# Frequent aberrant expression of p53 protein in gliomas but not in capillary hemangioblastomas and pheochromocytomas

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<sup>3</sup> Faculty of Natural Sciences, Vilnius University, Vilnius, Lithuania **Background**. Glioma is the most common and deadly malignancy of the central nervous system. Capillary hemangioblastoma (CHB) is a rare and usually benign tumour of brain, occurring as part von Hippel-Lindau (VHL) disease or as a sporadic entity. For an improved understanding of the molecular mechanisms of the pathogenesis of VHL-related tumours and gliomas, we analysed the expression of the tumour suppressor proteins p53 and p16, and promoter methylation of the tumour suppressor genes (TSGs) *MGMT* and *RASSF1A*.

Materials and methods. Expression of p53 and p16 was analysed in 27 cases of glioma, 5 cases of breast cancer, and 17 cases with VHL-related tumours, including CHBs and pheochromocytomas (PCCs), by means of immunohistochemistry. For the gene promoter methylation and deletion assessment, methylation-specific PCR and differential polymerase chain reaction combined with on-chip electrophoresis were used, respectively.

**Results.** Aberrant expression of the p53 protein was frequent (p < 0.01) in malignant tumours, including gliomas and breast carcinomas, but not in benign tumours (PCC and CHB). Protein p53 alterations were most frequent (65%) in glioblastomas, and a low number (20%) of patients with p53-positive gliomas survived more than one year after diagnosing the disease. Other biomarkers, including loss of p16 expression, promoter hypermethylation of the *MGMT* gene and promoter hypermethylation or deletion of the *RASSF1A* gene, were frequent in all groups of tumours.

**Conclusions.** Alterations of the tumour suppressors p16, MGMT and RASSF1A are frequent in VHL-related tumours, while aberrant expression of p53 is only detectable in malignant tumours – gliomas and breast carcinomas.

Key words: p53, glioma, capillary hemangioblastoma, pheochromocytoma, breast cancer, promoter methylation

# INTRODUCTION

Gliomas are the most common and deadly malignancies of the central nervous system (CNS) accounting for more than 70% of all primary CNS tumours (1). According to the cell of origin gliomas are subdivided into astrocytomas, oligodendrogliomas, ependymomas and mixed gliomas, where astrocytoma is the most common histological type (2). Among astrocytic tumours, glioblastoma (high grade glioma) is the most common entity which accounts for 50–60% of all astrocytic tumours. Glioblastoma may develop *de novo* (primary glioblastoma) or through progression from lowgrade tumours (secondary glioblastoma). In most European countries, the incidence of glioblastomas is approximately 2–3 new cases per 100,000 people per year (1). The median survival of glioblastoma patients treated with optimal therapy is approximately 12 months, and less than 10% of patients survive up to 5 years. Modern molecular analyses have demonstrated that the malignant progression of astrocytic tumours is caused by multiple genetic lesions, including inactivation of tumour suppressor genes and activation of oncogenes (1, 3, 4). Most extensively studied molecular prognostic markers for gliomas are alterations in the *TP53*, *PTEN*, *p16*, and *EGFR* genes. However, most of the studies

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did not show the independent prognostic value of these molecular changes (1, 5). Recent studies of epigenetic changes in glioma have revealed a novel group of DNA methylation biomarkers (6); their clinical value should be clarified in the nearest future.

Capillary hemangioblastoma (CHB) is a rare and usually benign tumour representing approximately 3% of all CNS tumours (7). About 20–30% of all CHB occur as part of von Hippel–Lindau (VHL) disease caused by inherited mutation of the VHL gene (7, 8). Pheochromocytoma (PCC) is a rare neuroendocrine tumour of adrenal gland, which occurs as part of several cancer syndromes, including VHL disease (9); however, most of PCCs cases (80–90%) occur sporadically. Collectively, CHBs and PCCs represent two most common types of VHL-related tumours. Mutations of the VHL gene have been observed not only in familial cases of CHB and PCC, but also in a part of sporadic tumours (10); however, the further molecular mechanisms of VHL-related tumours, especially sporadic cases, remain to be explored.

