High expression of DNA methyltransferases in primary human medulloblastoma

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Abstract

Epigenetic alterations have been implicated in cancer development. DNA methylation modulates gene expression, which is catalyzed by DNA methyltransferases (DNMTs). The objective of our study was to evaluate expression of DNMTs in medulloblastoma and analyze its correlation with clinical features. Nuclear expression of DNMT1, DNMT3A and DNMT3B was analyzed in human primary medulloblastoma of 44 patients using immunohistochemistry. Correlation of expression of DNMT levels with classical histological subtypes, novel molecular subgroups and survival of patients was analyzed. Elevated expression of DNMT1, DNMT3A and DNMT3B was observed in 63.64%, 68.18% and 72.73% of all cases, respectively. None of them showed a correlation with classical histology or survival. Concerning molecular subtypes, significantly higher expression of DNMT1 was observed in the SHH group compared to non-SHH samples (p = 0.02), but without significant difference in DNMT3A or DNMT3B levels between any subtypes. In conclusion, DNMT1, DNMT3A and DNMT3B are highly expressed in human medulloblastoma samples, suggesting that promoter hypermethylation may play a role in medulloblastoma development. Demethylation of tumor suppressor gene promoters may be considered as a possible future target in therapy of medulloblastoma.

Key words: medulloblastoma, DNA methyltransferases, survival, SHH.

Introduction

Medulloblastoma (MB) is the most frequent malignant brain tumor in childhood, with various histological appearances. There are non-overlapping histological (classic, desmoplastic, extensive nodular, large cell/anaplastic) and molecular classifications (SHH, WNT, Group 3 and 4) for medulloblastoma [3,6,11,23,25,27,34]. Despite multimodal therapies, the 70-75% long-term survival rate has not been exceeded yet in high-risk patients [13,29,34]. A more precise classification of patients would help to develop a more effective treatment strategy and reduce side effects [3,11,23].

There is emerging evidence that epigenetic changes contribute to carcinogenesis. The most important mechanisms of epigenetic alteration are DNA methylation, histone modification and microRNA regulation. DNA methyltransferases (DNMTs) are enzymes transferring methyl groups to cytosine in CpG dinu-

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Tímea Pócza, Tibor Krenács, Eszter Turányi, János Csáthy, Zsuzsanna Jakab, Peter Hauser

cleotides, resulting in gene silencing. The DNMT family has three active members: DNMT1, DNMT3A and DNMT3B. DNMT3A and DNMT3B are regarded as de novo methyltransferases, whilst DNMT1 reacts on hemimethylated DNA, copying the methylation pattern during replication. Generally, tumor cells are characterized by global hypomethylation and hypermethylation of certain gene promoters. Therefore, repetitive elements are hypomethylated and specific tumor suppressor genes are repressed due to hypermethylation. These may contribute to chromosomal instability and abnormal gene silencing that could promote tumor-initializing steps [2,9,14,28,38]. Promoter hypermethylation of tumor suppressor genes and DNA repair genes has been observed in a wide variety of tumors, such as colorectal, breast cancer, glioma and medulloblastoma [19,22,36]. Overexpression of DNMTs was detected at the mRNA or protein level. Increased DNMT1 expression was described in gastric cancers, along with DNMT3A in breast and pancreatic cancer, or along with DNMT3B in glioma [8,16,30,38,39]. Although there is emerging evidence that epigenetic events play a remarkable role in the development of adult cancers, our knowledge about their role in pediatric brain tumors is still very limited.

DNA methylation studies in medulloblastoma have focused on the role of tumor suppressor genes in the development of brain tumors, and shed light on contradictory results in the majority of genes, except for RASSF1A [37]. Recent studies indicate that the DNA methylation profile can be used for MB subgrouping and disease risk assessment [18,35].

The aims of our study are to characterize the expression of DNMTs in human medulloblastoma samples and their potential prognostic role in survival of patients. Possible correlations of DNMT proteins and clinicopathological features are also analyzed.

