Aberrant DNA hypermethylation and apoptotic defects in pediatric neuroblastomas
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Chapter 4

Clustering of hypermethylated genes in neuroblastoma

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Abstract
CpG-island hypermethylation of gene promoters is a frequent mechanism for gene inactivation in tumors. Many neuroblastomas have hypermethylation and down-regulation of CASP8, leading to resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). We recently found hypermethylation of the four TRAIL receptors in 9 neuroblastoma cell lines. Here, we analyzed methylation of 34 genes in 22 neuroblastoma cell lines. Of the 29 newly analyzed genes, only FLIP at 2q33 was methylated in 8/22 cell lines. The FLIP protein is a negative regulator of Caspase 8. FLIP maps adjacent to CASP8 and their methylation patterns showed a moderate correlation. Furthermore, co-methylation patterns were observed for the TRAIL receptor pairs DCR1 and DCR2 and between DR4 and DR5. All four receptors co-localize in chromosome band 8p21. The 6 genes methylated in neuroblastomas appeared to occur in pairs. The genes within each pair have a strong sequence homology, and originated from gene duplication. We found no evidence for regional spreading of methylation, as we did not observe de novo methylation in additional local CpG islands. However, the gene pairs showed a striking co-regulation at the mRNA expression level. Down-regulation of FLIP strongly corresponds with down-regulation of CASP8, and this was also found for DCR1 and DCR2. Only a subset of the down-regulated genes was methylated. This suggests a mechanism of co-regulated transcriptional silencing of the gene pairs, followed by a methylation event that is less penetrating. The methylation pattern therefore supports a model in which CpG islands are not randomly targeted by methylation in cancer. Specific transcriptional silencing probably marks genes that can become methylated.
Introduction

Epigenetic silencing of tumor suppressor genes plays an important role in the pathogenesis of most cancers. In normal cells, only a few genes are transcriptionally regulated by DNA methylation, including imprinted genes and genes on the silenced X-chromosome. Human cancer cells, however, display epigenetic instability, which is characterized by global CpG demethylation of the bulk chromatin, and hypermethylation of normally unmethylated promoter-associated CpG islands. Similarly to genetic aberrations in cancer, each cancer type appears to display a unique pattern of genes affected by hypermethylation of promoter CpG islands. For instance, methylation of MGMT and APC are frequently observed in colorectal cancer, p14ARF in gastric tumors, BRCA1 uniquely in breast and ovarian cancer, MLH1 in colorectal, endometrial and gastric tumors with microsatellite instability, and TP73 and CDKN2B methylation has been observed in hematological malignancies.\(^1\)-\(^2\)

Hypermethylation of promoter CpG islands is associated with formation of regional heterochromatin, and attraction of transcriptional repressors. The role of methylation in this model is supported by the physical interaction between key elements of repressed heterochromatin such as histone deacetylases, transcriptional repressors, methylated lys9 residues in histone H3 and several components of the methylation machinery, like the DNA methyltransferases (DNMT1, 3A and 3B) and the methyl-binding proteins (MECP2 and MBD1).\(^3\)

Despite the emerging patterns of aberrant methylation of cancer genomes and the biological model for transcriptional repression, the initiating mechanisms underlying CpG island hypermethylation are still poorly understood. Foremost is the question whether aberrant methylation is initiated by the repressor complexes in a gene-specific manner, or as a random process, initiated by an aberrant methylation machinery that randomly targets genomic sequences.

In pediatric neuroblastomas several potential tumor suppressor genes are frequently hypermethylated and down-regulated. This was particularly found for genes of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway to apoptosis (CASP8, and the receptors TNFRSF10A, TNFRSF10B, TNFRSF10C, and TNFRSF10D, further referred to as DR4, DR5, DCR1, and DCR2, respectively).\(^4\)-\(^6\) In this study we analyzed the methylation pattern of 34 genes at 12 different chromosomal regions in 22 neuroblastoma cell lines. The analysis included the mentioned genes of the TRAIL pathway as well as 7 additional genes involved in apoptosis. Furthermore, 18 genes in chromosome band 1p36 and 4 genes associated with carcinogenesis, like CDKN2A/p16INK4A, were analyzed.

