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ARTICLE

# Promoter Methylation Pattern of Caspase-8, P16$^{INK4A}$, MGMT, TIMP-3, and E-Cadherin in Medulloblastoma

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**Methylation of promoter regions of CpG-rich sites is an important mechanism for silencing of tumor suppressor genes (TSG). To evaluate the role of tumor suppressor genes caspase-8 (CASP8), TIMP-3, E-cadherin (CDH1), p16$^{INK4A}$, and MGMT in medulloblastoma tumorigenesis, 51 medulloblastomas (46 primary tumor specimens, 5 cell lines) were screened for methylation of promoter linked CpG-islands. For CASP8, we examined the 5′ UTR region that has been shown to be associated with expression of CASP8. As detected by methylation specific PCR, methylation rate was low for TIMP-3 (3% of tumor samples; 1/5 cell lines), for MGMT (0% of tumor samples; 1/5 cell lines), for p16$^{INK4A}$ (2% of tumor samples; 2/5 cell lines) and for CDH1 (8% of tumor samples; 1/4 cell lines). CASP8, however, was methylated in 90% of tumor samples and 4/5 cell lines examined. Screening other tumor entities for CASP8 methylation, we found a similarly high level in 6 neuroblastoma cell lines in contrast to 5 osteosarcoma-, 4 Ewing's sarcoma- and 6 non-embryonic tumor cell lines without any increased promoter methylation. From our results we conclude that methylation of the CASP8 5′ UTR region may play a role in inactivation of CASP8 in neural crest tumors.** (Pathology Oncology Research Vol 10, No 1, 17–21)

*Keywords:* aberrant promoter methylation, medulloblastoma, caspase-8, p16$^{INK4A}$, MGMT, TIMP-3, CDH1

## *Introduction*

Medulloblastoma is the most frequent malignant brain tumor in children, arising from the external granular layer of the cerebellum. The overall event-free survival is 50-70% in nationwide studies. Aggressive invasive growth and high risk of leptomeningeal dissemination requires an intensive multimodal therapy including neurosurgery, irradiation and chemotherapy, leading to long-term sequelae like neurological deficits, endocrine disorders or impaired psychological development, especially in younger children.[22]

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*Abbreviations:* CASP8, caspase-8; MGMT, O6-methylguanine-DNA-methyl-transferase; PNET, primitive neuroectodermal tumor; TIMP-3, tissue inhibitor of metalloproteinase 3; TRAIL, TNF-related apoptosis inducing ligand; TSG, tumor suppressor gene; UTR, untranslated region.

Molecular analysis of medulloblastoma may provide insights in molecular mechanisms of tumor development, and may reveal new tools to define markers of prognostic significance, leading to sophisticated therapeutic strategies.

Aberrant methylation of normally unmethylated CpG islands, located in the promoter regions, has been associated with transcriptional inactivation of tumor-related genes in human cancers.[18] On the basis of these findings, aberrant promoter methylation is suspected of participating in the pathogenesis and progression of neoplastic diseases. Information on the methylation status in a variety of malignant tumors is available, and this suggests that each tumor type may have a distinct methylation profile.[7] Recent studies demonstrate that methylation is a common phenomenon in medulloblastoma.[11]

To define the role of promoter methylation of different circumscribed TSGs in medulloblastoma development, we studied the methylation of gene promoter linked CpG-islands of TIMP-3, p16$^{INK4A}$, E-cadherin (CDH1), and MGMT in 46 medulloblastoma specimens and 5 medulloblastoma cell lines. For CASP8 we examined the methylation status of the 5' UTR region, which is strictly correlat-

*Table 1. Characteristics of 46 medulloblastoma patients*

| | |
|---|---|
| Mean age | 6.5 years (range 1.0 to 15.1) |
| Sex (female) | 16/46 (35%) |
| Metastatic disease | 5/46 (11%) |

ed with CASP8 expression.[2,16] Although methylation of the examined region is unlikely to be the direct cause of gene silencing, it may reflect methylation in the promoter region.

The TSGs examined in this study have been shown to be involved in the development of brain tumors. They were chosen on the basis of their critical cancer-related function because they are known to frequently be hypermethylated and silenced in other neoplasms. The target genes include typical tumor suppressor genes, including an apoptosis promoting factor, angiogenesis and invasion inhibitors, and a DNA repair gene.