Material and methods

Medulloblastoma tissue samples

Formalin-fixed, paraffin-embedded MB tumor samples were collected from the National Institute of Neurosciences (Budapest, Hungary) and 1st Institute of Pathology and Experimental Cancer Research, Semmelweis University (Budapest, Hungary). Forty-four primary medulloblastoma samples removed surgically between 2004 and 2010 and related clinical data of patients derived from the Hungarian Pediatric Cancer Registry were used. The study was approved by the Ethics Committee of the Institutional Ethical Review Board of Semmelweis University (TUKEB No. 30/2015).

Tissue microarray (TMA) construction and immunohistochemistry

Hematoxylin and eosin (HE) stained sections were reviewed by a neuropathologist to select representative areas for producing TMA. TMA blocks were created by a computer-controlled TMA Master (3DHISTECH, Budapest, Hungary) instrument. Depending on sample quality one or two cores of representative areas with a diameter of 2 mm were taken from each tumor sample. From the TMA blocks 4 μm sections were created. Sections were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked. Heat-induced antigen retrieval was performed with Tris-EDTA buffer (pH = 9.0) for 45 min in a microwave oven. Sections were incubated overnight using anti-DNMT1 antibody (dilution 1 : 200; Ab19905, Abcam, Cambridge, UK), anti-DNMT3A antibody (dilution 1 : 600; Ab13888, Abcam, Cambridge, UK) and anti-DNMT3B antibody (dilution 1 : 200; Ab13604, Abcam, Cambridge, UK) diluted in 5% BSA containing Tris-buffered saline (TBS; pH = 7.4). Antigen development was performed by Novolink Polymer Detection System (Novocastra, Wetzlar, Germany) following the manufacturer's instructions. Reactions were visualized by 3,3'-diaminobenzidine (DAB) (DAKO, Denmark) as a substrate, and then slides were counterstained by hematoxylin. Tonsil for DNMT1, and kidney for DNMT3A and DNMT3B were used as positive tissue controls.

For determination of the WNT subgroup anti-β-catenin antibody (dilution 1 : 150; M-3539, DAKO, Denmark) and of the SHH subgroup anti-secreted frizzled-related protein 1 (SFRP1) antibody (dilution 1 : 1500; ab-4193, Abcam, Cambridge, UK), characteristic proteins of these pathways, were applied. Immunohistochemical reactions were executed as described above, but antigen retrieval was performed in TE buffer boiled in a pressure cooker for 20 minutes.

Evaluation of immunostaining

Slides were digitalized by a Pannoramic Scan instrument and examined by Pannoramic Viewer

software (3DHISTECH, Budapest, Hungary). To evaluate DNMT expression only nuclear staining was considered. Intensity of staining and rate of positive cells were calculated from 1,000 cells in a representative area of each sample. According to the intensity of staining, four values were created: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The proportion of positive cells was ranked in five groups: 0 (negative), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The products of the two values (0-12) were divided into two groups: 0-3 regarded as no/weak expression and 4-12 regarded as moderate/strong expression. Samples were classified into WNT or SHH subgroups when the proportion of positive cells with nuclear β-catenin or negative nuclear β-catenin and positive SFRP1 staining was above 10% [7,27].

Statistical analysis

All statistical analysis was performed using Statistica software (StatSoft Inc., Tulsa, OK, USA). Survival of patients was calculated by the Kaplan-Meier method. Correlation of expression of DNMTs and survival was examined by the log-rank test. Correlation of expression of each DNMT was analyzed by the Spearman rank order test. Correlation of expression of DNMTs and age, gender, histological subtype or molecular subgroups was examined by the Mann-Whitney U-test and Fisher's exact test, when appropriate. Results were considered statistically significant when the p value was less than 0.05.

