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DNA methylation and gene expression of *COX7A1*, *SPINT1* and *KRT81* in glioblastoma and brain tissue

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Glioblastoma (GBM) is the most common primary brain tumor of the adult. Despite existing multimodal aggressive treatment regiments the prognosis of patients with GBM remains very poor. Most patients die within two years after diagnosis. Changes in individual gene expressions frequently accompany malignant cell transformation. Therefore comparative gene expression analyses of tumorous and non-tumorous tissue may help identify genes of significance in gliomagenesis. Epigenetic silencing of tumor suppressor genes is one of important mechanisms of gene inactivation during tumorigenesis. This study attempted to detect genes potentially regulated by promoter methylation in glioblastoma. To this end we analyzed the expression of *COX7A1*, *SPINT1* and *KRT81* in glioblastomas and human brain tissue and investigated their relation to promoter methylation of these genes.

We based candidate gene selection on bioinformatics, reverse transcription-polymerase chain reaction (RT–PCR) and bisulfite sequencing. *COX7A1*, *SPINT1* and *KRT81* mRNA expression was analyzed in glioblastomas (n = 22) and human brain tissue (n = 2). *COX7A1, SPINT1* and *KRT81* promoter methylation was analyzed in selected glioblastoma samples, human brain and human lymphocytes by bisulfite sequencing.

We found a differential gene expression of *COX7A1*, *SPINT1* and *KRT81* in the glioblastoma samples. Normal fetal and adult brain featured a robust expression of these genes. Analysis of bisulfite-modified DNA of brain tissue confirmed that the *SPINT1* promoter had only single scattered methylated CpG- dinucleotides while the CpG- islands of the *COX7A1* and *KRT81* promoters were abundantly methylated in brain tissue despite robust gene expression.

COX7A1 and *KRT81* gene expression is independent of promoter methylation in glioblastoma. The data suggest the possibility of epigenetic regulation of *SPINT1* in glioblastoma. Future work should focus on *SPINT1* gene function in glial cells to substantiate its potential tumor suppressor gene function in gliomas.

Key words: *COX7A1, KRT81, SPINT1,* glioblastoma, methylation, expression

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive form of malignant glioma. It is the most common primary human brain tumor in the adult. Diffuse brain tissue infiltration and molecular tumor cell properties of therapy resistance make treatment of these tumors especially difficult. Despite multimodal therapy with gross total resection in combination with adjuvant radio- and chemotherapy prognosis of patients with GBM remains very poor. Median survival is about one year following diagnosis. The analysis of gene expression and its regulation is one of the most intriguing fields in cancer research. Promoter methylation affects the expression of many genes in normal and cancer tissue and may play an important role in gliomagenesis. Pharmacologic manipulation of glioma cells with the demethylating agent 5'-aza-dC combined with genome wide expression profiling succeeded in unveiling novel candidate genes, which are epigenetically regulated in cancer in general (Yamashita et al., 2002) and in gliomas in particular (Mueller et al., 2007; Elias et al., 2009).

COX7A1 (cytochrome c oxidase subunit VIIa polypeptide 1), *KRT81* (keratin 81) and *SPINT1* (serine peptidase inhibitor, Kunitz type 1) were among promising candidate genes identified in a previous wide study of such genome (Mueller et al., 2007). These genes revealed robust expression in normal brain samples, their gene promoter harbored a CpG-rich island and 5'-aza-dC demethylation was accompanied by a significant up- regulation of their expression in glioma cells, suggesting epigenetic gene regulation (Mueller et al., 2007). One important indication for a potential role of a gene in the development of cancer is its expression alteration compared to healthy tissue. To elucidate potential epigenetic gene regulation of *COX7A1, SPINT1* and *KRT81*, we investigated their expression in human brain tissue and glioblastoma samples in relation to the methylation status of their gene promoter.

MATERIALS AND METHODS

DNA and RNA isolation

22 glioblastoma samples were obtained from Charité, Campus Virchow Klinikum from patients who underwent surgery in the Department of Neurosurgery and snap frozen. All GBM were histologically diagnosed according to the WHO criteria. Total RNA and DNA were isolated from snap frozen primary glioblastoma tissues using TRIzol (Invitrogen Life Technologies Inc) according to the manufacturer's recommendations. DNA was extracted from snap frozen primary glioblastoma tissues using Genomic DNA from Tissue Kit (Macherey-Nagel) according to the manufacturer's protocol.