We continued our studies (11, 12) on the molecular mechanisms of the pathogenesis of VHL-related tumours and gliomas by analysing the expression of the major tumour suppressor proteins p53 and p16, and promoter methylation of the tumour suppressor genes (TSGs) *MGMT* and *RASSF1A*. Several studies indicated a direct association between mutation in gene *TP53* and an increased expression of p53 protein (5). Similar evidence has been accumulating for the loss of p16 expression shown to be caused by deletions in the 9p21 locus (*CDKN2A* locus) or hypermethylation in the promoter region of the gene *p16* ( $p16^{INK4a}/CDKN2$ ).

Epigenetic processes, such as aberrant DNA methylation in the promoter region of TSGs, occur as early and ubiquitous alterations in human cancer. Promoter hypermethylation is recognized as an alternative to genetic mutation, as both events can inactivate expression of TSGs (13). Gene *MGMT* (O<sup>6</sup>-methylguanine-DNA-methyltransferase) promoter hypermethylation is detectable in approximately half of gliomas and is associated with a longer overall survival in glioma patients receiving alkylating chemotherapy. However, this valuable biomarker is poorly studied in VHL-related tumours, especially benign tumours of the brain CHBs. RASSF1A (Ras

imately 3% of all CNS	omarkers for discrimination between benign and malig-
IB occur as part of von	nant brain tumours and for prediction of aggressiveness of
l by inherited mutation	the diseases, we analysed four molecular biomarkers in 27
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VHL-related tumours.

the diseases, we analysed four molecular biomarkers in 27 malignant tumours of the CNS (gliomas) and in eight benign tumours of the brain (capillary hemangioblastomas). To increase the number of cases in the group of rare VHLrelated tumours, nine cases of the same VHL-related group diagnosed with pheochromocytoma were included in the study. As an example of the cancer with well known profile of analysed biomarkers, five cases with breast carcinoma were also analysed. In addition, to immunohistochemistry and DNA methylation analysis by means of methylationspecific PCR (MSP), DNA integrity in the promoter region, the *RASSF1A* was assessed by means of differential polymerase chain reaction (dPCR) combined with on-chip electrophoresis technology, enabling a quantitative comparison of PCR products.

association domain family 1 isoform A) is a recently dis-

covered tumour suppressor, its promoter hypermethylation

being frequently detectable in many human cancers, includ-

ing breast cancer, but quite limitedly studied in gliomas and

In order to assess the clinical relevance of these bi-

The study contributes to the understanding of the molecular pathways of the pathogenesis of glioma and two of the most common VHL-related tumours, hemangioblastoma and pheochromocytoma. In addition, the study for the first time analyses the epigenetic and genetic inactivation of the *RASSF1A* and *MGMT* genes in human capillary hemangioblastoma.

## MATERIALS AND METHODS

## Patients and samples

We analysed 27 cases of glioma, nine cases of PCC and eight cases of CHB. For comparison, five cases diagnosed with breast carcinoma were included in the study. Most of the cases of glioma were diagnosed as glioblastoma (74%). The main characteristics of the study group are presented in Table 1. The study was approved by the Lithuanian Ethics Committee.

Tumour	Number	Se	x	Age, years	
Tulliou	Number	Male	Female	mean (variation)	
Glioma	27	9	18	64.0 (38–80)	
<ul> <li>glioblastoma</li> </ul>	20	9	11	66.4 (49–80)	
<ul> <li>oligodendroglioma</li> </ul>	4	0	4	50.5 (38–61)	
<ul> <li>astrocytoma</li> </ul>	2	0	2	66.0 (57–75)	
<ul> <li>oligoastrocytoma</li> </ul>	1	0	1	75	
Hemangioblastoma <sup>a</sup>	8	4	2	36.2 (22–79)	
Pheochromocytoma	9	4	5	50.9 (25–64)	
Breast cancer	5	0	5	62.8 (51–88)	
All cases	49	17	30	58.0 (22–88)	

Table 1. Main characteristics of study groups

<sup>a</sup> Demographic data are missing for 2 cases of capillary hemangioblastoma.

