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Frequent aberrant DNA methylation of *CDKN2A* locus in capillary hemangioblastomas, pheochromocytomas and gliomas

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Background. Both capillary hemangioblastoma (CHB) and pheochromocytoma (PCC) are rare, usually benign tumours occurring sporadically or as part of familial cancer syndromes. The genetic background of most of the inherited cases is well established, but the molecular causes of sporadic cases remains poorly characterized. To better understand the molecular mechanisms of CHB and PCC pathogenesis, we analysed the genetic and epigenetic alterations of the *p16* and *p14* genes at the *CDKN2A* locus.

Materials and methods. Aberrant methylation of the *p16* and *p14* genes was analysed in 16 cases with CHB or PCC by means of methylation-specific PCR. The differential polymerase chain reaction was used to prove the occurrence of the genetic deletion of *p16*. For comparison, 28 cases of glioma – a highly malignant tumour of the brain – was included into the study.

Results. Data of our study show that gene *p16* is hypermethylated in 25% of CHBs and in 25% of PCCs, while in gliomas this alteration is more frequent (35%) and predominantly occurs in low-grade tumours (67%). Frequent hypermethylation of the *p14* gene was observed in PCCs (50%) and CHBs (37.5%), but was less prevalent in gliomas (26%). When all alterations in the *CDKN2A* locus were considered, including hypermethylation of *p16* and *p14*, and genetic deletion of *p16*, 75% of PCC, 62.5% of CHB, and 64% of gliomas had at least one alteration of this locus.

Conclusions. Our study adds new data to understanding the involvement of the *CDKN2A* locus in the pathogenesis of CHB and PCC – two of the most common VHL-related tumours. Furthermore, aberrant methylation in the *CDKN2A* locus is also frequent in gliomas.

Key words: *CDKN2A* locus, promoter methylation, glioma, capillary hemangioblastoma, pheochromocytoma

INTRODUCTION

The cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus on the human chromosome 9p21 encodes two important cell cycle regulators – the *p16* (*CDKN2A/INK4a*) and *p14* (*ARF*) genes. The tumour suppressor p16 is an inhibitor of cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and is a significant component of the retinoblastoma protein (pRB) regulatory pathway (1). The ARF protein encoded by *p14* is an important player in the p53 pathway; it is responsible for the stabilization and increased functionality of p53 (1). Inactivation of this locus through genetic deletion or aberrant promoter DNA methylation has been observed in various malignancies, including gliomas (2–4) and pheo-

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chromocytomas (5, 6). Moreover, the evidence obtained using a mouse model (7) strongly supports involvement of *CDKN2A* locus in the development of pheochromocytoma (PCC). Our previous study (8), conducted by means of immunohistochemistry, identified a frequent loss of the p16 protein expression in gliomas, PCC and capillary hemangioblastomas (CHB). We continued our studies on the pathogenesis of PCC and CHB (8, 9) by analysing genetic losses and DNA hypermethylation in the promoter region of the *p16* and *p14* genes at the *CDKN2A* locus. For comparison, cases with glioma – a highly malignant tumour of the central nervous system (CNS) – were included into the study.

Capillary hemangioblastoma (CHB) is a benign hypervascular tumour most frequently located in the CNS and in some cases in the retina (10). About 20–30% of all CHBs occur as part of familial von Hippel–Lindau (VHL) disease caused by inherited mutation of the *VHL* gene, but sporadic cases are also common among the patients (10, 11). Pheochromocytoma (PCC) is a rare tumour arising from neural crest-derived chromaffin cells in the adrenal medulla. The majority (80–90%) of PCCs are sporadic cases, but they may occur also as part of several cancer syndromes, including VHL disease (12). Mutations of the *VHL* gene have been observed not only in familial cases of CHB and PCC, but also are detectable in sporadic tumours (13). However, recent data have pointed to the involvement of other genes in the pathogenesis of CHB and PCC, especially in sporadic cases (14, 15).