Reverse transcription–PCR analysis

For cDNA synthesis 1 μg of total RNA was used for reverse transcription (random primer) using SuperScriptTM First Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The following primer sequences were used (Table 1).

All PCRs were performed in a 10-μl final volume using AmpliTaq Gold PCR Master Mix (Applied Biosystems) with the following amplification conditions: 95 °C for 5 min, and 36 cycles at 94 °C for 30 s, Tm for 40 s and at 70 °C for 30 s. Amplified products were electrophoretically separated on 1.2% agarose gels, and visualized with ethidium bromide.

Table 1. cDNA primers

Primer name	Sequence	Tm °C
KRT81_cDNAf	ATGCATCACCACCGTGTCGG	63
KRT81_cDNAr	GCGCACCTTGTCGATGAAGG	63
COX7A1_cDNAf	AGGACAAGGCAGAATGCAGG	63
COX7A1_cDNAr	GGATGTCATTGTCCTCCTGG	63
SPINT1_cDNAf	CATCAACTGCCTCTACGAGC	58
SPINT1_cDNAr	ATCCCAGACCCTCCAAAGCC	58
GAPDH_cDNAf	GGTTTTTCTAGACGGCAGGTCA	58
GAPDH_cDNAr	TGGCAAATTCCATGGCACCGTCA	58

Table 2. Bisulfite modified DNA primers

Primer name	Sequence	Tm °C
KRT81_promoter_bisulfite modified_Frag_1f	GGTGTTTTGAGGAGTATATTGGAGT	53
KRT81_promoter_bisulfite modified_Frag_1r	TAATAATACATAAAAAACTAAACCC	53
KRT81_promoter_bisulfite modified_Frag_2f	GGGTTTAGTTTTTTATGTATTATTAT	55
KRT81_promoter_bisulfite modified_Frag_2r	TATAATCCAAAACACCCACCTTATC	55
Cox_promoter_bisulfite modified_Frag_1f	TTTGTAAAAATGTATTTTTTGGTAT	51
Cox_promoter_bisulfite modified_Frag_1r	ACCTACATTCTACCTTATCCTCTTCC	51
Cox_promoter_bisulfite modified_Frag_2f	GTGAGGTTTTTATGGGTTGGGT	53
Cox_promoter_bisulfite modified_Frag_2r	AAAAAAATTTCCTAAATTAAAAAAAA	53
SPINT1_promoter_Frag_1_methyl_f	TTTTAGGTTTGGGGTTGGGAAAGTA	57
SPINT1_promoter_Frag_1_methyl_r	CCTAATTTCTAATAAAACTAAATCAAC	57

Bisulfite modification

Sodium bisulfite modification was performed using the CpGenome Universal DNA Modification Kit (Chemicon International) according to the manufacturer's protocol. Universally methylated DNA (CpGenome Universal Methylated DNA, Chemicon International) and unmethylated DNA from normal blood samples were treated with bisulfite by the same method.

Bisulfite sequencing

Initially, bisulfite-modified DNA of a target promoter region was amplified. Primer sequences and conditions are shown below in Table 2.

All PCRs were performed in a 20-µl final volume using AmpliTaq Gold PCR Master Mix (Applied Biosystems) with the following amplification conditions: 95 °C for 5 min, and 36 cycles at 94 °C for 30 s, Tm for 40 s and at 70 °C for 2 min and final extension 70 °C for 10 min. Amplified products were electrophoretically separated on 1.2% agarose gels, and visualized with ethidium bromide.

The PCR product was plasmidincorporated using One Shot Escherichia coli cells and the TOPO TA Cloning Kit (Invitrogen Life Technologies Inc). Cells were then plated and grown overnight on prewarmed LB plates containing X-gal, ampicillin and kanamycin. Colony PCR was performed on ten white colonies to validate the insert using primers: M13 Forward: GTAAAACGACGGCCAG; M13 Reverse: CAGGAAACAGCTATGAC. ExoSAP-IT reagent (Affymetrix, USA) was used for PCR Product Cleanup. Preparation for sequencing using SEQ Sequencing Reaction Kit (Montage, USA) according to the manufacturer's protocol. Fragments were sequenced and analysed using Sequencher Software version 4.2. (Gene Codes Corporation, Ann Arbor, MI, USA).