Paraffin-embedded tumour specimens were obtained from the archives of the National Centre of Pathology, Lithuania. All good-quality samples of CHB and PCC and representative samples of glioma and breast cancer, collected at the National Centre of Pathology (NPC) in 2002–2005, were included into the study. For the analysis of genetic and epigenetic alterations, genomic DNA was extracted from the specimens by digestion with proteinase K, followed by a standard phenol–chloroform purification and ethanol precipitation.

DNA extracted from leukocytes of healthy controls (n = 2) and DNA from cancer cell lines T24 (from American Type Culture Collection, USA), U87, U138, U251 and U373 (kindly provided by Prof. Tom Bohling, HUSLAB, Helsinki, Finland) were used as the controls in TSG hypermethylation studies.

#### Immunohistochemistry analysis

Monoclonal mouse anti-human p53 (clone DO-7, Dako Cytomation Glostrup, Denmark; dilution 1:700) and the immunohistochemical kit of p16INK4a were used as the primary antibodies. Immunohistochemistry was performed using the Dako REAL EnVision detection system with peroxidise / DAB+ (Dako). Antigen retrieval for p53 was performed by incubation of the slides in 10 mM of the sodium citrate (pH 6.0) and EDTA buffers (pH 9.0) for 5 min at 110 °C using a microwave processor (Microwave Vacuum Histoprocessor, Milestone). For the p16 antigen, retrieval was done by heating up to 100 °C for 15 min in a retrieval solution (CINtec\* Histology Kit; Dako Cytomation). Sections from breast carcinomas served as the positive controls. In addition, normal brain tissue adjacent to the glioma was stained with p53 and p16 antibodies. Immunostaining was scored as absent (weak staining in less than 10% of tumour cells), moderate (staining in 10 to 30% of tumour cells), and strong (more than 30% of cells stained).

#### Methylation analysis of RASSF1A and MGMT genes

Methylation-specific PCR (MSP (13) was used for analysis of the methylation pattern in the 5' region of the *RASSF1A* and *MGMT* genes. Genomic DNA (1  $\mu$ g) was exposed to bisulphite modification as described elsewhere (14). Briefly, 1  $\mu$ g of genomic DNA was denatured with 3 M NaOH for 15 min at 37 °C and then exposed to bisulphite modification with 2.3 M sodium metabisulphite and 10 mM hydroquinone (all chemicals from Sigma-Aldrich Inc., Gillingham, Dorsel, UK). Exposure to sodium metabisulphite was carried out at 50 °C for 16 h. Modified DNA was purified with the Wizard DNA Clean-up System (Promega, Madison, WI), desulphonated with 3 M NaOH treatment and precipitated with ethanol.

PCR primers specific for methylated (M) and unmethylated (U) sequences within the 5' region of the *RASSF1A* and *MGMT* genes were designed according to published sequences as indicated previously (14) and purchased from biomers.net (Ulm Donau, Germany).

The MSP reaction mixture for 25  $\mu$ l of the total reaction volume contained 1-2 µl of bisulphite-modified DNA template, PCR buffer, 0.4 mM of each deoxynucleotide triphosphate, 2.5 mM of MgCl., 1% of dimethylsulfoxide, sense and antisense primers at the final concentration of 6 ng/µl and 1.25 U of AmpliTaq Gold polymerase (Applied Biosystems, Piscataway, NJ). PCR was performed in a thermocycler (Mastercycler ep gradient S, Eppendorf AG, Hamburg, Germany) under the following conditions: hot start at 95 °C for 10 min, 35 to 37 cycles at 95 °C for 45 sec, 62 °C for 45 sec, 72 °C for 45 sec, and the final extension at 72 °C for 10 min. The PCR product was analyzed on non-denaturing polyacrylamide gel after ethidium bromide staining. The gene was recorded as hypermethylated when MSP amplification products were detected in both reactions with primers for the M and U sequences or for the M sequence only.

