
IMR-32	-	-	NB	-	-	-	3.9	0.5	44.0	0.4
SH-SY-5Y	-	-	NB	-	-	-	4.7	0.6	63.6	0.5
SK-N-AS	-	-	NB	0.6	0.0	2.5	5.5	0.7	70.3	0.6
SK-N-BE(2)	-	-	NB	1.1	0.8	1.6	5.6	0.7	79.5	0.7
SK-N-DZ	-	-	NB	-	-	-	1.9	0.2	77.9	0.6
SK-N-F1	-	-	NB	3.0	2.1	3.8	5.6	0.7	130.2	1.1
SK-N-SH	-	-	NB	5.4	3.3	10.6	2.5	0.3	62.6	0.5

MYCN, amplification of *MYCN*; 4, stage 4; DOD, dead of disease; NB, neuroblastoma; Gang, ganglioneuroma; mRNA expression in relation to *18S* as well as the mean value of tumors without 1p loss.

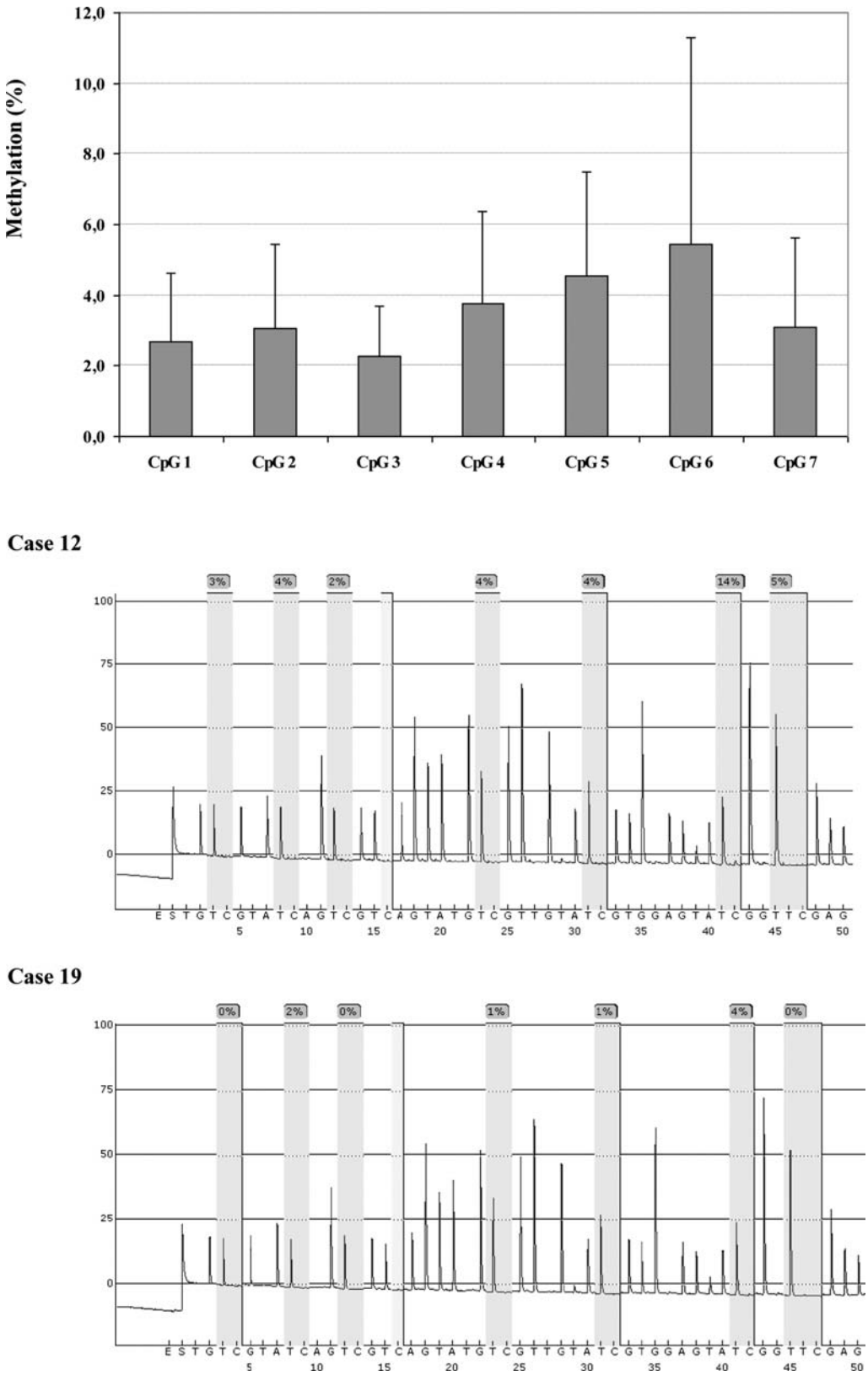

Figure 1. Quantification of *RIZ* promoter P1 methylation in neuroblastomas and ganglioneuromas. The diagram illustrates the mean methylation density at the 7 CpG sites assayed in tumors 2-40. The results are exemplified in the Pyrograms below. Tumor no. 12 shows low MetI of 5.2% with modestly increased methylation to 13.9% at CpG no. 6. Tumor no. 19 exhibit low MetI (1.3%) with methylation in the range 0-4.4% at individual CpGs.

using a standard method including proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. DNA samples (2 μg) were subsequently sodium bisulfite modified with the EZ DNA Methylation kit (Zymo Research Corp., Orange, USA) following the recommendations of the manufacturer.

Pyrosequencing. Methylation density of the *RIZ* promoter P1 was quantified using a Pyrosequencing approach and the following primers: GGTTGGGTGGTGGTTATT and AAAC CTACCAAACTAAAAAACTCC. First, a 100-bp segment of the *RIZ* promoter P1 (Ensemble ID: ENSG00000116731) was amplified from bisulphite treated DNA (1.5 μl) using Hot-start Taq polymerase, HotStar Taqs Master mix kit (Qiagen Ltd.) and the following conditions: 95˚C for 15 min, 45 cycles of (95˚C for 20 sec, 53˚C for 20 sec and 72˚C for 20 sec), and 72˚C for 10 min. After verification at 3% agarose gel electrophoresis, PCR products were subjected to Pyrosequencing using the PSQ™ HS96 system (Qiagen Ltd.), PyroGold reagents (Qiagen) and the sequencing primer GGGTGGT GGTTATTGG. The resulting Pyrograms were evaluated using Pyro Q-CpG software (Biotage AB), and