RESULTS

Reverse transcriptase polymerase chain reaction (RT-PCR) is commonly applied to monitor gene expression. By RT-PCR gene expression can be investigated with high accuracy and excellent reproducibility. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) amplification was used as an internal control for cDNA quality and quantity.

Expression array data for *COX7A1, KRT81* and *SPINT1* genes were verified by RT–PCR using cDNA isolated from 22 snap frozen glioblastoma samples. In addition, we investigated the expression of these genes in cDNA isolated from normal human brain.

Data showed that the housekeeping gene GAPDH was equally expressed in all samples confirming excellent cDNA quality and equal

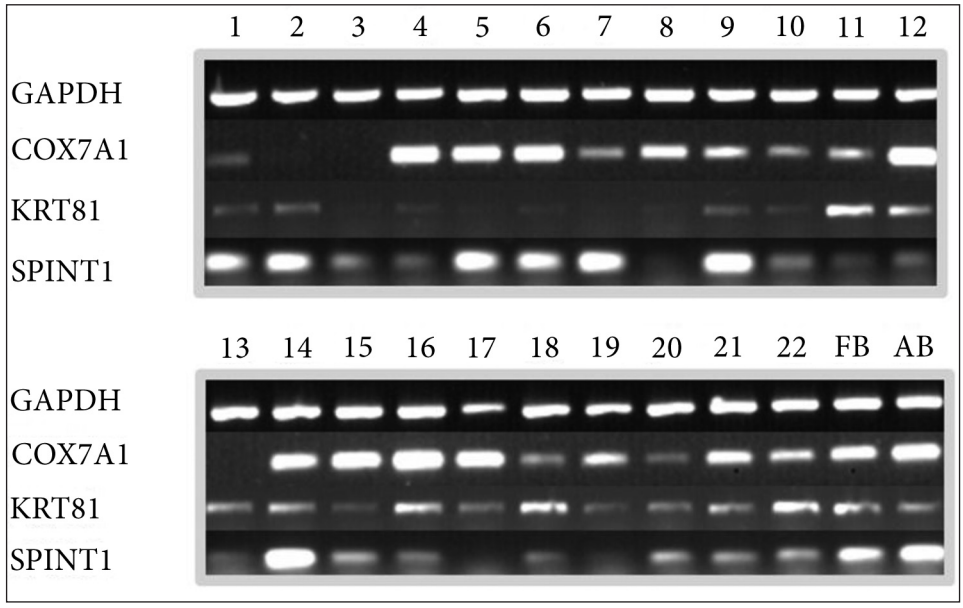

Fig. 1. Differential expression of *COX7A1*, *KRT81* and *SPINT1* in glioblastoma samples (1–22) and in normal fetal (FB) and adult human brain (AB) samples

cDNA quantity in these samples. It is noteworthy that *COX7A1*, *KRT81* and *SPINT1* revealed a differential expression pattern in the glioblastoma samples (Fig. 1). Gene expression was decreased in some of the glioblastoma samples. Normal fetal and adult human brain showed robust expression of *COX7A1*, *KRT81* and *SPINT1* suggesting that down regulation of these genes in glioblastoma samples was tumor-related. As promoter methylation of these genes might be one possible mechanism of *COX7A1*, *KRT81* and *SPINT1* down regulation expression analysis was followed by promoter methylation analysis by bisulfite sequencing.

COX7A1, _KRT81_ and SPINT1 genes promoter sequences analysis in glioblastoma tumors and control brain

In order to address the possibility of gene regulation by promoter methylation we decided to perform bisulfite sequencing of *COX7A1*, *KRT81* and *SPINT1* gene-promoters in selected glioblastoma samples, normal adult human brain and DNA derived from human leucocytes.

Bisulfite sequencing allows a precise analysis of methylation in a certain region by converting all non-methylated cytosines into thymine while methylated cytosines remain unchanged. The methylation status of individual cytidine residues in CpG- dinucleotides is then visualized in the sequencing data. The *SPINT1* gene promoter region is located on the chromosomal locus 15q15.1. We sequenced a large CpG- island fragment around the transcription start site. The fragment was 344 bp in size and covered 40 CpG- dinucleotides. Bisulfite sequencing results are shown in Fig. 2.