Glioma is the most prevalent tumour of the brain, which accounts for 2% of all cancers and results in a disproportionately high share of cancer morbidity and mortality (16). Gliomas are derived from glial cells (astrocytes, oligodentrocytes and ependymocytes) and comprised of astrocytomas, glioblastoma multiforme (GBM), oligodendrogliomas, and ependymomas. GBM is the most common (accounting for 60–70% of all malignant gliomas) and the most malignant variant of glioma, with most of the patients dying within 1 year after diagnosis (16, 17). Tumourigenesis of glioma is a complex process, and the p16-pRb and ARF-p53 cell cycle arrest pathways play a prominent role in glial transformation (16, 17).

Aberrant DNA methylation in the promoter region of tumour suppressor genes (TSGs) is an early and ubiquitous process in human cancer, which has a deleterious effect on gene expression and normal cellular functions. Altered epigenetic modifications contribute to many of the hallmarks of cancer cells, including the loss of cell cycle control due to inactivation of major regulatory proteins, such as p16 and ARF (18). Our previous studies (19–21) and data of other researchers have revealed a frequent inactivation of the genes on the *CDKN2A* locus through aberrant promoter methylation in a wide spectrum of tumours, including carcinomas of lung, bladder, head and neck, while data on DNA methylation status on this locus in human PCC and CHB are very scarce.

We extended our previous studies on the molecular pathways of hemangioblastoma and pheochromocytoma and analysed the genetic and epigenetic alterations of the *p16* and *p14* genes on the *CDKN2A* locus. For comparison, a set of cases with glioma, a highly malignant tumour of the brain, was included into the study.

MATERIALS AND METHODS

Patients and samples

The study analysed 8 cases of PCC, 8 cases of CHB and 28 cases of glioma, mainly diagnosed as glioblastoma (75%). The main characteristics of the study group are presented in Table 1. The study was approved by the Lithuanian Ethics Committee.

Paraffin-embedded tumour specimens were obtained from the archives of the National Centre of Pathology, Lithuania. All good-quality CHB and PCC samples and representative samples of glioma collected at the National Centre of Pathology (NPC) during 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 standard phenol–chloroform purification and ethanol precipitation.

Table 1. **Main characteristics of study groups**

Tumour	Number	Sex		Age, years (Mean (variation))
		Male	Female	
Glioma	28	9	19	64.0 (38–80)
• Glioblastoma	21	9	12	65.9 (49–80)
• Oligodendroglioma	4	0	4	50.5 (38–61)
• Astrocytoma	2	0	2	66.0 (57–75)
• Oligoastrocytoma	1	0	1	75
Hemangioblastoma[a]	8	4	2	36.2 (22–79)
Pheochromocytoma	8	4	4	49.5 (25–64)
All cases	44	17	26	57.3 (22–80)

[a] Demographic data are missing for two cases of capillary hemangioblastoma.

DNA extracted from leukocytes of healthy controls (n = 2) and DNA from the 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.

Methylation analysis of the *p16* and *p14* genes

Methylation-specific PCR (MSP; 22) was used for the analysis of the methylation pattern in the 5' region of the *p16* and *p14* genes (Fig. 1A). Genomic DNA (1 μg) was exposed to bisulphite modification as described elsewhere (20). Briefly, 1 μg 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 and precipitated with ethanol.

PCR primers specific to methylated (M) and unmethylated (U) sequences within the 5' region of the *p16* and *p14* genes were designed according to published sequences (22, 23) and purchased from biomers.net (Ulm Donau, Germany).