CASP8 is a cystein protease that induces apoptosis. Binding of TNF-related apoptosis inducing ligand (TRAIL) to death receptor (DR)4 and DR5 enables caspase-8 and the adapter protein FADD to bind to the receptor. Recruitment of caspase-8 leads to its proteolytic activation, which initiates a cascade of caspases leading to apoptosis.[6] A high methylation rate and thereby gene silencing for CASP8 in medulloblastoma has been published recently.[15,27] Tissue inhibitors of metalloproteinases (TIMPs) antagonize matrix metalloproteinase activity. TIMP-3 is bound to the extracellular matrix, inhibits TNF-alpha converting enzyme and induces apoptosis by stabilization of TNF-alpha receptors on the cell surface.[1,20] CDH1 plays an important role in cell-cell adhesion, and prevents invasive growth and metastasis.[4] Loss of $p16^{INK4A}$ may accelerate the transition through the cell cycle.[3] O6-methylguanine-DNA-methyl-transferase (MGMT) gene encodes the DNA repair enzyme O(6)-alkylguanine DNA alkyltransferase. Even though it represents a TSG, its loss improves the response of tumors to alkylating agents by the impairment of the cellular DNA repair capacity. Recently, an increased activity of MGMT in medulloblastoma has been shown.[5,9]

## *Materials and Methods*

Patients' characteristics are enlisted in *Table 1*. Staging procedures included MRI before and immediately after neurosurgery. Metastatic disease was detected by craniospinal imaging and lumbar puncture. Patients were treated according to German Standard therapy protocols of the HIT study group. Primary medulloblastoma tumor samples were obtained after neurosurgery of 46 pediatric patients, cell lines were obtained from ATCC (Daoy, D283, D341) or were kindly provided by T. Pietsch, University of Bonn, Germany (MHH1, MED-MEB-8A).

Genomic DNA was isolated from frozen cell material using a salting-out method.[21]

Aberrant promoter methylation was examined by methylation-specific PCR described previously.[17] Briefly, 1 g of genomic DNA was denatured with 2 M NaOH and modified with sodium bisulfite treatment. After purification, the DNA was treated with NaOH, precipitated with ethanol, and resuspended in water. Four microliters of bisulfite-modified DNA were amplified by PCR using primer-sets that were specific for unmethylated and methylated sequences of each gene as described previously.[1,8,19,25]

DNA was considered as methylated if a specific PCR product using methylation specific primers was seen, regardless of the signal obtained with the non-methylation specific primer (*Figure 1*).

To deal with the problem of incomplete bisulfite modification and of dilution of tumor cells by non-malignant cells, a serial dilution of methylated, bisulfite-modified DNA by unmethylated, bisulfite-modified DNA was performed. It was possible to detect 1% methylated DNA in 99% of unmethylated DNA (*Figure 2*). The non-bisulfite-modified form of $p16^{INK4A}$ was examined as a control for DNA integrity and was positive in all samples.

## *Results*

The methylation status of TIMP-3, CDH1, $p16^{INK4A}$, and MGMT were tested using methylation-specific PCR. Methylation rate in primary medulloblastoma specimens

*Table 2. Promoter methylation status of the indicated genes: number of methylated samples/total sample number*

| | *Tumor samples* | *Methylation of cell lines* | | | | |
|---|---|---|---|---|---|---|
| | | *MED-MEB-8A* | *MHH1* | *Daoy* | *D283* | *D341* |
| Caspase-8 | 36/40 (90%) | + | + | – | + | + |
| TIMP-3 | 1/38 (3%) | – | – | + | – | – |
| MGMT | 0/44 (0%) | + | – | – | – | – |
| $p16^{INK4A}$ | 1/46 (2%) | – | + | + | – | – |
| E-cadherin | 3/36 (8%) | ND | – | – | + | – |

| | *Cell line* | *Methylation rate* |
|---|---|---|
| Caspase-8 | Neuroblastoma | 4/6 |
| Caspase-8 | Osteoblastoma | 0/5 |
| Caspase-8 | Ewing's sarcoma | 1/4 |
| Caspase-8 | Non-embryonic tumors* | 0/6 |

*+, methylated loci; –, unmethylated loci; ND, not done. *6 non-embryonic tumor cell lines: 4 T-cell leukemias, 1 cervical carcinoma, 1 monocytic leukemia.*

was low for TIMP-3 (3% of tumor samples; 1/5 cell lines), for MGMT (0% of tumor samples; 1/5 cell lines), for $p16^{INK4A}$ (2% of tumor samples; 2/5 cell lines), and for CDH1 (8% of tumor samples; 1/4 cell lines) compared to normal tissue (*Figure 1*).[25,26] A slightly higher methylation rate was found for all examined TSGs in the medulloblastoma cell lines examined (*Table 2*).

