MMP-14 and TGFβ-1 methylation in pituitary adenomas

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Abstract. Pituitary adenoma (PA) is one of the most common abnormalities in the sellar region. Despite the fact that PA is a benign monoclonal neoplasm, it can cause serious complications, including ophthalmological, neurological and endocrinological abnormalities. Currently, the causes that increase the progression of tumors are unknown. Epigenetic silencing of the matrix *metalloproteinase-14* (*MMP-14)* and *transforming growth factor beta-1* (*TGFβ-1)* genes may be associated with the development of PA, since these genes are important in the processes of tumor metastasis and angiogenesis. The purpose of the present study was to determine if the methylation status of the *MMP-14* and *TGFβ-1* promoters is associated with PA development. In the present study, 120 tissue samples of PA were used. The methylation status of the *MMP-14* and *TGFβ-1* promoters was investigated by methylation specific-polymerase chain reaction. Statistical analysis was conducted to investigate the associations between the methylation status, age and gender of PA patients, PA tumoral activity, recurrence and invasiveness. The *MMP-14* gene was methylated in 30.00% (17/56 functioning and 19/64 non-functioning) of patients with PA, while the *TGFβ-1* gene was methylated in 13.33% (9/56 functioning and 7/64 non-functioning) of patients with PA. It was also observed that promoter methylation of *MMP-14* correlated with the male gender (58.8 vs. 35.7%, P=0.022), while unmethylated (non-silenced) *MMP-14* correlated with the female gender (64.3 vs. 41.7%, P=0.027). Associations between the promoter methylation status of the *MMP-14* and *TGFβ-1* genes and PA functioning or recurrence were not identified. The present study reveals that silencing of the *MMP-14* gene correlates with patients' gender. However, *MMP-14* and *TGFβ-1* promoter methylation cannot be considered as a prognostic marker in PAs.

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Introduction

Pituitary adenoma (PA) is a benign tumor originating in adenohypophyseal cells of the anterior lobe of the pituitary gland (1). The classification of PAs is based on the secretion of hormones; thus, PA can be a secreting (functioning) or a non-secreting (non-functioning) tumor (2). According to their size, adenomas are classified into microadenomas (≤10 mm) and macroadenomas (>10 mm) (2). Women have a 2-fold increased risk of developing PA in comparison with men (1). Most commonly, PA is a non-malignant tumor; however, it tends to recur (3). Usually, this tumor is soft and has no capsule that could isolate it from the surrounding mass of microglia (1). That is the reason why it can grow and infiltrate the surrounding structures. Adenomas may cause symptoms in two ways: i) Due to tumor-related hypersecretion or hyposecretion of hormones, in which case, the tumor causes compression to a normally functioning hypophysis; or ii) due to the compression exerted by the PA on the surrounding structures (2). Functionally active PAs cause less damage to the visual function than non-functioning PAs, since functioning PAs become symptomatic due to hormone secretion, whereas non-functioning PAs can grow slowly, compress the optic chiasm (which is located directly above the pituitary gland) and cause progressive visual loss (4).

The pathogenesis of PA is complex and poorly understood. It is considered that PA has a multifactorial etiology that includes genetic factors that have an impact on PA development (4,5).

Matrix metalloproteinases (MMPs) are a broad family of zinc-binding endopeptidases that aid to degrade the extracellular matrix, which is associated with cancer cell invasion, metastasis and angiogenesis (5). Human *MMP-14*, a membrane-bound zinc endopeptidase, is one of the most important cancer targets, since it plays central roles in tumor growth and invasion (6). *MMP-14* is a membrane-type metalloproteinase with collagenase activity and potential roles in numerous biological processes in both normal and cancerous tissues (7). The majority of studies on *MMP-14* primarily focus on angiogenesis and invasion (8,9). In addition to invasiveness, oncogenes and tumor suppressor genes associated with malignant transformation have received attention in the past years.