the C:T peak ratios at the 7 individual CpG sites reflect the proportion of methylated to non-methylated alleles. A non-CpG C within the assayed sequences served as internal control for efficiency of bisuphite conversion. In all runs *SssI* treated human lymphocyte DNA was included as positive control for hypermethylation. For each sample a methylation index (MetI) was calculated as the mean level of methylation recorded at the 7 CpGs examined in *RIZ* promoter P1, and in addition the methylation level at each individual CpG site was considered.

Statistical analyses. Statistical calculations were done using STATISTICA version 7 (Statsoft, Inc., Tulsa, OK, USA), and p-values ≤0.05 were regarded as statistically significant. Differences in *RIZ1* (*RIZ-PR*) and *RIZ1+2* (*RIZ-CR*) expression were compared by Mann-Whitney U test with regard to *MYCN* amplification status, loss in chromosomal arm 1p, stage 4 vs. non-stage 4 tumors, high-risk vs. low-risk status, female vs. male sex, and ganglioneuromas vs. neuroblastomas. To estimate whether there was a difference between the extent of expressional reduction of *RIZ1* and *RIZ1+2* in *MYCN* amplified tumors the mean expression values for *RIZ1* and *RIZ1+2* for *MYCN* non-amplified tumors were divided by the respective expression values observed in each individual tumor with *MYCN* amplification. These values would then reflect the fold difference, i.e., the level of reduction of *RIZ1* and *RIZ1+2* expression compared to the means of *RIZ1* and *RIZ1+2* expression in the *MYCN* non-amplified group. Mann-Whitney U test was then used to compare if there was a difference between the degree of reduction for *RIZ1* and *RIZ1+2* in *MYCN* amplified tumors. Similar analyses were also used to compare tumors with vs. without 1p loss. Kruskal-Wallis analysis was used to evaluate differences in *RIZ1*/*RIZ1+2* expressions between tumors of different clinical stages. Spearman Rank Order Correlations were used to assess correlation between *RIZ1*/*RIZ1+2* expression and age at presentation.