PCR reaction was performed together with controls specific for methylated (KM, *in vitro* methylated leukocyte DNA using bacterial SssI methylase; New England BioLabs Inc, Beverly, MA) and non-methylated (KL, leukocyte DNA from healthy controls) reaction. Non-template (water) controls were always included in PCR.

#### Gene deletion analysis by on-chip electrophoresis

Differential PCR (dPCR; (15) combined with on-chip electrophoresis (Agilent 2100 bioanalyzer, Agilent Technologies, Santa Clara, CA), enabling a quantitative comparison of PCR products, was carried out to detect homozygous or hemizygous deletion in the RASSF1A gene (3p21 locus). The occurrence of deletion was analysed in cases where the PCR product (either for U or M sequence) was not obtained by means of MSP. The sequence covering the gene region analysed in MSP was included in dPCR analysis. As a reference, the sequence for the gene  $\beta$ -actin was used (28). The PCR reaction was conducted in 25 µl of the total reaction volume that contained 1 µl of non-modified DNA template, PCR buffer, 0.4 mM of each deoxynucleotide triphosphate, 2.5 mM of MgCl<sub>2</sub>, primers for a gene and  $\beta$ -actin at final concentrations of 1 µM, and 1 U of AmpliTaq Gold polymerase (Applied Biosystems). DNA was amplified with 36 cycles of PCR and analysed with an Agilent 2100 bioanalyzer, using the DNA 7500 kit (Agilent Technologies). Samples showing a two or more times lower DNA concentration (ng/µl) of the target gene as compared to the  $\beta$ -actin signal were considered as having hemizygous deletion, while the absence of the target gene sequence in the dPCR was viewed as homozygous deletion.

## Statistical analysis

Two-sided Fisher's exact test was used for comparison of categorical variables. Odds ratios (OR) and the exact or Mantel–Haenszel 95% confidence intervals (CI) for two binomial samples were calculated. P < 0.05 was considered as statistically significant.

### RESULTS

## Expression of p53 and p16

Assessment of p53 expression (Fig. 1) revealed an aberrant expression (from moderate to high) of the protein in 56% (15 / 27) of the gliomas (Table 2). p53-positive cases were mainly glioblastomas (13 / 15), but a moderate expression of the protein was also observed in one astrocytoma case (1 out of 2 astrocytomas), and a strong expression was also detected in one oligoastrocytoma (1 out of 1 oligoastrocytomas). Three out of the five breast carcinomas included into the study had an aberrant expression of the p53 protein. In contrast, no alterations of p53 were detected in VHL-related tumours (Table 2).

Immunohistochemistry indicated a frequent loss of p16 expression in all types of cancer included in the study (Fig. 1, Table 2). The protein was not detectable in 67% (18 / 27) of gliomas, 94% (16 / 17) of VHL-related tumours and in all analysed cases of breast cancer. Among gliomas, no p16 was expressed in low-grade tumours, except one case with astrocytoma. The alteration was also observed in 60% (12 / 20) of glioblastomas.

Hypermethylation or deletion in promoter region of TSGs Aberrant methylation in promoter regions of the *MGMT* and *RASSF1A* genes was analysed by means of methylation-specific PCR (Fig. 2). The cases negative in MSP were assessed for the genetic deletion of the analysed region.