Transforming growth factor beta (TGF-β) signaling functions as a suppressor or a promoter in tumor development, depending on the tumor stage and type (10,11). TGFβ signaling is initiated by the binding of ligands (*TGFβ1*, *TGFβ2*

and *TGFβ3*) to type II TGFβ receptors (TGFβ RII), followed by the recruitment of type I TGFβ receptor (TGFβ RI) to form the complex (12-14). Next, TGFβ RII phosphorylates TGFβ RI to activate it (12-14). The clinical significance of TGFβ ligands and downstream signaling mediators has been studied in multiple types of tumors, and the results are discordant (12-14). It is known that *MMP-14* works in the activation of *TGF-β1* cytokine, but the *MMP-14* molecule does not directly activate the *TGFβ-1* cytokine by causing collagen degradation prior to the involvement of thrombospondin 1 (15).

To the best of our knowledge, there are no studies on the role of *TGFβ-1* and *MMP-14* in patients with PA. Therefore, the aim of the present study is to investigate the role of the *TGF-β1* and *MMP-14* signaling pathways in PA tumor development.

Materials and methods

Subjects and ethical statement. The promoter methylation status of *TGFβ-1* and *MMP-14* was analyzed in 120 PA tumor tissues. All PA tumor samples were surgically resected in the Department of Neurosurgery, Hospital of Lithuanian University of Health Sciences Kaunas Clinics (Kaunas, Lithuania), and were histologically diagnosed in the Department of Anatomical Pathology, Hospital of Lithuanian University of Health Sciences Kaunas Clinics. PA samples were snap-frozen in liquid nitrogen prior to DNA extraction. Approval (no. P2-9/2003) to undertake the study was obtained from the Kaunas Regional Biomedical Research Ethics Committee (Kaunas, Lithuania). The study was conducted in the Departments of Ophthalmology and Neurosurgery, Hospital of Lithuanian University of Health Sciences Kaunas Clinics between February 2010 and May 2015. Written patient consent under the approval of the Ethics Committee of Lithuanian University of Health Sciences was obtained for every patient.

The inclusion criteria were as follows: i) Determined and confirmed PA via magnetic resonance imaging (MRI); ii) patient's general good condition; iii) patient's consent to participate in the study; iv) patient's age ≥18 years; and v) no other tumors localized in the brain or other organs. The following data were determined for each patient: Age at the time of operation, gender, promoter methylation status of the genes *TGFβ-1* and *MMP-14*, hormones activity and recurrence of PA.

Invasiveness evaluation. All PAs were anatomically analyzed based on MRI findings. PA invasiveness was confirmed by a surgeon. The suprasellar extension and sphenoid sinus invasion by PAs were classified according to the Wilson-Hardy classification (Hardy classification modified by Wilson) (16). The degree of suprasellar and parasellar extension was graded as stages A-E. The degree of sellar floor erosion was graded as grades I-IV. Grade III, localized sellar destruction, and grade IV, diffuse destruction, were considered as invasive PA. The Knosp classification system was used to quantify invasion of the cavernous sinus, in which only grades 3 and 4 define true invasion of the tumor into the cavernous sinus: Grade 0, no cavernous sinus involvement; grades 1 and 2, the tumor pushes into the medial wall of the cavernous sinus, but does not go beyond a hypothetical line extending between the centres of the two segments of the internal carotid artery (grade 1) or it goes beyond such a line, but without passing a line tangent to the lateral margins of the artery itself (grade 2); grade 3, the tumor extends laterally to the internal carotid artery within the cavernous sinus; and grade 4, total encasement of the intracavernous carotid artery (17). Therefore, only grade III and IV tumors were considered to be invasive.

DNA extraction and modification. Tumor DNA was extracted from 50-100 mg of frozen PA tissue using the salting-out method (12-14). The methylation status of the *MMP-14* and *TGFβ-1* genes promoter was determined by bisulfite treatment of DNA. A total of 400 ng DNA was used for bisulfite modification, which was performed using an EZ DNA Methylation kit (Zymo Research Corporation, Irvine, CA, USA), according to the manufacturer's protocol. Bisulfite-treated DNA was eluted in 40 μl distilled nuclease-free water, and stored at -80˚C until subjected to methylation specific-polymerase chain reaction (MS-PCR).