Results

Patients' data

Demographic characteristics of the studied cohort are summarized in Table I. Median age of 44 patients was 8.5 years (range 1.1-28.7 years). The proportion of male patients was 55%. Histologically, 84.1%, 11.4% and 4.5% of patients showed classic, desmoplastic and large cell/anaplastic (L/A) type of medulloblastoma, respectively. Survival data were available in 40 patients (90.9%). Median follow-up time was 5.6 years (range 0-9.7 years). Patients were treated according to the Hungarian MBL2004/2008 schedules [15]. There is no standard treatment for adults with medulloblastoma in Hungary. Patients are treated according to the individual decision of each treating physician, and these treatment details are poorly accessible in Hungary. Therefore, adult patients were omitted from the survival analysis.

Expression of DNMTs in human medulloblastoma

Elevated (moderate/strong) expression of DNMT1, DNMT3A and DNMT3B was observed in 63.64%, 68.18% and 72.73% of patients, respectively (Table I). Representative stains are shown in Figure 1. The relationship of histology and DNMT expression was evaluated only in the classic and desmoplastic type. Moderate/strong expression of DNMT1 was detected in 62.2% of classic and 60.0% of desmoplastic, of DNMT3A in 70.3% of classic and 60.0% of desmoplastic, and of DNMT3B in 70.3% of classic and 80.0% of desmoplastic samples. We could analyze only two samples of L/A subtype. Both of them showed moderate/strong expression for DNMT1 and DNMT3B, but only one for DNMT3A.

Correlation between DNMT expression and patients' data

There was no correlation between the expression of DNMT1, DNMT3A and DNMT3B and age at disease onset or gender or histological subtype (Table II). Kaplan-Meier curves, based on different expression of DNMTs, did not show a significant difference in overall survival (Fig. 2). None of the DNMTs could be used as a prognostic marker for medulloblastoma in our cohort. DNMT1 and DNMT3A ($p = 0.08$), or DNMT1 and DNMT3B ($p = 0.17$) or DNMT3A and DNMT3B ($p = 0.69$) did not show a co-expression pattern. Expression of nuclear β-catenin was shown only in one patient (2.3%) with weak staining. There were 12 (27.3%) patients belonging to SHH subgroup according to their cytoplasmic, membranous or secreted SFRP1 expression and lack of nuclear β-catenin (Table I). Expression of DNMT1 in the SHH subgroup compared to the non-WNT/non-SHH subgroup of patients was significantly higher ($p = 0.02$), whereas expression of DNMT3A and DNMT3B did not differ significantly between different groups ($p = 0.78$ and $p = 0.17$, respectively, Table II).

Discussion

In recent years a new prognostic classification of medulloblastoma was introduced by molecular findings, whilst there are still several twists in this classification system. It is still motivating to find further prognostic markers [3,6,11,23,25,27,34]. Nowadays, an exact diagnosis of pediatric brain tumors can be established based on the methylation profile of dif-

Tímea Pócza, Tibor Krenács, Eszter Turányi, János Csáthy, Zsuzsanna Jakab, Peter Hauser

Table I. Characteristics of patients and results of immunohistochemical staining of DNMTs, β-catenin, and SFRP1