**Fig. 1.** Immunohistochemistry analysis for p16 and p53 protein expression. (A) Strong expression of p16 detected in the nuclei and cytoplasm of glioblastoma; normal brain tissue on the right side was mainly negative for p16. (B) Week p16 signal in the nuclei and cytoplasm of glioma cells (glioma 27). (C) Sample of breast carcinoma with a clear positive nuclear p53 signal in tumour cells used as a positive control. (D) Moderate nuclear expression of p53 protein in glioma cells (glioma 4). Original magnifications:  $B - \times 400$ , A, C,  $D - \times 200$ 



**Fig. 2.** Gene *RASSF1A* and *MGMT* promoter methylation analysis by means of methylation-specific PCR (MSP; A); gene *RASSF1A* deletion detection assessed by differential PCR using  $\beta$ -actin (*ACTB*) as a reference gene and analysed by on-chip electrophoresis (dPCR; B and C). In MSP analysis, methylated cases are indicated by an arrow; in dPCR analysis KL, GL7 and GL19 cases had no losses of *RASSF1A*, while other cases show reduced product from gene *RASS1A* as illustrated by on-chip gel photo (B) or fluorescence intensity peaks (C). Sm – size marker, m – methylated, u – unmethylated PCR product, Pd – peak from primer dimmers, KL – leukocyte DNA, KM – *in vitro* methylated DNA, U138 and U373 – glioma cell line, GL – glioma cases, HB – capillary hemangioblastoma cases

Table 2. Summary of molecular analysis in gliomas, capillary hemangioblastomas, pheochromocytomas and breast tumours. p53 and p16 expression was analysed by means of immunohistochemistry, status of the *MGMT* and *RASSF1A* genes promoter was evaluated by means of methylation-specific PCR and differential PCR

	Number of cases	Survival more than 1 yr (%)	Frequency of molecular alterations (%)			
Tumour type			Increased p53 expression	Loss of p16 expression	Hypermethylation or deletion of <i>RASSF1A</i>	Hypermethylation of <i>MGMT</i>
Glioma	27	26	56	67	63 <sup>b</sup>	87.5°
low-grade	7	57	29	86	71	83
high-grade	20	15	65	60	58	89.5
VHL-related tumours	17	83	0	94	43 <sup>b</sup>	64
hemangioblastoma	8	83	0	100	43	50
pheochromocytoma	9	nd	0	89	43	83
Breast cancer	5	nd	60	100ª	nd	nd

<sup>a</sup>Three cases of breast cancer were analysed for expression of p16; <sup>b</sup> 19 cases of gliomas and 14 cases of VHL-related tumours were analysed for alterations in RASSF1A; <sup>c</sup>25 cases of glioma, 14 cases of VHL-related tumours were analysed for alteration in MGMT; nd – breast cancer cases were not involved in *RASSF1A* and *MGMT* analysis; survival data were not collected for pheochromocytomas and breast cancer.

Aberrant methylation in the promoter region of the MGMT gene was frequently observed in gliomas (21 / 24), but was identified also in VHL-related tumours (9 / 14; Table 2). Among gliomas, gene hypermethylation was equally detectable in high-grade and low-grade tumours. In VHL-related tumours, hypermethylation of MGMT was more prevalent in pheochromocytomas (5 / 6) than in hemangioblastomas (4 / 8).

No hypermethylation of the *RASSF1A* promoter was observed in CHBs or PCCs tumours, but frequent occurrences of deletions in the 3p21 locus were shown by means of dPCR (Fig. 2). Deletion of the analysed *RASSF1A* locus was observed in three out of seven hemangioblastomas and in three out of seven pheochromocytomas. In total, the prevalence of the *RASSF1A* gene inactivation in VHL-related tumours was 43% (Table 2).

In gliomas, both hypermethylation and deletion were observed in the promoter region of *RASSF1A*. Hypermethylation occurred in low-grade tumours – in two cases with oligodendrogliomas and one case with oligostrocytoma. Deletion of the analysed region occurred in 7 of 12 glioblastomas. Collectively, the gene was inactivated by promoter hypermethylation or deletion in 63% (12 / 19) of gliomas (Table 2).

