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Tumor suppressor gene *ZAC/PLAGL1:* altered expression and loss of the nonimprinted allele in pheochromocytomas

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ZAC/PLAGL1 is a novel imprinted tumor suppressor gene encoding an important inducer of cell cycle arrest and apoptosis, and found to be lost during tumorigenesis. We analyzed the significance of *ZAC* in the development of a rare, usually benign tumor of the adrenal gland: pheochromocytoma (PCC). Twenty-four PCCs were analyzed for the loss of the active nonimprinted allele of *ZAC,* and nine of the twenty-four PCCs were also assayed for expression of the protein. In thirteen of the cases, a paired nonmalignant tissue was available for analysis. Methylation-specific polymerase chain reaction revealed frequent (15 of 23, 65%) loss of unmethylated DNA in the imprinting control region of *ZAC*. Immunohistochemistry identified reduced ZAC expression in 56% (5 of 9) of the subset cases. Four of the five PCC cases where reduced expression of ZAC was observed were also positive for the loss of the active *ZAC* allele. Additionally, the loss of ZAC expression was also found to be frequent in a series of capillary hemangioblastomas and gliomas (6 of 6, 100%, and 17 of 27, 63%, respectively) examined for comparison. In conclusion, our study suggests the involvement of the imprinted *ZAC* gene in the pathogenesis of PCC.

Keywords Imprinting, pheochromocytoma, promoter methylation, *ZAC* gene

Pheochromocytoma (PCC) is a rare tumor arising from neural crest–derived chromaffin cells in the adrenal medulla or in the paraganglion system. Usually PCCs are benign tumors, but about 10% are malignant (1). Because of the lack of histological criteria for differentiation of benign cases from malignant ones, only metastasized tumors are classified as malignant. A set of histological features has been proposed (2) for prediction of the malignant potential of PCC; however, molecular biomarkers are needed for more precise identification of cases of aggressive disease course.

Although a majority (80–90%) of PCCs develop as sporadic cases, they can also occur as part of several cancer syndromes such as von Hippel–Lindau (VHL) disease, multiple endocrine neoplasia type 2 (MEN 2) syndrome, paraganglioma syndromes, and neurofibromatosis 1 (1,3). Mutations of the *VHL, RET, SDHB, SDHC, SDHD,* or *NF1* genes are the most frequently detected alterations in the familial cases of PCC, while little is known about the molecular background in the sporadic cases. Recent data have pointed to the involvement of other gene loci in the pathogenesis of PCC, especially in sporadic cases (4,5). In our previous work, frequent genetic loss on the 6q chromosome arm was identified in PCC (6), similar to capillary hemangioblastoma (CHB), another type of tumor that may occur as part of VHL disease (7,8). Subsequent analysis (6,7) of the loss of allele in the 6q23–q25 chromosomal region highlighted the possible

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involvement of the *ZAC* gene (also called *PLAGL1* or *LOT1*)—a novel imprinted tumor suppressor gene located at 6q24∼q25—in the pathogenesis of PCCs and CHBs. Our recent study on CHB (9) suggested the loss of the paternal allele of *ZAC* as the main mechanism of gene inactivation in CHB, a benign tumor of the central nervous system. However, to our knowledge, the imprinting status of the *ZAC* gene or its expression has not yet been studied in PCCs.

ZAC is a *z*inc finger transcription factor capable of inducing apoptosis and cell-cycle arrest (10). The *ZAC* gene was identified as a gene which was lost during spontaneous transformation of rat ovarian cells in a tissue culture model (11). Later, it was shown that forced expression of ZAC inhibits cancer cell proliferation *in vitro* and suppresses tumor formation in nude mice (12). As a transcription factor, ZAC can induce and regulate expression of genes related to tissue development and differentiation (13), while its antiproliferative and pro-apoptotic functions are mainly related to co-regulation of p53-responsive promoters (12,14). In humans, ZAC is widely expressed in adult tissues, with the most abundant expression detected in the pituitary gland, kidney, placenta, and adrenal gland (15). Loss of ZAC expression has been reported in a number of human tumors, including breast, ovary and prostate cancer, pituitary adenoma, squamous cell carcinoma of the head and neck, basal cell carcinoma, and hemangioblastoma (9,16–21).