Materials and methods

Cell Culture

Cell lines were cultured at 37 °C, 5% CO\(_2\), using Dulbecco’s Modified Essential Medium (DMEM; Gibco) containing 10% Fetal Calf Serum, 292 μg/ml L-Glutamine, 1% 100x MEM (Non-essential amino acids medium, Gibco) and 0,5% Penicillin/Streptomycin solution.
RT-PCR Detection of mRNA

Total RNA was isolated from cell lines using RNAzol™ B (Cinna, Biotec Laboratories Inc) according to manufacturer’s protocol. First strand cDNA synthesis was performed on 2 μg of total RNA in a volume of 20 μl using Superscript™ II (GibcoBRL) and oligo (dT). The specific primers used for mRNA amplification were as follows:

**FLIP** (accession nr GI: 4505246) F (235): GTTCAAGGAGCAGGGACAAG; R (436): TGCAATC GATTATCAGGCAG; Primers for additional genes have been described previously⁶, or available on request.

Analysis of expression was performed in a 25 μl PCR reaction containing 1 μl of cDNA, 1 μl dNTP’s (2.5 mmol/l each), 0.5 μl each of the specific primers (150 ng/μl), and 0.25 μl Taq DNA polymerase (5 U/μl; Boehringer). PCR conditions were as follows: 1 cycle, 5 min/95 °C; 35 cycles, 1 min/95 °C, 1 min/60 °C, 1 min/72 °C, and one cycle 5 min/72 °C. PCR products were loaded on a 4% agarose gel (Metaphor; BioWhittaker Molecular Applications, USA), stained with Gelstar nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA) and directly visualized under UV illumination.

Methylation-specific PCR (MSP)

Genomic DNA was isolated from cell lines and primary tissues, using standard procedures. Procedures for bisulfite treatment and PCR analyses were performed as described⁶,⁷. The primer sequences for **FLIP** are (5’ to 3’): TGGTTATTTGTTAGTTTTTTTGGAGT (unmethylated sense); GTTATTTGTAGTTTTTTCGGAGTC (methylated sense); AATAACAAACAACAAAAATCCA (unmethylated antisense); CAACGCACAAAAAAATCCCG (methylated antisense). Expected PCR products 211 bp (U) / 203 bp (M).

The specific MSP primer sequences for **TNFRSF10A/DR4**, **TNFRSF10B/DR5**, **TNFRSF10C/DCR1**, **TNFRSF10D/DCR2**, and **CASP8** were as described⁶. Expected PCR products for **DR4**: 91 (methylated)/102 (unmethylated) bp, **DR5**: 199/208 bp, **DCR1**: 125/135 bp, **DCR2**: 138/145 bp, **CASP8**: 321/322 bp. Primer sequences of additional genes available upon request.

PCR reactions were hot started at 95 °C for 15 min, by using 0.25 μl (5 U/μl) of HotStarTaq DNA polymerase (Qiagen). Reactions were performed at 60 °C annealing temperature. Each PCR reaction was loaded on a 6 % non-denaturing polyacrylamide gel, stained with ethidium bromide and directly visualized under UV illumination. Genomic DNA treated with Sss1 methylase (New England Biolabs; as instructed by manufacturer’s protocol) and after bisulfite modification was used as positive control for methylated DNA.

Statistical Analysis

Statistical significance was determined by the Fisher’s exact, two-tailed probability test. The analysis of the correlation between expression of **FLIP** and **CASP8** was complicated by faint expression bands for **FLIP** in several cell lines. The Fisher’s exact test reached p=0.001 if low expressing cell lines were
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considered to lack expression of FLIP (LAN-6, SJNB-1, 110B, IMR32, TR14, SK-N-BE, LAN-2, NMB). It reached a p=0.12 if these same cell lines were considered as expressing the FLIP mRNA. The association between methylation and expression of FLIP was significant (p=0.05) for both considerations of the FLIP expression pattern. The significance of the correlation between amplification of MYCN and the expression of FLIP varied between p=0.004 and p=0.05.