The analysis of bisulfite-modified DNA of brain tissue confirmed that the *SPINT1* promoter had only single scattered methylated CpG-dinucleotides in healthy brain tissue. An unmethylated promoter is compatible with the robust *SPINT1* expression in normal human brain.

As can be seen from the bisulfite sequencing results in our investigation of tumor samples *SPINT1* promoter methylation was not frequently observed. Samples with high expression of *SPINT1* were unmethylated. Glioblastoma samples with low *SPINT1* expression accompanied by promoter methylation were observed, however, not very frequently. Nevertheless, our results encourage more detailed analyses of the whole *SPINT1* promoter sequence in a larger group of glioma samples to clarify the true relationship between promoter methylation and gene expression.

We sequenced two fragments of the *COX7A1* promoter. The first fragment was 309 bp in size and covered 27 CpG-dinucleotides. The second fragment was 349 bp in size and harbored 17 CpG-dinucleotides. Bisulfite sequencing results of these two fragments are shown in Fig. 3.

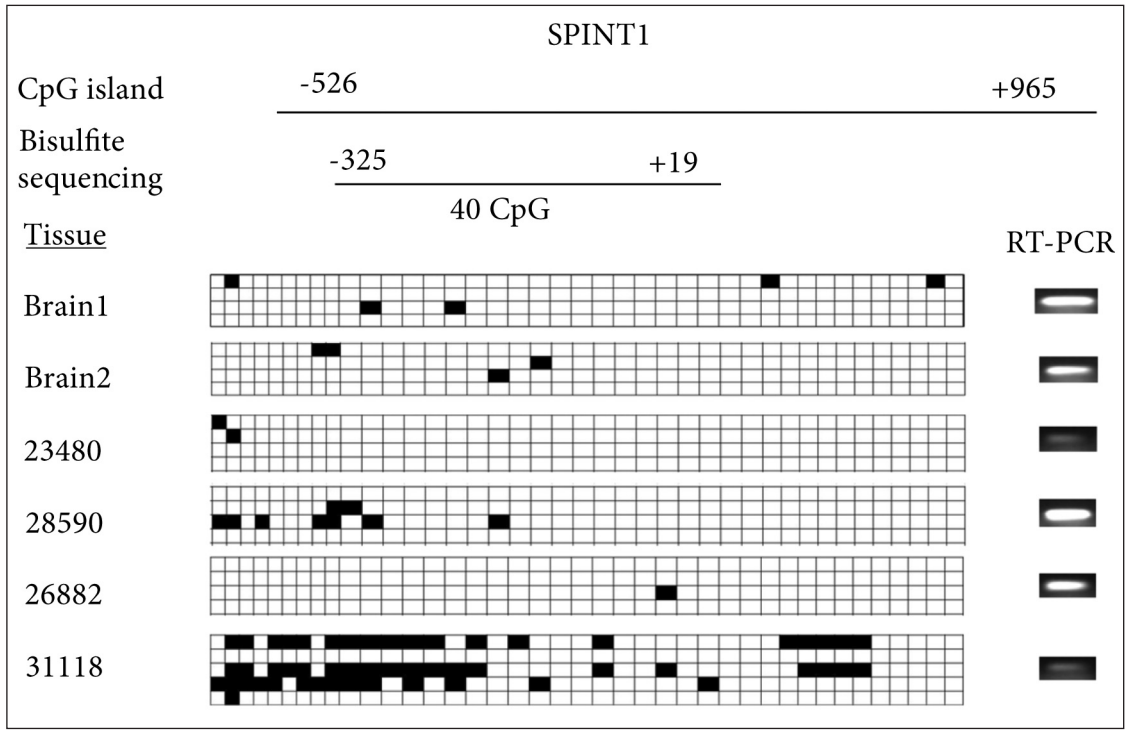

Fig. 2. *SPINT1* promoter CpG-island: bisulfite sequencing and RT-PCR results in brain and glioblastoma samples. Tumor samples with promoter methylation (ID31118) compared to unmethylated tumor samples and unmethylated promoter in normal adult brain (*left*). Sequenced samples are accompanied by RT-PCR results (*right*). Each square represents one CpG-dinucleotide within the promoter sequence. Black squares indicate methylated CpG-dinucleotides and white squares indicate unmethylated CpG-dinucleotides