In glioma cell lines included into the study, hypermethylation of the *RASSF1A* gene was detected in U138, U251 and U373 and of the *MGMT* gene in the cell lines U87, U251, U373. Leukocyte DNA from healthy controls was negative for methylation or deletion in either of the genes.

## Biomarkers and clinico-pathological features of the tumours

A comparison of survival data among the tumour groups was made by evaluating the proportion of cases that survived for more than one year after diagnosing the disease. This value was significantly (p = 0.02) higher in the group of benign tumours of brain (CHBs) than in the group with glioma. In addition, in low-grade gliomas the survival was higher than in high-grade gliomas (p = 0.05; Table 2).

Comparison of protein p53 expression in different groups of tumours revealed a marked difference between malignant tumours, including gliomas and breast carcinomas, and benign tumours CHB and PCC (Fig. 3). Protein p53 expression was more frequently identified in gliomas (56%) and breast cancer (60%) than in VHL-related tumours (0%). The difference was highly significant when VHL tumours were compared to gliomas (p = 0.0001; OR .4; 95% CI 2.4-795.8) or breast carcinomas (p = 0.006; OR 49.0; 95% CI 1.9-1259.7), but with vide confidence intervals caused by comparison to 0 value. Loss of p16 expression did not assist in the discrimination between benign and malignant tumours. The alteration was frequent in breast carcinomas (100%) and gliomas (67%), but was identified also in almost all (94%) cases with CHB and PCC. Similarly, no difference between the frequency of genetic or epigenetic alteration in the MGMT and RASSF1A genes was observed between gliomas and VHL-related tumours.



Fig. 3. Frequency of p53 and p16 protein expression changes in gliomas, VHLrelated tumours (capillary hemangioblastomas and pheochromocytomas) and breast carcinomas

Association analysis with clinical and demographic variables was done only in gliomas - the homogeneous and best represented group of tumours in the study. p53-positive gliomas tended to be of older age at the diagnosis of diseases (mean age 66.0 years) as compared to p53-negative gliomas (mean age 61.5 years), and the p53-positive group was mainly composed of high-grade tumours (87% of glioblastomas). However, a comparison of patient survival did not reveal any significant difference between p53-positive and p53-negative gliomas. The proportion of cases who survived for more than 1 year after the diagnosis was 20% in the p53-positive group and 33% in the p53-negative group. No significant associations were detected between the p53 status and gender of the patients; also, no significant correlations occurred between p53 and other biomarkers included into the study. No significant associations were observed among the other biomarkers (loss of p16 expression, MGMT hypermethylation, and deletion or hypermethylation of RASSF1A) and clinical-demographic variables of gliomas.

## DISCUSSION

In the present study, four molecular biomarkers were assessed for the frequency of detection and clinical relevance in 27 cases of glioma, the malignant tumours of the CNS, and in 17 cases of rare VHL-related tumours, including 8 capillary hemangioblastomas, benign tumours of brain. The assessed molecular biomarkers represent important tumour suppressor genes involved in cell-cycle control, apoptosis and DNA repair and frequently inactivated in a variety of tumours. Protein expression analysis revealed a frequent (56%) aberrant expression of the p53 protein in gliomas, while no such alterations were detected (0%) in the group of benign tumours, PCC and CHB. An altered expression of the p53 protein was more frequent in high-grade gliomas (glioblastomas; 65%) than in low-grade tumours (29%). A low number of patients (20%) with p53-positive gliomas survived for more than one year after the diagnosis of disease. Other biomarkers, including the loss of p16 expression, promoter hypermethylation of the *MGMT* gene and promoter hypermethylation or deletion of the *RASSF1A* gene, were frequent in all groups of cancer analysed and did not favour discrimination between benign tumours and malignancies.