MS-PCR. The methylation status of the *TGFβ-1* and *MMP-14* promoters region was determined by MS-PCR. Primers distinguishing unmethylated and methylated alleles were designed using 'MethPrimer' (18), and their sequences are presented in Table I.

MS-PCR was performed in a total volume of 15 μl, using Maxima Hot Start Green PCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with Hot Start Taq DNA polymerase and 10 pmol of each primer (Invitrogen; Thermo Fisher Scientific, Inc.). Each MS-PCR incorporated ~20 ng of bisulfite-treated DNA as a template. Human blood lymphocyte DNA treated with bisulfite served as an unmethylated DNA control, while CpG Methylated Human Genomic DNA (Thermo Fisher Scientific, Inc.) was used as a positive methylation control. A blank water control was also included. The conditions for *MMP-14* MS-PCR were as follows: 95˚C for 5 min, followed by 39 cycles of 95˚C for 15 sec, 57˚C for 30 sec and 72˚C for 30 sec, and a final step at 72˚C for 5 min. The conditions for *TGFβ-1* gene amplification were almost identical to those for *MMP-14*, with the exception of the number of cycles (38) and the temperature used in the second step of each cycle (60˚C). The amplified products were analyzed on 2% agarose gels with 0.5 μg/ml (final concentration) of ethidium bromide and visualized under ultraviolet light. The presence of PCR products of the correct molecular weight indicated the presence of either methylated or unmethylated alleles.

Statistical analysis. For statistical analyses, SPSS version 20 (IBM SPSS, Armonk, NY, USA) was used. Statistical analyses were conducted to investigate the association between methylation status, age, gender, PA tumoral activity and recurrence. Associations between gene methylation data and clinical features of PA patients were evaluated using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of PA patients. The median age at PA diagnosis was 56.0 years, with an age range from 18 to 84 years. The male to female ratio was 1:1.35. The median age of males

Table I. Methylation specific-polymerase chain reaction primers.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
MMP-14 (U)	TTTATTGAAGATAAACGTGTTTTGA	CATAAACAAAAAAAACAAAAAACAACA
MMP-14 (M)	TTTATCGAAGATAAAGGCGTTTC	GTAAACAAAAAAAACAAAAAACAACG
TGFβ-1 (U)	GTGGGTTTTTATTATTAGTATGTGG	AAATCCTATCCAAACTACAACTCAC
TGFβ-1 (M)	TTGTGGGTTTTTATTATTAGTACGC	AAATCCTATCCAAACTACGACTCG

U, unmethylated; M, methylated; MMP, matrix metalloproteinase; TGF, transforming growth factor.

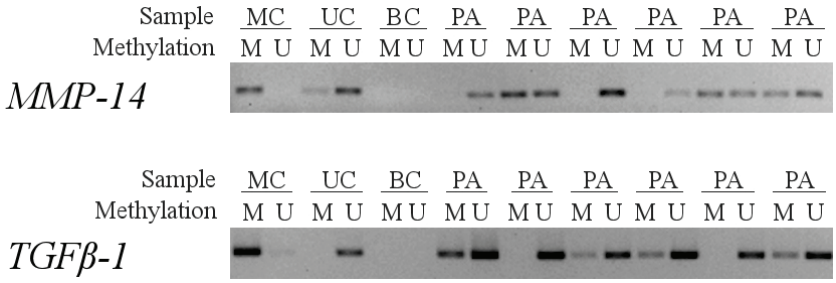

Figure 1. Genes promoter methylation status in PA by the methylation-specific polymerase chain reaction assay. MC, methylated control; UC, unmethylated control; BC, blank control; M, methylated; U, unmethylated; PA, pituitary adenoma sample; MMP, matrix metalloproteinase; TGF, transforming growth factor.

(n=51) was 57.0 years and of females (n=69) 56.0 years. The samples were divided into two groups: Functioning (n=56) and non-functioning (n=64) PAs. Recurrence was observed only in 12 cases out of 120 (3/56 functioning and 9/64 non-functioning) PAs. Invasiveness was evaluated in 104 (35 non-invasive and 69 invasive) cases of PA.