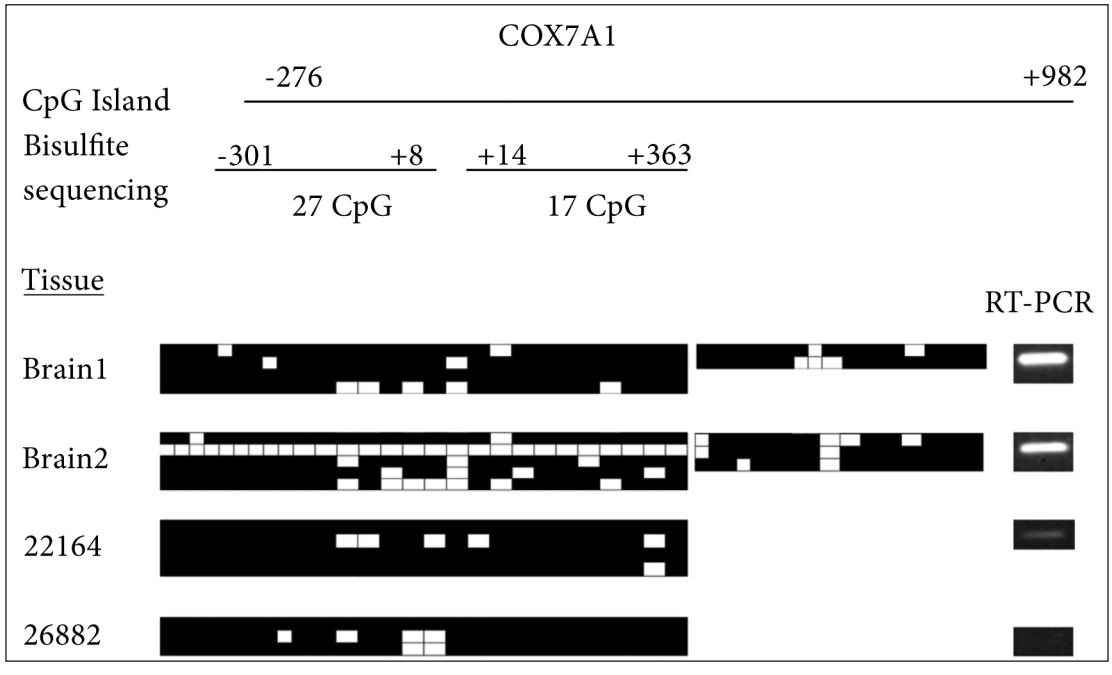

Fig. 3. CpG-island of *COX7A1* promoter: bisulfite sequencing and RT-PCR results in brain and glioblastoma samples. Glioblastoma samples compared to brain tissue samples. Selected samples RT-PCR results (*alongside*). Black squares indicate methylated CpG- dinucleotides and white squares indicate unmethylated CpG-dinucleotides in promoter sequences