The tumour suppressor p53 (also known as protein 53 or tumour protein 53) is an important regulator of many central cellular processes, such as cell cycle, response to DNA damage, and apoptosis (5, 16). The gene TP53 is inactivated in about half of all human cancers. In our study, 56% of gliomas showed an aberrant expression of p53; this is in agreement with the data on the frequency of TP53 mutations in these tumours (3, 4). Data of other studies (17, 18), in agreement to our results, have demonstrated that the proportion of tumour cells with abnormal p53 immunoreactivity increases with the grade of glioma and is predominant in glioblastomas. In addition, Zolota etal. (18) detected a significant association of p53-positivity with the expression of cell proliferation biomarker Ki67 and with the reduced survival of patients. In our study, no alterations of p53 expression were observed in CHBs and PCCs; this is in agreement with the other studies of these rare tumours (19, 20).

The tumour suppressor gene p16 (p16<sup>INK4a</sup>/CDKN2A) encodes the p16 protein, an important regulator of the cell cycle. It is a molecular component of the retinoblastoma protein (pRB) regulatory pathway frequently altered in human malignancies (21). Inactivation of genes of the pRB pathway is commonly observed in glioblastomas, with 50% of primary cases having these alterations (3, 4). Similarly to our observation, a high frequency of p16 inactivation in gliomas with an alteration prevailing in low-grade tumours was reported in (18). In another study (22), the loss of p16 expression was shown to be associated with a higher Ki67 and a poor survival in low-grade astrocytomas. In PPCs, a frequent loss of p16 expression was shown in our study and in a study of other authors (23) which showed that the loss of p16 expression in PCCS was caused by deletions or aberrant promoter methylation of the p16 gene. No other studies have been published on p16 status in CHBs, but our study shows a frequent loss of protein expression in these tumours. In addition, our previous study (12) has indicated an involvement of aberrant promoter methylation in the inactivation of the p16 gene in CHBs.

Hypermethylation in the promoter regions of tumour suppressor genes is an early and frequent event associated with the transcriptional silencing of regulatory genes during carcinogenesis (13). Our recent studies and studies of other authors (13, 14, 24, 25) revealed a significant association between hypermethylation of particular TSGs and the extent of carcinogen exposure, aggressiveness of disease and sensitivity to anticancer treatment in different tumours. The clinical importance of hypermethylation markers has been shown in various malignancies, including glioma (6). The epigenetic inactivation of the MGMT gene by promoter hypermethylation is emerging as a significant biomarker predicting response to treatment in glioma patients. The MGMT protein is involved in the repair of the alkyl adducts caused by chemotherapy and is responsible for the resistance of tumour cells to alkylating drugs. Gene inactivation by promoter hypermethylation reduces the DNA repair activity of glioma cells overcoming resistance to alkylating agents. Different studies (15, 26, 27) revealed various frequencies of MGMT hypermethylation in glioma, ranging from 30 to 90% depending on the grade of glioma. Our study has shown a high rate (87.5%) of gene inactivation in gliomas from Lithuanian population, but a study in a larger group of patients is desirable. In addition, our study reports a high frequency of MGMT hypermethylation in CHB (50%) and PCC (83%), for the first time showing a frequent inactivation of this enzyme in these usually benign tumours.

RASSF1A is a recently discovered tumour suppressor; its inactivation is involved in the development of numerous human malignancies (28). RASSF1A contains a Ras association domain and is potentially a target of the Ras oncoprotein. RASSF1A is involved in cell cycle regulation and apoptosis induction. In cancer, the gene is most frequently inactivated by aberrant promoter methylation, but deletions of the 3p21 locus are also frequent in various tumours. In the pathogenesis of VHL-related tumours, the 3p chromosomal arm is of high importance as it also encodes the VHL gene. In agreement with other studies (29, 30), we detected a frequent (63%) inactivation of RASSF1A not only in gliomas, but also in VHL-related tumours (43%). However, in VHL-related tumours the gene was inactivated by genetic deletion possibly caused by the bigger genetic losses in the 3p chromosomal arm of cancer cells.