MMP-14 and TGFβ-1 gene promoter methylation frequency in PA. The methylation status of the *MMP-14* and *TGFβ-1* promoters in PA tumor samples was detected by MS-PCR. The methylation status of the *MMP-14* and *TGFβ-1* promoters was evaluated in 120 PA tumors. Representative samples are shown in Fig. 1. The detection of bands with both primer sets was observed in a number of samples but not in all PA tumor samples, probably due to the existence of non-malignant cells in a fraction of the samples or to the methylation of only one allele of the gene. *MMP-14* promoter hypermethylation was detected in 30.00% (36/120) of PAs, while the *TGFβ-1* gene promoter was methylated in 13.33% (16/120) of patients with PA. Both genes were methylated in only 5 samples.

Statistical analysis of age and genes methylation revealed no correlation between them (χ^2 test; age and *MMP-14* methylation, P=0.952; and age and *TGFβ-1,* P=0.971).

MMP-14 methylation was significantly associated with male gender (58.8 vs. 35.7%, P=0.022), while unmethylated *MMP-14* was associated with female gender (64.3 vs. 41.7%, P=0.027). It was also observed that 45/51 males had non-silenced *TGFβ-1* gene. However, this was not significant (χ^2 test, P=0.664). Associations between methylation status and PA functioning or recurrence were not identified (P>0.05) (Table II).

Invasiveness and methylation of both genes had no correlation (χ^2 test, P=0.166 and P=0.308). However, >50% (62/104) of PAs were invasive and had an unmethylated *TGFβ-1* gene. A correlation between invasiveness and age groups was also observed, since 40/69 PAs corresponded to patients who were ≤56 years old (χ^2 test, P=0.037).

Discussion

PA is a common benign monoclonal neoplasm accounting for 15-20% of all primary intracranial tumors (19). Pituitary tumors are benign, but not uncommonly, they invade locally into adjacent tissues such as the cavernous sinus and dura (20). Early prediction of which pituitary tumors will recur and/or exhibit an invasive phenotype remains difficult despite the introduction of several tissue-based molecular markers (20).

It is known that promoter hypermethylation silences gene transcription, resulting in a shortage of expression of one or another protein (21). Membrane type-1 (MT1)-MMP is an activator of soluble MMP-2 (22). The activity of both MMPs is regulated by their physiological inhibitor, tissue inhibitor of metalloproteinase (TIMP)-2 (22). An MT1-MMP/MMP-2/TIMP-2 axis plays a key role in the invasive behavior of numerous cell types (22). It was observed that hypermethylation of the *MMP-14* and *MMP-2* genes correlates with non-invasive tumor behavior (22). To the best of our knowledge, there are no studies that have explored the association between *MMP-14* methylation status and the development of PA. Previously, Altaş *et al* (23) investigated single nucleotide polymorphism (SNP) in the promoter of another gene of same MMP family, and analyzed how that polymorphism is linked to PA development. It is known that *MMP-14* and *MMP-1* function similarly in the degradation of collagen fibers (22). In agreement with this, Altaş *et al* observed that SNP of the *MMP-1* gene promoter in both alleles caused a higher risk of PA development and invasiveness than SNP of the *MMP-1* gene promoter in only one allele (23). In addition, high expression of the *MMP-14* gene was detected in invasive PA and was associated with invasiveness (24).

Table II. Associations between clinical data of patients and genes methylation.