The methylation rate completely differed for the CASP8 gene: 90% of the tumor samples and 4 of 5 cell lines showed positive PCR signals for methylation of the promoter-linked CpG island. With the small number of 4 unmethylated samples there was no significant correlation between methylation status and other relevant parameters like age, gender, histologic subtype, metastatic disease or outcome: 8.8 years (range 4.0-13.3); 2/4 of the patients were female; 0/4 metastatic diseases; 0/4 relapses or deaths in this group, no desmoplastic histological pattern. Although the number of patients allows no statistically significant statement, it is remarkable that none of the patients with unmethylated tumors showed metastatic disease or had a tumor relapse.

We examined the CASP8 promoter methylation status of other embryonic tumor cell lines and found methylation of the promoter in 4 of 6 neuroblastoma cell lines, 0 of 5 osteosarcoma- and 1 of 4 Ewing's sarcoma cell lines. Moreover, in 6 non-embryonic tumor cell lines (4 T-cell leukemias, 1 cervical carcinoma, 1 monocytic leukemia) no methylation of the CASP8 promoter was detectable.

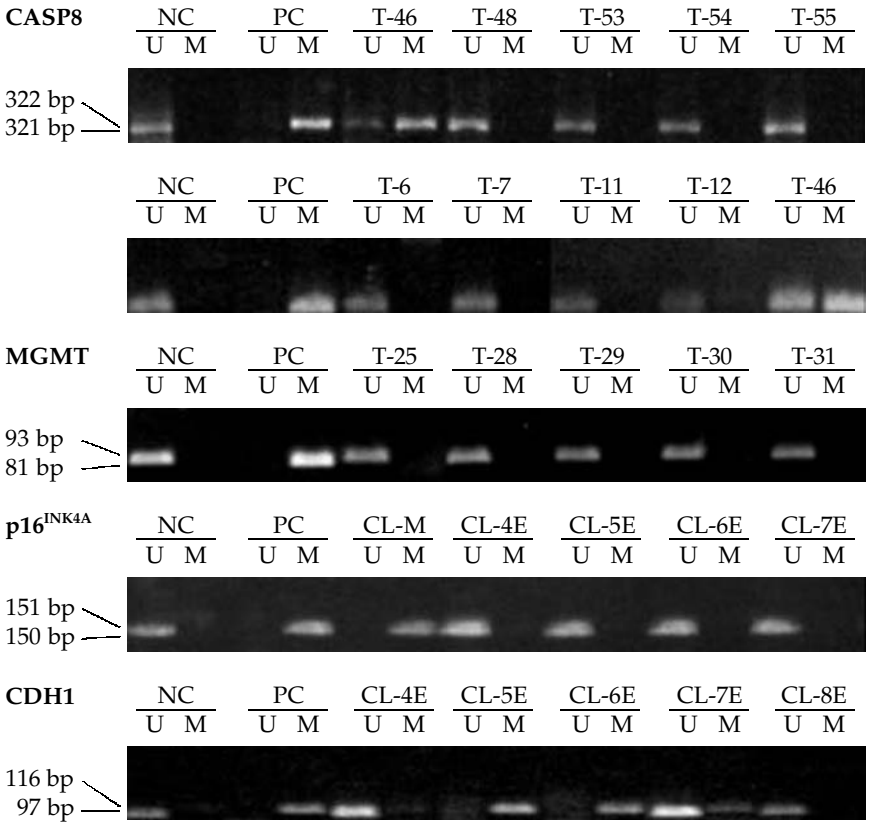


***Figure 1.*** *Methylation status as determined by MSP. The gene studied is given on the left of each panel. Lane U: amplified product with primers recognizing unmethylated sequence; lane M: amplified product with primers recognizing methylated sequence. NC, normal control; PC, positive control for methylation. The PCR product sizes of all of the genes are shown on the left. Many of the methylation positive tumors showed both M- and U-signal, due to incomplete methylation of the DNA in the tumor tissue and/or contamination with non-malignant cells (e.g. CASP8, TIMP, T-46).*