The study contributes to the understanding of the molecular pathways of the pathogenesis of poorly studied VHLrelated tumours - hemangioblastoma and pheochromocytoma - and shows a frequent loss of p16 expression as well as inactivation of the genes MGMT and RASSF1A in these tumours. Alterations of these genes can be especially important in the pathogenesis of sporadic CHB and PCC cases which may arise without inherited VHL mutation. In addition, the study reports the aberrant expression of p53 protein in a significant set of gliomas and especially in glioblastomas. This biomarker is a potent indicator of a malignant outcome of diseases, as it distinctly discriminates between malignant gliomas and benign tumours of the brain (capillary hemangioblastomas). Although this study was done in quite small groups of patients, it provided valuable information for the further development of the system of molecular biomarkers relevant for an improved management of brain tumours and tumours of other localization.

## CONCLUSIONS

The tumour suppressor protein p53 is aberrantly expressed in malignant tumours such as glioma and breast cancer, but not in usually benign VHL-related tumours, capillary hemangioblastoma and pheochromocytoma. A loss of p16 expression as well as epigenetic or genetic inactivation of the genes *MGMT* and *RASSF1A* occur frequently in glioma, hemangioblastoma and pheochromocytoma.

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## P53 BALTYMO RAIŠKOS POKYČIŲ TYRIMAI GLIO-MOSE, KAPILIARINĖSE HEMANGIOBLASTOMOSE IR FEOCHROMOCITOMOSE

#### Santrauka

Įvadas. Glioma yra dažniausiai pasitaikantis ir daugiausia mirčių sukeliantis piktybinis centrinės nervų sistemos navikas. Kapiliarinė hemangioblastoma (CHB) – retas, paprastai gerybinis smegenų navikas, pasireiškiantis sporadiškai arba kartu su von Hippel-Lindau (VHL) liga. Norėdami geriau ištirti su VHL liga susijusių navikų ir gliomų molekulinius patogenezės mechanizmus, analizavome naviką slopinančių p53 ir p16 baltymų raišką bei naviką slopinančių *MGMT* ir *RASSFIA* genų promotorių metilinimą.

Medžiagos ir metodai. Tirti 27 gliomos, 5 krūties vėžio ir 17 VHL grupės (CHB ir feochromocitomos, PCC) ligonių navikų mėginiai. p53 ir p16 baltymų raiška tirta imunohistochemijos metodu, genų promotorių metilinimas analizuotas metilinimui jautrios PGR metodu, o delecija nustatyta lyginamosios PGR metodu, produktą vertinant *On-Chip* elektroforeze.

**Rezultatai.** Pakitusi p53 baltymo raiška dažniau (p < 0,01) nustatyta piktybiniuose (gliomose ir krūties karcinomose) nei gerybiniuose navikuose (PCC ir CHB). p53 baltymo raiškos pokyčiai dažniausiai (65 %) stebėti glioblastomose. Tik 20% pacientų su p53 teigiamomis gliomomis išgyveno ilgiau nei vienerius metus po diagnozės. Kiti biožymenys – p16 raiškos praradimas, *MGMT* geno promotoriaus hipermetilinimas ir *RASSFIA* geno promotoriaus hipermetilinimas ar delecija – dažni visose tirtų navikų grupėse.

**Išvados.** Naviko supresorių p16, *MGMT* ir *RASSFIA* pokyčiai dažnai nustatomi VHL gupės navikų mėginiuose, tuo tarpu p53 raiškos pokyčiai stebimi tiktai piktybiniuose navikuose – gliomose ir krūties karcinomose.

**Raktažodžiai:** p53, glioma, kapiliarinė hemangioblastoma, feochromocitoma, krūties vėžys, promotorių metilinimas