Results

Hypermethylation of RIZ promoter P1 is a rare event in neuroblastoma. Methylation indices MetI representing mean level of methylation for the 7 CpG sites investigated ranged from 0.7 to 8.9% (Table II; exemplified in Fig. 1). An arbitrary cut-off at 10% MetI was applied for hypermethylation, which is in general agreement with most studies applying Pyrosequencing for methylation quantification. In addition, a normal adrenal medulla showed MetI at 2.6% (range 0.8-5.7%). Nine neuroblastomas and one cell line exhibited increased methylation at one or more individual CpG sites although the MetI was still <10%. Methylation levels >10% were predominantly recorded at CpG 6 (Fig. 1). The remaining tumors and cell lines showed low methylation <10% at all individual sites examined.

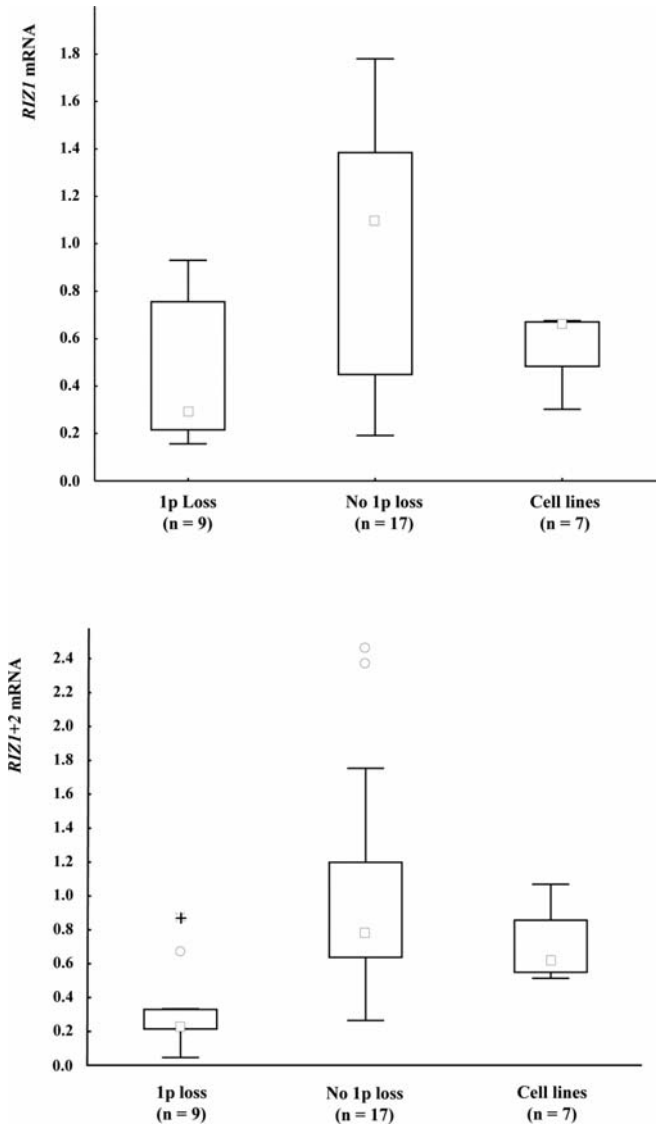

Figure 2. Boxplots illustrating relative expression of *RIZ1* (top) and *RIZ1+2* (bottom) in neuroblastoma tumors and cell lines. Presented expression values were normalized to *18S* and subsequently related to the arbitrary mean of 1.0 assigned to neuroblastomas without 1p loss. Significantly reduced expression is observed in biologically unfavourable tumors with 1p loss as compared to those without 1p loss.