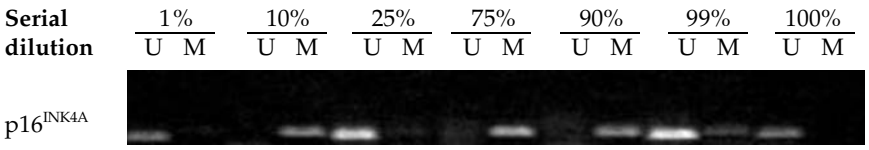


***Figure 2.*** *MSP analysis of a serial dilution of methylated DNA by unmethylated DNA after modification by bisulfite treatment. Indicated is percentage of methylated DNA in total DNA (50 ng).*

## *Discussion*

While de novo methylation of CpG islands is rare in normal tissues, tissue culture cells and malignant tumor cells both display a genome-wide increase in CpG island methylation.[18] Promoter CpG island methylation is a powerful mechanism of gene silencing: a high density of methylated cytosine residues leads to reduction of transcriptional activity. Aberrant methylation of individual CpG islands may result from selective pressure, or may reflect loss of protection of CpG islands against DNA methylation.[18]

*Costello et al.* screened the methylation status of 1184 unselected CpG islands in 98 primary human tumors of 7 different entities including PNET, using the technique of

Restriction Landmark Genomic Scanning, and were able to demonstrate a specific methylation pattern for each of the 7 tumor types. For PNET, they predicted aberrant methylation at an average of 420 CpG islands, assuming 45,000 CpG islands per genome.[7] In another study using the same technique, even the methylation pattern differed between medulloblastoma and other PNET.[11] In our study of medulloblastoma tumor specimens, four tumor suppressor genes (TIMP-3, CDH1, $p16^{INK4A}$, and MGMT) displayed a low level of methylation, comparable to non-malignant tissues.[26] The CASP8 5' UTR region, however, was frequently methylated in these tumors. Our data are congruent with the hypothesis that methylation of circumscribed genes is a specific phenomenon in medulloblastoma, which does not occur randomly.

Recent studies support our data of a high methylation rate of CASP8 5' UTR, and showed loss of CASP8 mRNA in medulloblastoma.[15,16,27] The high methylation rate of 90% in the present study may be due to the method generating qualitative, but not quantitative results (see *Figure 2*). *Harada et al*. have published a similar rate (81%) using the same technique.[16]

Methylation of the CASP8 5' UTR region was found in 4 out of 6 neuroblastoma lines, another malignant tumor of neuroectodermal origin, but only in 1 out of 4 Ewing's sarcoma and none of 5 osteosarcoma cell lines. Furthermore, in 6 non-embryonic tumor cell lines no methylation of the CASP8 promoter was seen. From our results we conclude that methylation of the CASP8 5' UTR region may play a role in inactivation of CASP8 in neural crest tumors. This observation is supported by a recent study by *Harada et al*.[16]

The CASP8 gene codes for a cystein protease and is located on human chromosome 2q33.[14] This region is not associated with long-range loss of heterozygosity in medulloblastoma, as shown by comparative genomic hybridization studies. In neuroblastoma, another tumor with a high rate of CASP8 methylation, no functional mutations of CASP8 have been described, but the absence of mRNA and gene product in methylated cell lines suggest that methylation is a crucial mechanism of gene silencing of CASP8.[25]

Chemotherapeutic agents act via death receptors and activation of the caspase cascade.[10,12] Silencing of CASP8 in medulloblastoma may result in resistance to drug-induced apoptosis, and may be important for further therapeutic strategies. Inhibitors of DNA methylation may provide a useful tool for restoring sufficient CASP8 activity in medulloblastoma. Demethylation by 5-aza-2'-deoxycytidine (Decitabine) leads to restoration of CASP8 mRNA and TRAIL-sensitivity in D458 and CHOP707 human PNET cells.[15] Introduction of inhibitors of methyltransferase,[23] or gene transfer of functional CASP8 may be another way to restore proper CASP8 activity. Co-application of an apoptotic agent and INF again induces apoptosis by up-regulation of CASP8 through the Stat1 pathway, even in cell lines with CASP8-silencing by methylation.[13]

Therefore, our data, as well as results of other groups,[15,16,27] may shed light on new therapeutic options to treat medulloblastoma according to its specific molecular properties.

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