**Results**

Methylation Pattern of 34 Genes in Neuroblastomas

Promoter-associated CpG islands of 34 genes were analyzed by MSP in a panel of 22 neuroblastoma cell lines and 6 normal tissues. Eighteen genes were selected from chromosome band 1p36, a region of frequent loss of heterozygosity in neuroblastoma. Furthermore, we analyzed several potential tumor suppressor genes and a series of genes of the TRAIL pathway (Table 1). There were 28 genes unmethylated in all cell lines (Table 1). We detected methylation of one gene, FLIP, that was not previously identified as a target of methylation. FLIP was hypermethylated in 8/22 (36%) neuroblastoma cell lines (Fig. 1), but not in any of the 6 analyzed normal tissues (Figure 1 and Table 1). Some cell lines were hemi-methylated (AMC106, SJNB-8, NMB). The FLIP gene maps next to the CASP10 and CASP8 genes in chromosome band 2q33 (Fig. 2a). Previously, we established

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Table 1. Methylation Pattern of 34 genes in 22 neuroblastoma cell lines and 6 normal tissues.
Black boxes: Methylated; White boxes: Unmethylated

<table>
<thead>
<tr>
<th>Chromosomes</th>
<th>Cell Lines</th>
</tr>
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<tbody>
<tr>
<td>1p36</td>
<td>NN-1, SK-N-SH, SK-N-FI, SK-N-AS, LAN-6, SJNB-1, GI-ME-N, 110B, ABC166, SJNB12, IMR32, SJNB8, KNCN, TR14, SK-N-BE, N-206, SJNB6, LAN-2, LAN-1, SJNB 10, NMB</td>
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<tr>
<td>Lung</td>
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<td>Fibroblast</td>
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<td>PBLs</td>
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the methylation patterns for CASP8 in 9 neuroblastoma cell lines\(^6\). In the present study, we expanded the analyses to the complete panel (Fig. 1 and Table 1). CASP8 was methylated in 17/22 (77\%) cell lines, including LAN-6, which has a faint unmethylated band. Three cell lines were hemi-methylated for CASP8, and two cell lines (SK-N-AS and LAN-2) completely unmethylated (Fig. 1). The frequent methylation of the two neighboring genes strongly contrasts to the lack of methylation in most other analyzed genes and suggests a shared underlying mechanism. However, it is remarkable that CASP8 and FLIP are not concomitantly methylated in all cell lines. Of the 8 cell lines methylated for FLIP, 7 have methylation of CASP8, but 10 additional cell lines with CASP8 methylation lack methylation of FLIP.

We furthermore analyzed the methylation of the four TRAIL receptors DCR1, DCR2, DR4 and DR5 in the cell line panel, as previous analysis of a limited cell line series detected methylation of these genes\(^6\). The four TRAIL receptors map directly adjacent to each other in a small region in chromosome band 8p21. DCR1 and DCR2 were methylated in 11/22 and 14/22 cell lines respectively (Table 1). Although the methylation of these two genes is more strictly correlated (p=0.02, Fisher’s exact two-tailed test) than in the case of CASP8 and FLIP, also here several cell lines show methylation of one of the neighboring genes only. DR4 and DR5 were methylated in 4 and 3 cell lines respectively (Table 1). Two cell lines showed methylation of both DR4 and DR5. None of the TRAIL receptors were methylated in six analyzed normal tissues.

**Analysis of Loco-regional Spreading of Methylation**

The analysis of 34 genes on 12 chromosomal regions showed clustering of de novo methylation in chromosomal bands 2q33 and 8p21, which strongly contrasts with the absence of methylation in the remaining 28 genes from 10 chromosomal regions. The co-methylation of neighboring genes suggests that the adjacent position of the genes plays a role in the mechanism of methylation. One possibility is that co-methylation results from loco-regional spreading of methylation to neighboring genes. We therefore analyzed the methylation status of promoter-associated as well as non-promoter CpG islands in the FLIP/CASP10/CASP8 cluster at 2q33 and the DCR1/DCR2/DR4/DR5 cluster at 8p21. At 2q33, there was only one additional non-promoter CpG island, 14 kb upstream of CASP10, which was not subject to de novo methylation since it was densely methylated in all cell lines and normal tissues. At 8p21, four additional CpG islands were identified, each
spanning more than 0.3 kb, and with a more than 50% GC content (Fig. 2). The density of methylation per CpG island was consistent throughout the tumor cell line panel and the 6 normal tissues (Fig. 2). Moreover, their methylation was equal in the cell lines with or without methylation of the TRAIL receptor gene(s). To complete the analyses, we tested the methylation status of the genes flanking the methylated gene clusters. The genes *NDUFB3* and *ALS2CR3* flank the cluster at 2q33 and the cluster at 8p21 is flanked by IMAGE clone 5093453 and the gene *RHOBTB2*. The promoter-associated CpG islands of the four genes were completely unmethylated in all neuroblastoma cell lines and normal tissues (Fig. 2). Therefore, the aberrant de novo methylation of the two clusters at 2q33 and 8p21 was restricted to the promoter-associated CpG islands of structurally related genes, without evidence of spreading to neighboring CpG dense areas or genes. These data do not support a model of regional spreading of methylation from one CpG island to another.