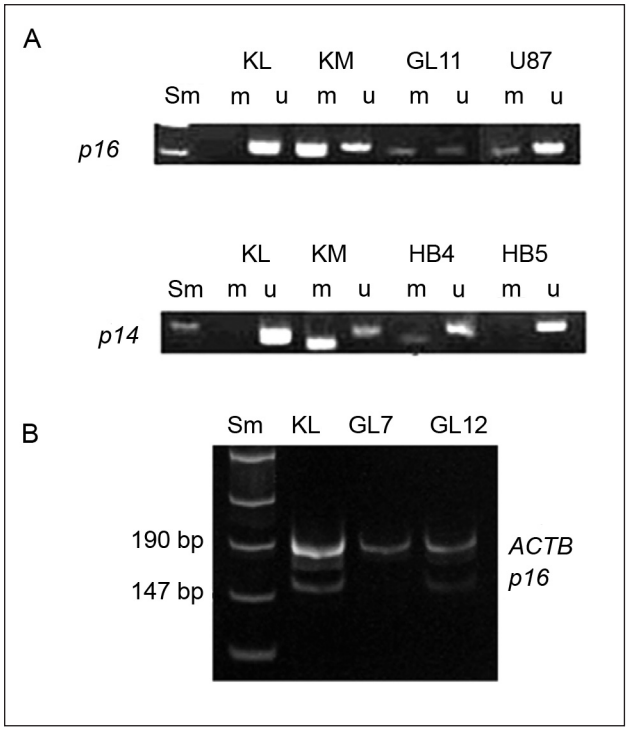

Fig. 1. An example of *p16* and *p14* promoter methylation analysis by methylation-specific PCR (MSP; A) and *p16* deletion detection by differential PCR, using β-actin (*ACTB*) as a reference gene (B). Sm – size marker, m – methylated, u – unmethylated PCR product, KL – leukocyte DNA, KM – *in vitro* methylated DNA, U87 – glioma cell line, GL – glioma, HB – capillary hemangioblastoma

The MSP reaction mixture for 25 μl of 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 $MgCl_2$, 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). The 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 s, annealing at 64 or 66 °C (*p16* and *p14*, respectively) for 45 s, 72 °C for 45 s, 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 being hypermethylated when MSP amplification products were detected in both reactions with the primers for M and U sequence, or for M sequence only (Fig. 1A).

Bisulphite-modified DNA from leukocytes of healthy donors was used as a negative control and *in vitro* methylated DNA (SssI methylase, New England BioLabs Inc, Beverly, MA) or DNA from cancer cell lines as a positive control. Non-template (water) controls were included in each PCR.

Gene deletion analysis by differential PCR

Cases negative in the MSP reaction (neither methylated nor unmethylated MSP product detected) were analysed by means of the differential PCR (dPCR; (2)) to confirm the occurrence of genetic deletion. Negative MSP reactions were mainly observed in the *p16* gene region, thus only this gene was included in the dPCR analysis. The sequence covering the *p16* gene region analysed in MSP was included in the dPCR analysis. As a reference, the sequence for the gene β-actin was used (2). 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 $MgCl_2$, primers for the analysed gene and β-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 on polyacrylamide gel after ethidium bromide staining. Samples positive for β-actin reaction, but negative for *p16* or showing a week signal were considered as having a deletion of the *p16* locus (Fig. 1B).

Statistical analysis

Two-sided Fisher's exact test was used for a 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.

Table 2. **Summary of molecular analysis in gliomas, capillary hemangioblastomas, and pheochromocytomas. Status of the *p16* and *p14* genes promoters was evaluated by means of methylation-specific PCR and differential PCR**

Tumour type	Number of cases	Frequency of alterations (%)			
		p16 methylation	*p16* deletion	*p14* methylation	≥1 alteration in *CDKN2A* locus
Glioma	28	9 / 26; 35%	6 / 26; 23%	7 / 27; 26%	16 / 25; 64%
Low-grade	7	4 / 6; 67%	1 / 6; 17%	3 / 7; 43%	5 / 6; 83%
High-grade	21	5 / 20; 25%	5 / 20; 25%	4 / 20; 20%	11 / 19; 58%
Hemangioblastoma	8	2 / 8; 25%	1 / 8; 12.5%	3 / 8; 37.5%	5 / 8; 62.5%
Pheochromocytoma	8	2 / 8; 25%	0 / 8; 0%	4 / 8; 50%	6 / 8; 75%

RESULTS

Genetic and epigenetic inactivation of the *p16* and *p14* genes

Aberrant promoter methylation of the *p16* gene (Table 2) was observed in two cases with CHB (2/8; 25%) and two cases with PCC (2/8; 25%). Similarly, the gene *p14* promoter was hypermethylated in three cases with CHB (3/8; 37%) and four cases with PCC (4/8; 50%). In total, the prevalence of hypermethylation in these tumours was 25% for the *p16* gene and 41% for the *p14* gene. One case with CHB (1/8; 12.5%) was negative in the *p16* MSP reaction and was analysed by dPCR. Occurrence of the genetic deletion was proved by this method. In total, 69% (11/16) of the cases with CHB or PCC were identified either with genetic (deletion) or epigenetic (DNA methylation) alterations of genes on the *CDKN2A* locus.