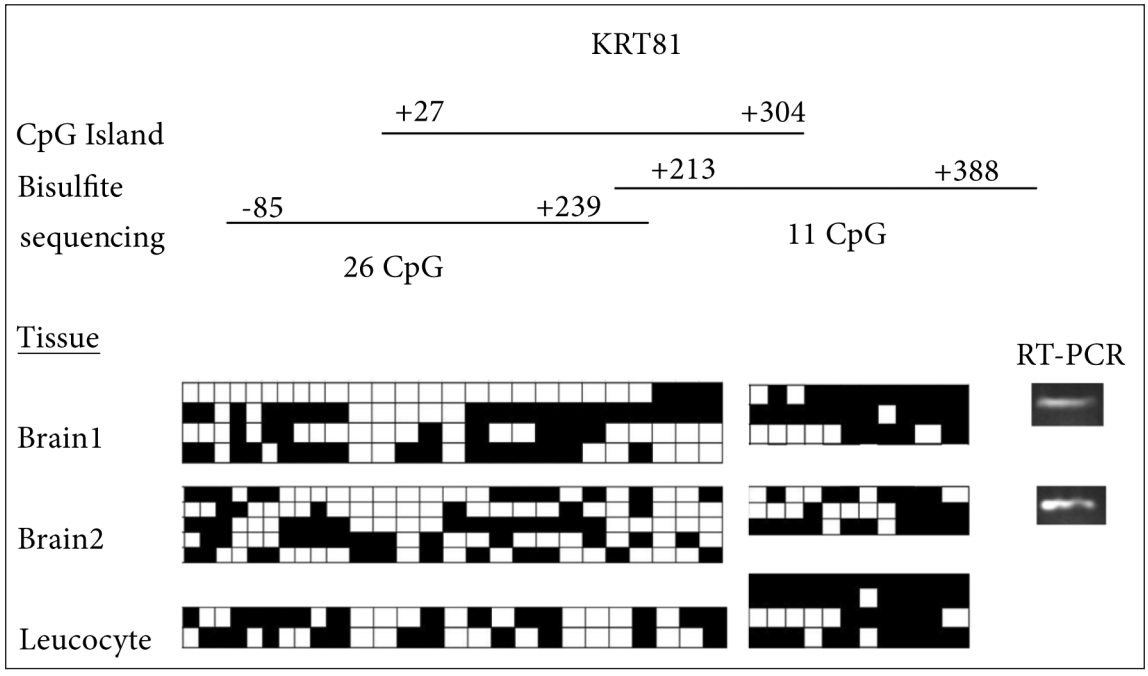

Fig. 4. CpG-island of *KRT81*-promoter: bisulfite sequencing and RT-PCR results in brain and leukocyte samples. Black squares indicate methylated CpG-dinucleotides and white squares indicate unmethylated CpG-dinucleotides in promoter sequences

The two fragments of the *COX7A1* promoter exhibited almost complete methylation of all analysed sequence-clones derived from normal human brain. It was decided to investigate the methylation status among different glioblastoma samples. The results revealed that the CpG-islands of the *COX7A1* promoter are most abundantly methylated in both glioblastoma samples and normal human brain tissue, irrespective of its gene expression level.

Selected RT-PCR results (*alongside*)

KRT81 gene promoter region with CpG islands is located on chromosome 12q13. Two potential *KRT81* promoter fragments were sequenced around the transcription start site. The first fragment was 324 bp in size and contained 26 CpG-dinucleotides. The second fragment was 175 bp in size and harbored also 26 CpG-dinucleotides. Firstly, we sequenced samples of human brain and leucocyte DNA. Bisulfite sequencing results of both fragments are shown in Fig. 4.

The results revealed that the *KRT81* promoter is frequently methylated in leucocytes and normal human brain tissue. We refrained from bisulfite sequencing of the promoter of this gene in glioblastoma tumor samples, as we did not expect that the data could be informative and suitable for future research. The data suggest that the expression of *KRT81* is not regulated by promoter methylation in gliomas.

DISCUSSION

Microarray analysis coupled with pharmacologic demethylation of cultured tumor cells is a useful tool for identifying genome-wide epigenetic events and has been successfully implemented for this purpose in other cancer types in the past (Yamashita et al., 2002; Lodygin et al., 2005). However, genes identified by this method must necessarily be examined in more detail to validate potential epigenetic gene regulation and to elucidate their importance in cancer development. Multiple independent techniques should be implemented to validate these candidate genes. To this end we investigated *COX7A1*, *KRT81* and *SPINT1* expression, first in normal human brain tissue. Normal fetal and adult human brain showed robust expression of *COX7A1, KRT81* and *SPINT1* suggesting that down regulation of these genes in glioblastoma samples was tumor related. Up-regulation of their expression following 5'-aza-dC mediated DNA-demethylation suggested promoter methylation