	Gender	Histology	Age at disease onset (years)	State	Survival (years)	DNMT1*	DNMT3A*	DNMT3B*	β-catenin	SFRP1
1	F	Classic	1.18	Dead	0.18	0	0	0	Negative	Negative
2	M	L/A	7.84	Dead	1.47	9	4	6	Negative	Positive
3	F	Classic	5.99	Dead	4.56	4	9	3	Negative	Negative
4	F	Classic	14.04	Dead	0.00	4	6	8	Negative	Negative
5	M	Classic	10.81	Dead	7.98	4	8	8	Negative	Positive
6	F	Classic	3.31	Alive	9.38	6	8	12	Negative	Negative
7	M	Classic	13.54	Alive	9.74	8	12	12	Negative	Negative
8	M	Classic	17.09	Alive	8.99	2	0	8	Negative	Positive
9	F	Classic	10.09	Dead	3.07	2	8	8	Negative	Negative
10	M	Desmoplastic	14.54	Alive	9.08	1	2	3	Negative	Negative
11	M	Desmoplastic	5.14	Dead	0.59	4	4	6	Negative	Negative
12	F	Classic	17.90	Dead	5.02	4	2	8	Negative	Negative
13	M	Classic	3.11	Dead	0.92	2	1	4	Negative	Negative
14	F	Classic	4.71	Alive	8.54	4	6	12	Negative	Negative
15	M	Classic	15.30	Alive	7.80	2	4	2	Positive	Negative
16	F	Classic	8.48	Alive	7.24	12	2	2	Negative	Negative
17	F	Classic	14.28	Alive	8.09	4	8	8	Negative	Negative
18	M	Desmoplastic	6.63	Alive	7.90	3	12	8	Negative	Positive
19	M	L/A	13.84	Dead	1.94	4	3	12	Negative	Positive
20	M	Desmoplastic	4.34	Dead	0.09	9	12	4	Negative	Negative
21	M	Classic	8.56	Alive	7.00	2	4	12	Negative	Negative
22	M	Classic	15.46	Alive	6.52	2	8	12	Negative	Negative
23	M	Desmoplastic	2.59	Alive	6.52	4	2	4	Negative	Positive
24	M	Classic	4.50	Dead	1.92	4	12	3	Negative	Negative
25	F	Classic	9.30	Dead	5.54	8	12	12	Negative	Negative
26	M	Classic	6.81	Alive	6.63	4	2	3	Negative	Negative
27	F	Classic	8.50	Alive	6.07	8	0	12	Negative	Negative
28	M	Classic	7.70	Alive	6.02	2	8	8	Negative	Negative
29	F	Classic	1.77	Alive	6.76	9	8	8	Negative	Positive
30	F	Classic	4.92	Alive	6.56	6	8	6	Negative	Negative
31	M	Classic	22.90	NA	NA	6	4	8	Negative	Positive
32	M	Classic	10.76	Dead	5.22	2	2	4	Negative	Negative
33	F	Classic	21.43	NA	NA	6	0	12	Negative	Positive
34	F	Classic	16.71	Alive	5.67	2	12	1	Negative	Negative
35	M	Classic	7.92	Alive	5.48	1	1	4	Negative	Negative
36	F	Classic	10.75	Alive	5.65	4	4	3	Negative	Negative
37	F	Classic	1.78	Dead	0.48	2	8	6	Negative	Negative
38	M	Classic	4.63	Alive	4.99	4	3	4	Negative	Negative
39	M	Classic	28.25	NA	NA	9	4	6	Negative	Positive
40	F	Classic	1.07	Alive	3.23	6	12	3	Negative	Negative
41	F	Classic	2.84	Alive	3.12	2	8	2	Negative	Negative
42	M	Classic	13.18	Alive	2.80	2	8	8	Negative	Negative
43	F	Classic	28.69	NA	NA	9	12	2	Negative	Positive
44	M	Classic	9.68	Alive	2.12	9	12	12	Negative	Positive