**Expression of DCR1 and DCR2 is Highly Correlated**

An alternative explanation for the observed co-methylation of the gene pairs was suggested by an expression analysis of these genes in the cell line panel. We previously established an association between hypermethylation and down-regulation of expression for the TRAIL-receptors *DR4*, *DR5*, *DCR1* and *DCR2* in 9 neuroblastoma cell lines. We extended the expression studies by semi-quantitative RT-PCR of the complete cell line panel. There appeared to be a striking correlation
between the expression of DCR1 and DCR2 (p=0.01) (Fig. 3). Only three of the 22 cell lines showed a discordant expression, while 19 cell lines were concordant with 6 expressing and 13 non-expressing cell lines. There was also a good correlation between down-regulation of the expression and methylation for DCR1 (p=0.012) and DCR2 (p=0.008). Methylated genes were always down regulated, except for the cell lines SK-N-BE and N206, which showed variable expression of DCR2 in repeat experiments, while the gene was consistently methylated. However, down-regulation was not always associated with hypermethylation of the genes. As a result, in some cell lines, like SJNB1 and TR14, DCR1 and DCR2 are both switched off, while only DCR2 is methylated. The strong correlation between the expression of DCR1 and DCR2 suggests that both genes are coordinately down regulated. The incomplete correlation with methylation suggests that methylation is not a prerequisite for this down-regulation, but rather an additional effect that may or may not occur.

Expression of FLIP and CASP8 is Highly Correlated
The co-regulation of DCR1 and DCR2 urged us to analyze whether the other pair of methylated genes, CASP8 and FLIP show a co-regulation as well. Semi-quantitative RT-PCR indeed showed highly correlated expression patterns of FLIP and CASP8 (p=0.001; see Materials and Methods) (Fig. 3). Six of 22 cell lines showed expression of both genes, while 7 cell lines showed a complete lack of expression of both genes. Eight CASP8 negative cell lines had a very weak FLIP expression. Only SJNB-10 showed a clearly discordant pattern, as it expressed FLIP but not CASP8. Methylation is significantly associated with down-regulation of CASP8 (p=0.009), but for FLIP the significance is dependent on the interpretation of the low-expressing cell lines (see Materials and Methods). Similar to the methylation patterns of DCR1 and DCR2, methylation was found in a subset of the down-regulated genes only. CASP8 was silenced but not methylated in one cell line and this situation was found for FLIP in 7 cell lines. The inverse situation, expression of methylated alleles, was only found in two cell lines for CASP8 and never for FLIP. The expression and methylation patterns of CASP8 and FLIP suggest that the down-regulation of the expression is highly coordinated,
while not all down-regulated genes become methylated. To our knowledge, no specific repressors of CASP8 and FLIP have been identified. Previous studies have suggested a correlation between MYCN amplification and CASP8 down-regulation. The MYCN transcription factor is frequently amplified in neuroblastoma\textsuperscript{8,9}. In our panel, we found a strong correlation between amplification of MYCN and the down-regulation of CASP8 (p=0.004) and FLIP (p=0.004 to p=0.051; see Materials and Methods). Fourteen of 15 MYCN amplified cell lines lacked expression of CASP8 while 5/7 MYCN single copy cell lines expressed CASP8. There was no correlation between amplification of MYCN and DCR1 and DCR2 expression. Our data support a model in which methylation of CpG islands does not randomly affect CpG islands in cancer cells, but is associated with specific transcriptional silencing of genes.