In gliomas, the frequency of hypermethylation was 35% (9/26) in the *p16* gene and 26% (7/27) in the *p14* gene. Hypermethylation of these genes was more prevalent (Table 2) in low-grade gliomas (astrocytomas, oligoastrocytomas, oligodendrogliomas) as compared to high-grade tumours – glioblastomas. However, this difference did not reach statistical significance ($p = 0.13$ for the *p16* gene and $p = 0.33$ for the *p14* gene). Deletion of the *p16* gene was detected in one case (17%) with low-grade glioma and in 25% (5/20) of high-grade gliomas. In total, 64% (16/25) of gliomas had at least one alteration of the analysed locus. Interestingly, hypermethylation of the genes in the *CDKN2A* locus or *p16* deletion were identified in all but one cases with low-grade glioblastoma, showing the significance of this locus in this type of malignancies.

In glioma cell lines, hypermethylation of the *p16* gene was detected in the cell line U87, while no hypermethylation of the gene *p14* was observed in any of glioma cell lines analysed. Leukocyte DNA from healthy controls was negative for methylation in both genes.

A comparison of benign tumours (PCC and CHB) with gliomas did not reveal statistically significant differences in the frequencies of epigenetic or genetic changes of the *CDKN2A* locus (Fig. 2). However, hypermethylation of the *p16* gene was most frequent in low-grade gliomas (67%; $p = 0.06$ as compared with other tumours), while the *p14* gene hypermethylation was predominant in PCC (50%; $p = 0.19$ as compared with other tumours).

Associations to clinical and molecular variables

No significant associations were observed between the genes' methylation or mutation and clinical-demographic variables of the cases in the total group of tumours. A separate analysis of gliomas as the most homogeneous and numerous group of the study revealed some important tendencies. Hypermethylation of *p14* was prevalent in female patients with glioma and not found in men ($p = 0.059$; OR 12.39; CI 0.62–246.32). The proportion of those who

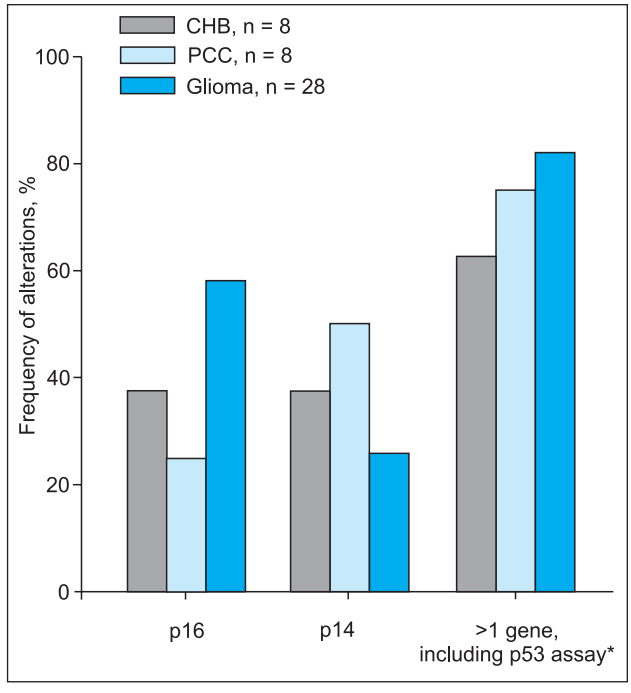

Fig. 2. Frequency of promoter methylation and deletion of the *p16* gene, promoter hypermethylation of the *p14* gene and alterations of at least one gene, including p53. * Data on p53 are published in (8)