Product of intensity (0-3) and rate of positive cells (0-4), maximal value: 12 SFRP1 – secreted frizzled-related protein 1, DNMT – DNA methyltransferase, M – male, F – female, L/A – large cell/anaplastic, NA – not available
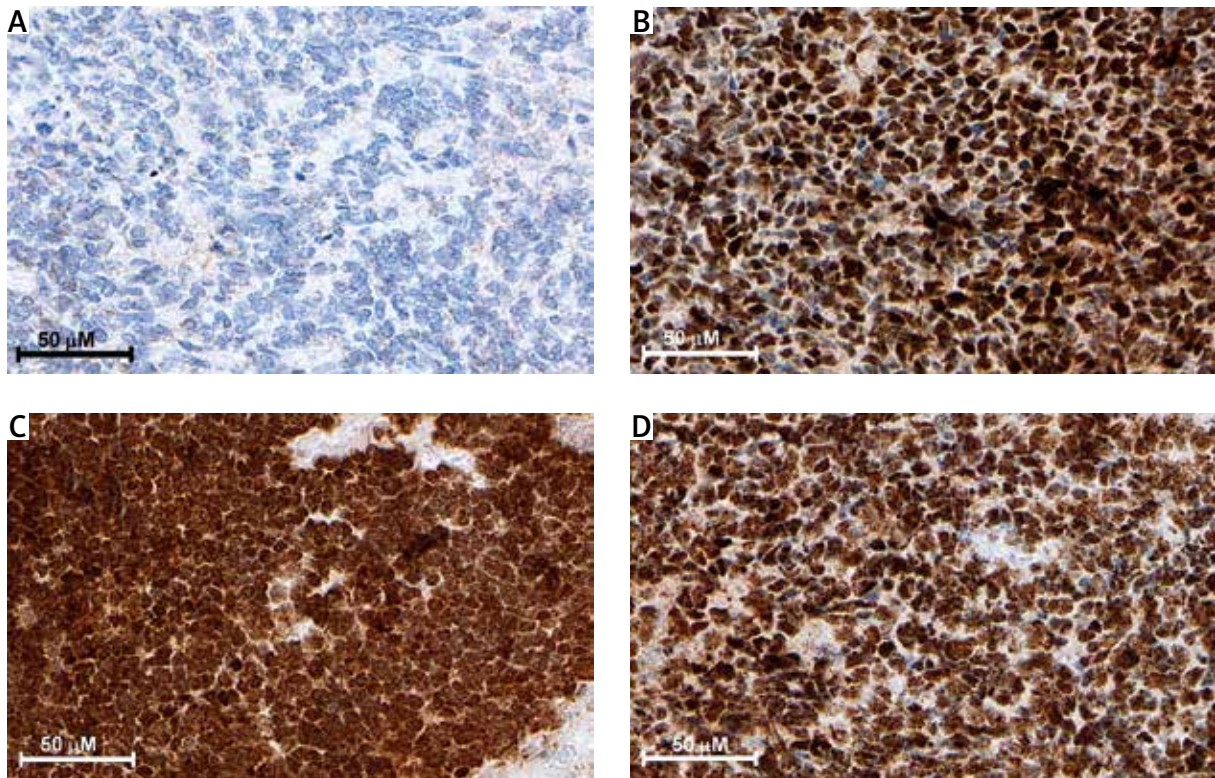
Fig. 1. Representative immunohistochemical staining of DNMTs in medulloblastoma. **A**) Negative staining of DNMT3A. **B**, **C**, **D**) Moderate/strong immunopositivity of DNMT1, DNMT3A and DNMT3B, respectively.

Table II. Evaluation of patients' characteristics and expression of different DNMTs. Correlation of DNMTs and clinical features are shown by probability value (*p*)

	All	DNMT1		*p*	DNMT3A		*p*	DNMT3B		*p*
		No/ Weak	Moderate/ Strong		No/ Weak	Moderate/ Strong		No/ Weak	Moderate/ Strong	
Number of patients	44	16 (36.36%)	28 (63.64%)	0.91[a]	14 (31.82%)	30 (68.18%)	0.91[a]	12 (27.27%)	32 (72.73%)	0.65[a]
Median age of patients (years)	8.53	9.33	8.49		8.49	8.93		7.65	8.93	
Gender										
Male	24	11	13	0.21[b]	9	15	0.52[b]	4	20	0.10[b]
Female	20	5	15		5	15		8	12	
Histological subtype										
Classic	37	14	23	1.00[b]	11	26	0.64[b]	11	26	1.00[b]
Desmoplastic	5	2	3		2	3		1	4	
Large cell/anaplastic	2	0	2	#	1	1	#	0	2	#
Molecular subtype										
Wnt	1	1	0	#	0	1	#	1	0	#
Shh	12	2	10	0.02[a] *	4	8	0.78[a]	1	11	0.17[a]
Non-Wnt/non-Shh	21	13	18		10	21		10	21	

[a]*Mann-Whitney U-test,* [b]*Fisher's exact test, two-tailed,* # *– not applicable,* * *– statistically significant*