Suppression of RIZ1 and RIZ1+2 expressions in aggressive neuroblastoma. Expression of *RIZ* transcripts were measured for *RIZ1* and *RIZ1+2* separately by qRT-PCR in tumors and cell lines (Table II). Comparison of *RIZ1* and *RIZ1+2* expression with clinical and genomic tumor characteristics revealed several significant associations suggesting suppressed expression in high-risk disease. Significantly lower levels of *RIZ1* as well as *RIZ1+2* expressions were detected among

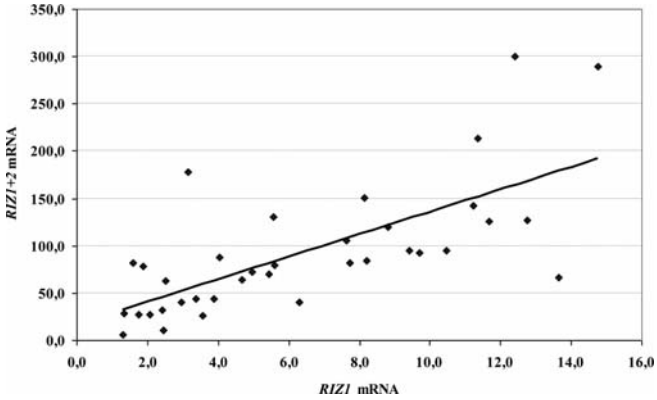

Figure 3. Comparison of *RIZ1* and *RIZ1+2* mRNA expression levels in tumors and cell lines.

neuroblastomas with 1p loss as compared to those without this abnormality ($p<0.005$; Mann-Whitney U test). Similarly, significantly lower levels of *RIZ1* and *RIZ1+2* were found in tumors with *MYCN* amplification compared to those without amplification, and in neuroblastomas classified as INSS stage 4 compared to tumors of lower stages ($p<0.005$; Mann-Whitney U test). No significant difference was observed in the extent of reduction for *RIZ1* as compared to *RIZ1+2* in tumors with 1p loss or *MYCN* amplification. No difference was demonstrated between ganglioneuromas and neuroblastomas with regard to *RIZ1* and *RIZ1+2* expressions. Lower *RIZ1* expression was observed in tumors from patients with a later age at presentation ($p<0.05$; Spearman Rank Order Correlations). There was also a tendency towards higher *RIZ1* and *RIZ1+2* expressions in stage 4S tumors (cases 12, 18 and 29) compared to low stage (stages 1 and 2) cases. However, this did not reach the level of statistical significance.

Overall the 7 neuroblastoma cell lines examined showed low levels of *RIZ1* and *RIZ1+2* expressions. To simplify comparisons between groups of samples, the expression levels were related to an arbitrary mean of 1.0 assigned to the group of neuroblastomas without 1p loss (Table II). As illustrated in Fig. 2 this revealed relatively high expression levels in tumors without 1p loss, and low levels in tumors with 1p loss as well as in neuroblastoma cell lines.

Discussion

Here we report substantially reduced *RIZ1* and *RIZ2* mRNA levels in 1p deleted neuroblastomas, a subset which is highly associated with unfavourable prognosis (2). At an average, a 2-fold *RIZ1* mRNA reduction was seen in 1p deleted cases, compared to the mean expression in tumors without 1p loss. *RIZ1+2* also showed a similar relative decrease, suggesting that not only *RIZ1* but also *RIZ2* expression is suppressed. Two 1p deleted cases showed no major decrease in *RIZ* expression (cases 3 and 6).