**Discussion**

Aberrant methylation of promoter-associated CpG islands is a well-recognized mechanism for down-regulation of tumor suppressor genes in cancer. Hypermethylation of the apoptotic genes CASP8 and the TRAIL receptors seems to play a role in the pathogenesis of neuroblastomas\textsuperscript{6,10}. Here, we describe the methylation pattern of 34 genes in neuroblastomas. De novo methylation was only observed in one additional gene, FLIP, and not in 28 other genes. The complete lack of methylation in several tumor suppressor genes from important regions such as 1p36 and others implies that aberrant methylation does not spread randomly over the genome, but seems to follow a clear, non-random pattern of distribution.

The six methylated genes described here include only intermediates of the TRAIL pathway to apoptosis, and cluster in two chromosomal regions. DCR1 and DCR2 are structural and functional homologues. FLIP, the gene with de novo methylation described here is a structural homologue of CASP8, but the FLIP protein acts as a functional inhibitor of the Caspase 8 protein. In view of its function, it is unclear how down-regulation of FLIP could alter or influence the phenotype of a tumor cell with down-regulated CASP8. In the absence of the Caspase 8 protein, the apoptotic route is blocked in all instances, regardless of the FLIP expression. The reverse, expression of both, most probably reflects the normal cellular homeostasis in pro- and anti-apoptotic signals. Down-regulation of FLIP alone was not observed in any cell line. Interestingly, we observed strikingly similar expression patterns for the gene pairs CASP8/FLIP and DCR1/DCR2 respectively, suggestive of co-regulation of transcription. The co-methylation of FLIP and CASP8 or DCR1 and DCR2 was not correlated as strong as the pattern of co-expression. For the four analyzed genes in the 22 cell lines, we observed a total of 15 silenced genes that were not methylated. In contrast, only three cases were observed of a methylated gene that was expressed. How do these data reflect the current models describing the relationship between gene hypermethylation and transcriptional repression? Two opposing models can be envisaged. Aberrant methylation randomly targets CpG islands, and occasionally inactivates a tumor suppressor gene. Alternatively, aberrant methylation
is an exponent of a suppressor-complex that targets specific genes. Support for the first model in which methylation has an initiating role in the silencing of genes comes from studies on DNA methyltransferases (DNMT's). Over-expression of ectopically expressed DNMT's can lead to de novo methylation of promoter CpG islands and gene silencing\textsuperscript{11, 12}, whereas demethylation can induce re-expression of silenced methylated genes\textsuperscript{13}. Another observation in line with this model is that in tumors caused by an inherited mutation in one allele of a tumor suppressor gene, the other allele can be inactivated by methylation\textsuperscript{14, 15}. This argues against methylation as an endpoint of silencing by transcriptional repressors, as this would likely act on both alleles of the tumor suppressor gene. These data support the view that methylation alone is sufficient for silencing of genes and not a consequence of other mechanisms of gene silencing. However, the alternative model, which explains aberrant methylation in cancer as a result of transcriptional silencing, is supported by recent data concerning the role of methylation in normal expression regulation. They suggest that DNA hypermethylation marks an endpoint of transcriptional silencing which locks the gene into a permanent state of silencing. This is based on the observation that hypermethylation of promoter regions is strongly associated with deacetylation of histone tails\textsuperscript{16}, and the attraction of transcriptional repressor complexes like human Sin3 and histone deacetylation complexes\textsuperscript{17}. Deacetylation of histones changes the so-called histone code, a process that includes methylation of histone residues as well. Especially methylation of histone 3 at lysine 9 (H3-K9) seems important, which is in a variety of organisms an important bridging step towards DNA methylation\textsuperscript{18-21}. In this model, epigenetic silencing of genes is a multi-step process with DNA methylation as an endpoint, leading to a permanent gene repression. The unusual clustering of silenced genes in neuroblastoma cell lines reported here best fits the latter model, in which silencing by transcriptional repressors precedes methylation. Methylation would in this model not be fully penetrant, leaving some genes in a silenced but unmethylated state. This interpretation of our data predicts that the gene pairs share a transcriptional repressor that is active in the tumor cell lines. Others and we have observed a strong correlation between silencing of \textit{CASP8} and amplification of \textit{MYCN}.\textsuperscript{10} We observed the same correlation between \textit{FLIP} and \textit{MYCN}. The \textit{MYCN} transcription factor has been implicated in transcriptional silencing\textsuperscript{22, 23}, warranting a further analysis of its role in the silencing of \textit{CASP8} and \textit{FLIP}.
References