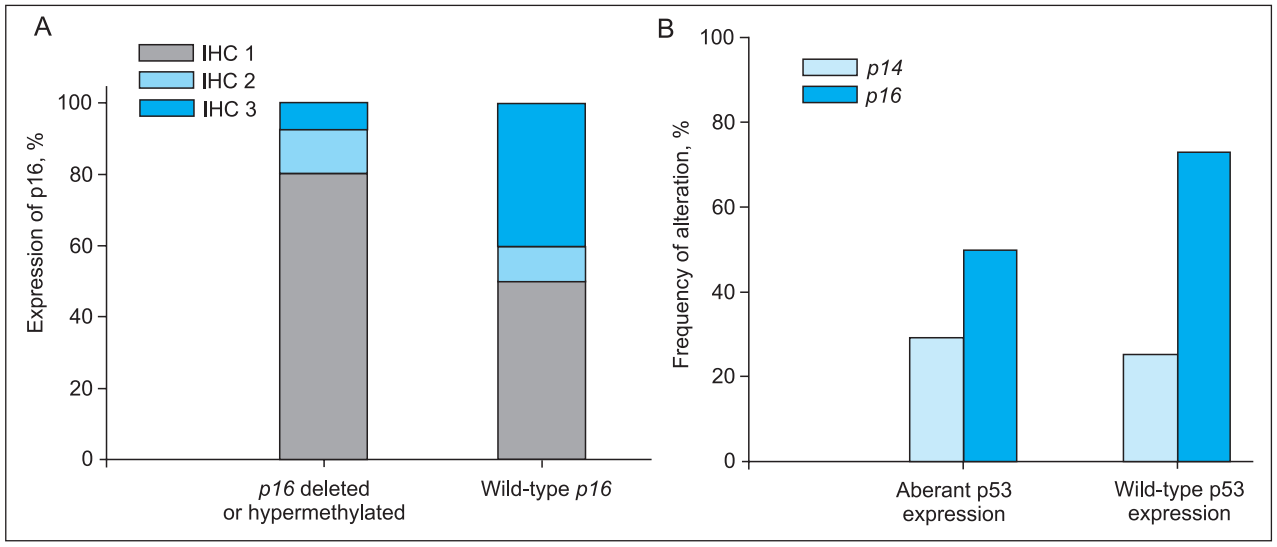

Fig. 3. Associations between the p16 protein expression and deletion or hypermethylation of the *p16* gene (A); associations between aberrant p53 expression and alterations of the *p16* or *p14* genes

survived longer than one year after the diagnosis was higher among cases with *p16* hypermethylation or mutation than in patients without these alterations (83% vs 50%).

The genetic and epigenetic alterations of the *CDKN2A* locus were correlated with the p16 and p53 protein expression assessed by means of immunohistochemistry (IHC) in our previous study (8) on the same set of cases. In PCC and CHB, neither p16 nor p53 expression were detected. In gliomas, only 7% of cases with *p16* deletion or hypermethylation showed a strong IHC signal (IHC3) for p16, while 40% of cases without genetic or epigenetic inactivation of *p16* expressed p16 abundantly (Fig. 3A). In gliomas, *p14* hypermethylation almost equally occurred in cases with an aberrant and normal p53 expression (29% and 25%, respectively), while *p16* alterations were more prevalent among p53 wild-type cases (73% vs 50% as compared with p53-mutant cases; Fig. 3B).

When alterations in all tree genes (*p16, p14* and *TP53*) were considered, 82% of gliomas were positive for at least one of these biomarkers (Fig. 2). In contrast, aberrant expression of p53 was rare in benign tumours (PCC and CHB) and did not improve the diagnostic value of the molecular system of biomarkers analysed in our study.

DISCUSSION

In the present study, we report a frequent inactivation of genes on the *CDKN2A* locus through aberrant methylation or genetic deletion in the rare and usually benign human neoplasms – pheochromocytoma and hemangioblastoma. At least one alteration of this locus was observed in 75% of PCC and in 62.5% of CHB, but was also frequent (64%) in gliomas – malignant tumours of the brain. The study provides first data on the frequency of aberrant methylation in the promoter region of the *p16* and *p14* genes in CHB.