Low levels of *RIZ1* expression were demonstrated by He *et al* in a panel of 7 neuroblastomas and 7 cell lines. Interestingly the two cases showing low *RIZ1* levels were classified as stages 2 and 4S (23) - a feature associated with favourable outcome. In contrast, we found reduced *RIZ1* expression in a subset of the cases characterized by high-stage and biologically unfavourable behaviour. A more recent study that compared global gene expression pattern differences between 1p deleted and non-deleted neuroblastomas, also noted differential expression of *RIZ* (38). However, no data were presented concerning the degree of expressional reduction and the particular *RIZ* transcript involved. Our data provide quantitative corroboration of the above reports on *RIZ1* suppression, on an independent well characterized series. Furthermore, our mRNA expression results provide indication that levels of both RIZ transcripts are reduced (Table II and Fig. 3). This contrasts the scenario seen in most other tumor types, where usually only *RIZ1* is reduced (23,24) and in some neoplasias RIZ2 may even show relative over-expression (37). The preferential expression of RIZ in fetal and adult neuroendocrine tissues, as well as expressional suppression in cancer, indicate a role for these molecules in both normal development and pathogenic processes of neuroendocrine tissues (31). To gain insight into the significance of this observation, further investigation of the role of *RIZ2* in normal neuroendocrine development and carcinogenesis is required.

A number of other candidate neuroblastoma tumor suppressor genes at 1p36.2-3 show expressional reduction in 1p deleted neuroblastomas (38-42). Therefore it is likely that transcriptional alteration in multiple genes cooperate in the pathogenesis of neuroblastomas. Several lines of evidence suggest a tumor suppressor role for *RIZ1* (19). One of the known cellular functions of RIZ1 is methylation of the lysine 9 residue on Histone H3, which is a chromatin modification associated with heterochromatinization and transcriptional repression (16,17). It is thus possible that reduced *RIZ1* expression may lead to the activation of genes favouring cell growth.

Various mechanisms could potentially lead to suppressed mRNA expression, including structural mutations, deletions, epigenetic modifications and other diverse regulatory mechanisms at the transcriptional or post-transcriptional levels. Epigenetic inactivation by promoter methylation has been implicated as a common mechanism underlying *RIZ1* silencing (20,26-30). In view of these findings, and our present observation of frequent suppression of *RIZ1* mRNA expression we have quantitatively assessed *RIZ1* promoter methylation in the tumor panel and four neuroblastoma cell lines. None of the tumors or cell lines analyzed showed MetI at >10% in the *RIZ1* promoter.

Previously, a few studies have undertaken to analyze *RIZ1* promoter methylation in neuroblastoma (33,43). All of these reports utilized a non-quantitative method, methylation specific PCR (MSP), and the results reveal some discrepancies. Alaminos *et al* observed 26% *RIZ1* methylation frequency in a series of 45 neuroblastomas (32). In contrast Hoebeeck *et al* found no methylation in a panel of 42 tumors (33). Divergent findings were also seen in neuroblastoma cell lines. Hoebeck *et al* and Alaminos *et al* observed *RIZ1* methylation in 9 out of 33 and 5 out of 10 neuroblastoma cell lines, respectively (32,33). In contrast Van Noesel *et al* found no methylation in 22 neuroblastoma cell lines (43). Furthermore, three of the cell lines reported to be methylated by Hoebeck and colleagues did not show methylation in the work by van Noesel *et al* (33,43). These discrepancies could

potentially arise due to differences in assay sensitivity and different CpGs assessed. MSP is generally a highly sensitive method (44). This feature together with the fact that the technique is non-quantitative may result in classifying a tumor as methylated on the basis of a minor proportion of methylated template. Besides technical restraints, conflicting methylation data in cell lines may also possibly arise due to epigenetic plasticity associated with propagation of cells in culture (45). Here we have quantified methylation density of the *RIZ1* promoter for several consecutive CpGs by Pyrosequencing. Our findings indicate that *RIZ1* promoter hypermethylation is typically present only in a small proportion of tumor cells (<10%). None of the 33 neuroblastomas showed high methylation levels, suggesting that *RIZ1* P1 methylation is unlikely to contribute to tumor progression.

Taken together, our data provide further evidence that *RIZ* is a target tumor suppressor gene in 1p36.2 in unfavourable neuroblastomas. Although *RIZ1* promoter methylation frequently occurs in other neoplasias, it is uncommon in neuroblastomas. It is highly possible that a more aggressive behaviour and poor prognosis for 1p deleted neuroblastomas is a composite result of expressional suppression of several tumor suppressor genes in this chromosomal region, of which, *RIZ1* represents one attractive target.

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