as a mechanism for gene silencing. Surprisingly partial bisulfite sequencing of the *COX7A1* and the *KRT81* promoter revealed abundant promoter methylation in both normal human brain and glioblastoma samples, irrespective of their individual expression levels. Our results do not fit the classical hypothesis that promoter hypermethylation leads to gene silencing. They might suggest that the expression of these genes might follow other regulations than promoter methylation in gliomas. However, these results might also be explained by other hypotheses. Some reports have shown that histone deacetylase inhibitors (HDACi) produce gene reactivation from hypermethylated promoters without any changes in DNA methylation at the promoter level or that inhibition of *SIRT1* reactivates silenced cancer genes without loss of promoter DNA hypermethylation (Raynal et al., 2012; Pruitt et al., 2006). Another hypothesis suggests that there are some transcription factors that are capable to bind to methylated DNA, i. e. SP1 or NRF1. *COX7A1* and *KRT81* transcription might be regulated by one of these transcription factors, explaining their expression despite methylated promoter sequences (Harrington et al., 1988; Smith et al., 2004). The third hypothesis relates to the bisulfite sequencing method itself. Bisulfite sequencing cannot discriminate 5-methylcytosine from 5-hydroxymethylcytosine. The recent discovery that the three members of the TET protein family can convert 5-methylcytosine (5 mC) into 5-hydroxymethylcytosine (5 hmC) has provided a potential mechanism leading to DNA-demethylation (Williams et al., 2012; Branco et al., 2012). Maybe in our result we detected not 5 mC but rather 5 hmC. All these hypotheses have to be addressed by investigations in future in order to elucidate the functional contribution of these genes to gliomagenesis.

CONCLUSIONS

COX7A1 and *KRT81* expression is independent of promoter methylation in glioblastoma. The data encourage extended promoter methylation analysis of *SPINT1* in order to elucidate its potential as a molecular glioblastoma marker.

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COX7A1, *SPINT1* IR *KRT81* GENŲ EKSPRESIJOS IR DNR METILINIMO TYRIMAI GLIOBLASTOMŲ IR SMEGENŲ AUDINIUOSE

Santrauka

Įvadas. Glioblastoma (GBM) yra dažniausias pirminis smegenų auglys. Nepaisant multimodalinio GBM gydymo, pacientų prognozė išlieka labai prasta. Dauguma pacientų miršta per dvejus metus po nustatytos diagnozės. Atskirų genų raiškos pokyčiai dažnai yra susiję su piktybinių ląstelių transformacija, todėl genų ekspresijos palyginimas sveikame ir auglio audiniuose gali padėti nustatyti gliomos genezei svarbius genus. Vėžio supresorinių genų epigenetinis „nutildymas“ yra vienas svarbiausių genų išjungimo mechanizmų. Šiuo tyrimu bandyta aptikti genus, kurių ekspresija gali būti reguliuojama genų promotorių metilinimu glioblastomose. Tyrimo tikslas – ištirti *COX7A1*, *SPINT1* ir *KRT81* genų raišką ir jos ryšį su šių genų promotoriaus metilinimu.

Metodai. Genus tyrėme taikydami bioinformatikos, atvirkštinės transkripcijos daugiagrandininę reakciją ir bisulfitinio sekvenavimo metodus. *COX7A1*, *SPINT1* ir *KRT81* genų mRNR raiška buvo tirta 22 glioblastomose ir 2 žmogaus smegenų audiniuose. *COX7A1*, *SPINT1* ir *KRT81* genų promotorių metilinimas buvo analizuojamas bisulfitinio sekvenavimo metodu pasirinktuose glioblastomos mėginiuose, žmogaus smegenų audinyje ir žmogaus limfocituose.

Rezultatai. *COX7A1*, *SPINT1* ir *KRT81* genų ekspresijos skirtumai buvo aptikti glioblastomos mėginiuose, o smegenų audinio mėginiuose buvo nustatyta ryški šių genų raiška. Šių genų promotorių bisulfitinės modifikuotos DNR tyrimas rodo, kad *SPINT1* geno promotoriuje metilinti tik pavieniai CpG dinukleotidai, o *COX7A1* ir *KRT81* promotoriai buvo gausiai metilinti net ir smegenų audinyje, kuriame buvo nustatyta šių genų ekspresija.

Išvados. *COX7A1* ir *KRT81* genų raiška glioblastomose nepriklauso nuo promotoriaus metilinimo. Duomenys rodo, kad *SPINT1* genas gali būti epigenetiškai reguliuojamas glioblastomose. Tolesnius tyrimus reikėtų sutelkti į *SPINT1* geno, kaip galimo naviko supresoriaus, funkcijos nustatymą gliomose.

Raktažodžiai: COX7A1, KRT81, SPINT1, glioblastoma, metilinimas, ekspresija