Tímea Pócza, Tibor Krenács, Eszter Turányi, János Csáthy, Zsuzsanna Jakab, Peter Hauser
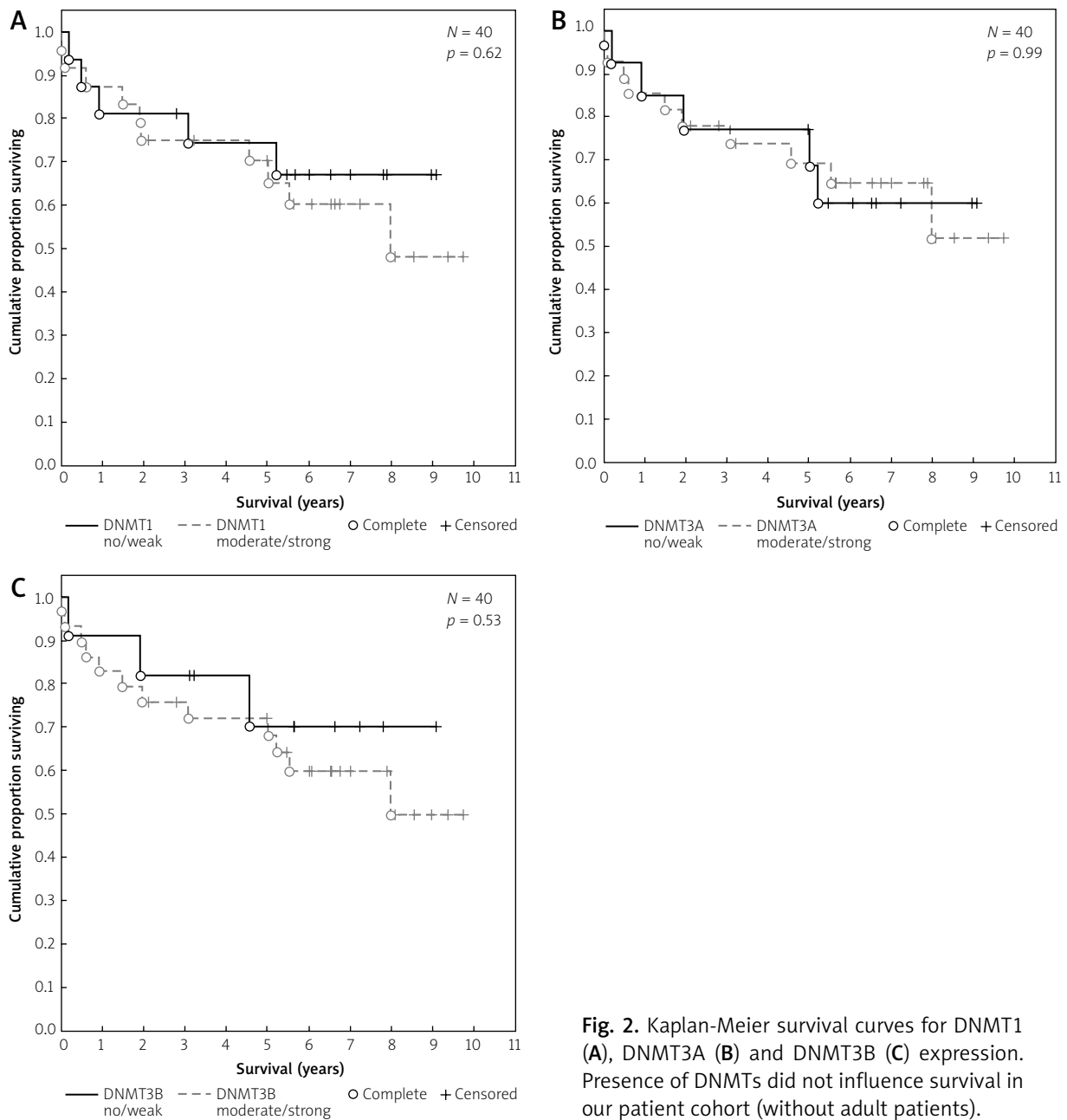

Fig. 2. Kaplan-Meier survival curves for DNMT1 (**A**), DNMT3A (**B**) and DNMT3B (**C**) expression. Presence of DNMTs did not influence survival in our patient cohort (without adult patients).

ferent tumor types [18,21,35]. DNMTs are responsible for DNA methylation, an important epigenetic regulation process, which contributes to development of cancer. DNMT1 is responsible for maintenance of methylation patterns during DNA replication. DNMT3A and DNMT3B are regarded as de novo methyl-transferases and abundantly expressed during embryonic development [2,9,14,19,22,28,38].