The *CDKN2A* locus on chromosome 9p21 encodes two important regulators from the pRB and p53 pathways – the *p16* (*CDKN2A/INK4*a) and *p14* (*ARF*) genes. The *p16* gene encodes an inhibitor of the cyclin-dependent kinases CDK4 and CDK6; inactivation of this inhibitor causes an uncontrolled proliferation of cancer cells. ARF protein regulates p53 stability in response to aberrant cell growth induced by oncogenic stimuli and, like the p16 protein, is also frequently inactivated during tumourigenesis. These two genes have different promoters and exon 1, but share the exons 2 and 3 that are transcribed in alternative reading frames (1). In cancer cells, both promoters can be inactivated through aberrant DNA methylation independently of each other. However, deletion of the 9p21 locus can inactivate both genes, or even include additional TSGs residing in this locus.

Previous studies (6, 24), including our own data (8), revealed a frequent loss of p16 expression in gliomas and PCCs. Also, our recent study (8) for the first time showed a frequent loss of p16 expression in CHB. Both events, genetic deletion and promoter hypermethylation, are shown to be involved in the inactivation of p16 expression in gliomas and PCCs. Nakamura et al. (2) detected a frequent (13/50; 26%) deletion of the *p16* gene in glioblastomas, but no such deletion was found in low-grade gliomas (2, 25). Similarly, in our study, deletion of the *p16* gene was detected in 25% of high-grade gliomas, but was rare (1/6) in low-grade gliomas. Genetic deletion of the *p16* locus occurred also in one case with CHB, but was not observed in PCC.

Numerous studies of various human tumours have supported the observation that p16 expression can be lost

due to the aberrant DNA methylation in the promoter of the gene. In our study, aberrant promoter methylation occurred in 25% of CHBs and in 25% of PCCs. This epigenetic alteration was also frequent (35%) in gliomas, and predominantly occurred in low-grade tumours (67%). In glioblastomas, the published frequency of *p16* promoter hypermethylation varies considerably among studies – from less than 10% up to 90% (2, 26–28). In agreement with our data, frequent hypermethylation of *p16* in low-grade gliomas has been shown in several studies (29, 30). In PCC, our data on the moderate frequency of *p16* hypermethylation are in line with other two studies (5, 6) in which the rates of this alteration were 24% and 35.5%, respectively.

In general, our present study on the inactivation of *p16* through genetic deletion or aberrant promoter methylation is in good agreement with our previous data (8) on p16 expression. In gliomas, 14 out of 15 cases with the genetic or epigenetic inactivation of *p16* had also reduced the expression of the p16 protein. Interestingly, all cases with CHB and PCC showed a reduced or no p16 staining in our previous study (8), despite DNA methylation or mutation status, showing the importance of additional regulators for the p16 expression in these tumours. Similarly, the study of Muscarella et al. (6) showed a reduced p16 expression in all but one cases with PCC, while the genetic and epigenetic alterations occurred only in a set (68%) of the cases. In CHB, no data on the frequency of genetic deletions or promoter hypermethylation of the *p16* gene have been published up to date.

The reported frequencies of *p14* promoter methylation in gliomas range from 20 to 40% (3, 4, 29, 31). Furthermore, aberrant methylation of *p14* was shown to be associated with the progression of astrocytic tumours. In PCC, only one study (32) analysed *p14* hypermethylation, and no such studies have been conducted in CHB. Kiss et al. (32) reported frequent (73%; 32/44) *p14* methylation using an MSP assay, but a quantitative analysis revealed low levels of the gene methylation. In the present study, *p14* gene promoter methylation was frequent in PCCs (50%) and CHBs (37.5%), but less abundant in gliomas (26%).