ZAC expression is regulated by genomic imprinting, a mode of epigenetic control that includes allele-specific gene expression regulated by DNA methylation and other epigenetic modifications in the imprinting control region of the gene (12). ZAC is expressed from the paternal allele where the imprinting control region is not methylated. In contrast, the regulatory region is hypermethylated on the maternal allele, thus leading to transcriptional inactivation of this sequence. Analysis of this differentially methylated region (DMR) located within a CpG island in the gene promoter revealed preferential loss of the active (paternal) allele of *ZAC* to be a frequent mechanism of gene inactivation in CHB (9).

In the present study, we analyzed the imprinting status of the *ZAC* gene and ZAC protein expression in PCC tumors from samples collected from two study centers, one in Helsinki, Finland, and the other in Vilnius, Lithuania. On the basis of our previous findings (9), a set of cases with CHB from the same VHL-related group of tumors and a set of gliomas—highly aggressive malignant tumors of the central nervous system—were included in the study for comparison.

Materials and methods

Patients and samples

The study analyzed 24 cases of PCC, with 9 paraffin-embedded tumor specimens obtained from the archives of the National Centre of Pathology, Lithuania (NCP), and another set of PCCs ($n = 15$) from the Department of Pathology, Helsinki University Central Hospital, Finland (HUCH). From the latter center, 13 samples of non-tumor adrenal medulla tissue located adjacent to the PCC and obtained from the same patients were also available for study. The ethics committees at both study centers approved the study. All tumors were located in the adrenal gland. None of the cases with PCCs had metastases. In the HUCH group, tumors without metastases but having at least one of the histological features typical to malignant PCC (2) were categorized as borderline PCC. Data on *VHL* mutation status was known for the HUCH cases, while no such data were available for the cases from NCP. As a result of limitations in the amount of available tissue material, the PCC cases from HUCH were analyzed for the loss of the active *ZAC* allele but were not included in the protein expression analyses.

For comparison, 8 cases with CHB and 28 cases of glioma, mainly diagnosed with glioblastoma (75%), were included in the study. All these cases were from NCP, where they were collected during the years 2002 to 2005. The main characteristics of the study group are presented in Table 1.

DNA extracted from the leukocytes of healthy controls ($n = 2$) and normal brain tissue adjacent to the glioma were used as the wild-type controls. DNA from cancer cell lines T24 and H157 (from ATCC, Manassas, VA), and primary glioma cell lines (from HUCH) served as controls in the *ZAC* methylation analysis. Genomic DNA was extracted from the specimens by digestion with proteinase K followed by standard phenol–chloroform purification and ethanol precipitation.

Immunohistochemistry analysis

Paraffin-embedded tissue from 9 cases of PCC, 6 cases of CHB, and 27 cases of glioma was available for immunohistochemical analysis of the ZAC protein. Polyclonal rabbit anti-human ZAC antiserum (H-253; Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:100) was used as the primary antibody. Immunohistochemistry was performed by the Real EnVision detection system with peroxidase/DAB+

Table 1 Compilation of the findings on reduced ZAC expression (immunohistochemistry) and the loss of the active (unmethylated) *ZAC* allele (MSP)

Tumor	No.	Gender		Age (yr), mean (range)	Reduced ZAC expression (%)	Loss of active *ZAC* allele (%)
		Male	Female			
Pheochromocytoma	24	5	19	47.0 (25–72)	5/9 (55.6)	15/23 (65.2)
Hemangioblastoma[a]	8	4	2	36.2 (22–79)	6/6 (100)	6/8 (75.0)
Glioma	28	9	19	64.0 (38–80)	17/27 (63.0)	21/27 (77.8)
Glioblastoma	21	9	12	65.9 (49–80)	13/20 (65.0)	16/20 (80.0)
Low-grade gliomas[b]	7	0	7	58.0 (38–75)	4/7 (57.1)	5/7 (71.4)

[a] Demographic details are missing for two cases of CHB.
[b] This group was composed of four oligodendrogliomas, two astrocytomas, and one oligoastrocytoma.