		MMP-14		*TGFβ-1*	
Factor	N (%)	U, N (%)	M, N (%)	U, N (%)	M, N (%)
Age, years					
≤56	60 (50.0)	42 (70.0)	18 (30.0)	52 (86.7)	8 (13.3)
>56	59 (49.2)	41 (69.5)	18 (30.5)	51 (86.4)	8 (13.6)
			P=0.952		P=0.971
Gender					
Females	69 (57.5)	44 (78.3)	15 (21.7)	59 (85.5)	10 (14.5)
Males	51 (42.5)	30 (58.8)	21 (41.2)	45 (88.2)	6 (11.8)
		P=0.027	P=0.022		P=0.664
Hormones activity					
None	64 (53.3)	45 (70.3)	19 (29.7)	57 (89.1)	7 (10.9)
Appear	56 (46.7)	39 (69.6)	17 (30.4)	47 (83.9)	9 (16.1)
			P=0.936		P=0.409
Recurrence					
None	108 (90.0)	76 (70.4)	32 (29.6)	94 (87.0)	14 (13.0)
Appear	12 (10.0)	8 (66.7)	4 (33.3)	10 (83.3)	2 (16.7)
			P=0.751		P=0.662
N (%)	120 (100.0)	84 (70.0)	36 (30.0)	104 (86.7)	16 (13.3)
Invasiveness					
None	35 (33.7)	27 (77.1)	8 (22.9)	29 (82.9)	6 (17.1)
Appear	69 (66.3)	44 (63.8)	25 (36.2)	62 (89.9)	7 (10.1)
			P=0.166		P=0.308
N (%)	104 (100.0)	71 (68.3)	33 (31.7)	91 (87.5)	13 (12.5)

U, unmethylated; M, methylated gene; MMP, matrix metalloproteinase; TGF, transforming growth factor.

The present study examined whether alterations in a gene promoter are associated with PA occurrences. The current study analyzed 120 PA patients for *MMP-14* promoter methylation. The results demonstrated that promoter methylation of *MMP-14* correlated with male gender (58.8 vs. 35.7%, P=0.022), while unmethylated (non-silenced) *MMP-14* correlated with female gender (64.3 vs. 41.7%, P=0.027). It is known that the incidence of PA is double in females compared with males (1). That is why it can be assumed that the non-silenced *MMP-14* gene may be associated with PA cases in females.

The present study also investigated the methylation status of the promoter of the *TGFβ-1* gene. However, no associations between the promoter methylation status of the *TGFβ-1* gene and the age and gender of PA patients, PA hormone activity or recurrence were identified. By contrast, it was observed that the majority of the males' PA samples were in the *TGFβ-1* unmethylated group. In other studies on PA using animal models it was noticed that there was a higher expression of the *TGF-β1* cytokine in male than in female individuals (15). Therefore, the expression of this gene may be associated with male gender, although further research is required. There are no studies on potential associations between methylation of the *TGFβ-1* gene and the secreting function of PA. However, *TGF-β1* activity was observed in hormonally active PA (15,25). In other studies, which compared healthy and unhealthy pituitary tissue, low *TGFβ-1* gene expression was detected in both non-invasive and invasive hormonally inactive PA (26). This demonstrates that *TGFβ-1* gene transcription happens depending on the type of cancer and the stage of the cancer process; therefore, further research is required to identify how this gene is effected by regulating its expression in the cell.

In another study, Elenkova *et al* (27) reported that *TGF-β1* could be a predictive blood serum marker for the invasiveness of PA. The *TGFβ-1* gene has been widely studied in other tumors. Chen *et al* studied the expression of this gene in patients with breast cancer and established links with poorer patient clinic outcome (28). Studies involving lung tumor cell lines have demonstrated that this gene is important in cell proliferation (29). *TGF-β1* is also commonly used in cancer studies. There are *in vitro* studies on *TGF-β1* function in cancer cell lines other than lung cancer and pituitary adenoma cells (30). Xiao *et al* analyzed the risks of radiation-induced pneumonia manifestation in patients with thoracic tumors, and *TGF-β1* SNP was considered as a possible risk factor (31).

The role of the aforementioned genes has been studied in cancer and various other disorders. Previous studies have reported that MMP-14 is associated with tuberculosis by regulating monocyte migration and collagen destruction (32), and with macrophage activation in atherosclerosis (33). TGF-β1 function has been observed in brain and nerve damage (34).

Thus, these genes appear to be responsible for a number of molecular mechanisms that are important in the etiology of various pathological processes.

In conclusion, to the best of our knowledge, the present study is the first to examine in a large population with PA (n=120) the association between the *MMP-14* and *TGFβ-1* promoters methylation status and the development of PA. The present study has demonstrated for the first time that promoter methylation of *MMP-14* correlates with male gender and unmethylated (non-silenced) *MMP-14* correlates with female gender. Further investigation is required to evaluate the effect of promoter methylation of *MMP-14* on the development of PA.

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