In our study, expression of three members of the DNMT family (DNMT1, DNMT3A, DNMT3B) in me-dulloblastoma samples and their possible effect on disease outcome were examined. During the development and progression of MB, the biological role of DNMTs, crucial regulators of the methylation pattern, is not completely understood yet. In our study, the correlation of expression of these enzymes at the protein level and clinical data of MB patients were investigated.

In our experiments elevated expression of all DNMTs was observed in most of the MB samples.

Correlation of expression of DNMTs and histological subtype of MB, age at disease onset or gender was not proven in our experience. Increased DNMT1 expression indicates worse outcomes in several types of cancers, such as gastric cancer, hepatocellular carcinomas and pancreatic ductal adenocarcinoma [10,26,33]. In contrast, our analysis showed no difference in disease outcome when comparing patients with no/weak and moderate/strong expression of three DNMTs, so DNMTs are not predictive markers for medulloblastoma survival. The different result may be due to the different biological nature of medulloblastoma and above-mentioned tumor types. While there are some common dysregulated signaling pathways (WNT, SHH, Notch) in medulloblastoma and in these other cancer types, differences could also be observed in regulation of the following pathways: in gastric cancer altered EGFR, ErbB, mTOR, VEGF, HGF/MET pathways, etc.; in hepatocellular carcinoma altered Ras/Raf/MAPK, mTOR, etc.; and in pancreatic ductal adenocarcinoma FGF, Notch, TGF-β, EGF and retinoid signaling [1,31,32].

The SHH subgroup of MB is characterized by the expression of SFRP1 and lack of nuclear β-catenin [17,27]. Our results indicate that SHH pathway activation is associated with elevated DNMT1 expression, which could be a consequence of upregulation of DNMT1 by SHH in SHH-associated medulloblastomas. Similar observations were made in pancreatic cancer and in myelodysplastic syndrome (MDS) [16,40]. DNMT1 and DNMT3A could be regulated by Gli1, an effector of the SHH signaling pathway. The SHH pathway was inhibited in pancreatic cancer and MDS cell lines, resulting in decreased DNMT1 expression, whereas induction of the SHH pathway resulted in higher DNMT1 expression [16,40]. In contrast, DNMT1 inhibition resulted in increased expression of SFRP1 in MB cell lines [20]. One explanation could be that investigated MB cell lines do not belong to the SHH subgroup, since they showed lower levels of SFRP1 compared to the SHH subgroup. Among non-WNT/non-SHH patients, other mechanisms could result in DNMT1 activation. Further studies are needed to clarify the relationship between regulation of DNA methylation and SHH signaling pathway.

Theoretically, inhibition of DNA methylation is a therapeutic opportunity by reversing gene silencing [9,12,14,38]. An in vitro experiment showed that the DNMT inhibitor 5-aza-2'-deoxycytidine (decitabine) reactivates the tumor suppressor PTCH1 gene in the MB cell line [4]. A combination of DNMT and histone deacetylase (HDAC) inhibitors was effective to prevent MB development in Ptch knockout mice, while in advanced stage tumors this therapy was less efficient [5]. Epigenetic modulators combined with multi-kinase inhibitor could enhance apoptosis in vitro in medulloblastoma cells [24].

In conclusion, this is the first study to analyze DNMT expression in medulloblastoma in terms of expression level and correlation with clinical data. Elevated expression of DNMTs in human medulloblastoma, and association of DNMT1 and SHH subgroups were observed, without an effect on survival. Based on increased expression of DNMTs in medulloblastoma, their inhibition has potential for further investigation to optimize therapy of medulloblastoma.

Acknowledgments

Disclosure

Authors report no conflict of interest.

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