ARF, encoded by *p14*, is a protein of the p53-regulated pathway. When the results of our previous study (8) were considered in combination with the present data, we noted an exclusive nature of the occurrence of p53 alterations and *p14* hypermethylation. In PCC and CHB, where no aberrant expression of p53 was detected by means of immunohistochemistry, aberrant methylation of *p14* was a frequent event that occurred in 44% of cases. In contrast, in glioblastomas where p53 alterations were obviously predominant (65%), *p14* hypermethylation was not a common event (20%). The exclusive nature of the inactivation of genes from the same pathway is a well known phenomenon. As an example, in gliomas He et al. (31) observed a negative correlation between *p14* methylation and p53 protein expression. Moreover, Watanabe et al. (3) have provided an evidence that alternative alterations of the *p53* and *p14* genes can serve as a significant predictor of a shorter progression-free survival in glioma. No such association with the survival of patients was detected in our study.

In summary, when all data on the genetic and epigenetic alterations of the *CDKN2A* locus were combined with the results on p53 expression, at least one alteration in the *CDKN2A* locus and p53 was found in 82% of gliomas. The alterations of the *CDKN2A* locus were also abundant in CHBs (62.5%) and PCC (75%), but p53 mutations were rare in these tumours. Our study supports the finding of other studies (5, 6, 32) that aberrant methylation of the *p16* and *p14* genes is highly important in PCC pathogenesis and for the first time reports a frequent inactivation of these genes in CHB.

CONCLUSIONS

The study contributes to the understanding of molecular pathways in the pathogenesis of poorly studied tumours – hemangioblastoma and pheochromocytoma – and shows a frequent inactivation of the genes *p16* and *p14* through aberrant methylation and genetic deletion in these tumours. In addition, our study supports data on the frequent involvement of the *CDKN2A* locus in the pathogenesis of gliomas – highly aggressive tumours of the CNS.

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CDKN2A LOKUSO HIPERMETILINIMO TYRIMAI KAPILIARINĖSE HEMANGIOBLASTOMOSE, FEOCHROMOCITOMOSE IR GLIOMOSE

Santrauka

Įvadas. Kapiliarinė hemangioblastoma (CHB) ir feochromocitoma (PCC) yra reti, dažniausiai gerybiniai navikai, pasireiškiantys sporadiškai arba kaip šeiminiai vėžio sindromai. Šeiminių susirgimų genetinės priežastys yra pakankamai gerai ištyrinėtos, kiek mažiau žinoma apie sporadinių navikų molekulines priežastis. Norėdami geriau ištirti CHB ir PCC molekulinius patogenezės mechanizmus, šiuose navikuose analizavome *CDKN2A* lokuso *p16* ir *p14* genų genetinius ir epigenetinius pakitimus.

Medžiagos ir metodai. CHB ir PCC (n = 16) *p16* ir *p14* genų promotoriuose DNR hipermetilinimas buvo tiriamas naudojant metilinimui jautrios PGR metodą, *p16* geno delecija nustatyta lyginamąja PGR. Palyginimui tie patys *CDKN2A* lokuso tyrimai atlikti piktybiniuose smegenų navikuose – gliomose (n = 28).

Rezultatai. *p16* geno hipermetilinimas nustatytas 25 % CHB ir 25 % PCC atvejais. Šis pakitimas rastas 35 % gliomų ir dažniausiai (67 %) buvo nustatytas žemo piktybiškumo laipsnio navikuose. Dažnas *p14* geno hipermetilinimas nustatytas PCC (50 %) ir CHB (37,5 %) ir kiek rečiau – gliomose (26 %). Bent vienas iš tirtų pakitimų, įskaitant *p16* ir *p14* hipermetilinimą bei *p16* deleciją, buvo nustatytas 75 % PCC, 62,5 % CHB ir 64 % gliomų.

Išvados. Tyrimu nustatyta, kad *CDKN2A* lokuso pakitimai yra dažni gerybiniuose VHL grupės navikuose, feochromocitomose bei hemangioblastomose, ir patvirtino šių pakitimų svarbą gliomų patogenezei.

Raktažodžiai: *CDKN2A* lokusas, glioma, kapiliarinė hemangioblastoma, feochromocitoma