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LOW O6–METHYLGUANINE-DNA METHYTRANSFERASE (MGMT) AND PAN-CYTOKERATIN (PAN-CK) EXPRESSION VIA IMMUNOHISTOCHEMISTRY IN PITUITARY ADENOMAS

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Abstract

Introduction. Pituitary adenomas (PA) are the third most common intracranial tumors, with an incidence rate of 10-15%. More than half are invasive, infiltrating adjacent structures. The primary objective of this project was to determine whether MGMT expression is associated with the invasiveness of PA.

Material and Method. All patients who underwent surgical decompression consecutively between 2007-2012 were included. All data were obtained from the case records. Formalin-fixed paraffin-embedded (FFPE) tissue specimens were stained with hematoxylin and eosin (HE) and then examined via light microscope. Paraffin blocks that lacked necrosis and hemorrhage were chosen for histologic examination. In addition to an immunoprofile battery that consisted of Ki-67 and p53, MGMT, S-100 and Pan-CK were evaluated as well.

Results. The subjects included 25 women and 15 men. The mean age was 48.9 ± 14.5 years. Of these, 63% of cases involved the invasion of adjacent structures. Of the PA, 17 (42%) were non-functioning pituitary adenomas (NFPA). There was a statistically significant relationship between the invasiveness and Ki-67, p53, MGMT expression, and prolactinoma. Gonodotropinomas were mostly non-invasive. FPAs presented invasive features more frequently than NFPAs. Pan-CK was positive in GH-secreting adenomas but negative in FSH- and LH-secreting adenomas.

Conclusion. Ki-67 and p53 in lower expression level can be used for evaluating invasiveness but not for recurrence. MGMT expression can be a useful IHC indicator for invasiveness. However, Pan-CK cannot be used for invasiveness or aggressiveness.

Key words: pituitary adenoma, invasiveness, MGMT, cytokeratin, Ki-67, p53, immunohistochemistry.

INTRODUCTION

Pituitary adenomas are the third most common intracranial tumors, with an incidence rate of 10-15% (1). Their clinical behavior varies widely. The majority are indolent, slow-growing neoplasms, but they have the potential to cause devastating morbidity through hormonal hypersecretion or hyposecretion. About 5% of all pituitary adenomas become locally invasive. The histology of invasive tumors is similar to that of non-invasive tumors but clinical course may be different (2). More than half of surgically managed pituitary tumors are invasive, infiltrating structures surrounding the sella, including bone, dura, and cavernous sinuses (3). Radiologic classification of Hardy, revised by Wilson, is performed according to tumor size, extensions, and the degree of local invasion (4). The size, invasion, and surgical excision of tumors are the most important parameters defining their recurrence and progression (5).

The WHO categorizes pituitary tumors as typical adenomas, atypical adenomas and pituitary carcinomas (6). Histological parameters affecting tumor progression, which are important for other kind of tumors, such as mitotic activity and pleomorphism, are not always important for pituitary adenomas (7). However, the WHO classification does not provide an accurate correlation between histopathological findings and clinical behavior. In 2004, the WHO developed a new classification system for atypical adenomas based on tumor markers thought to correlate with more aggressive pituitary tumor biology, including pituitary carcinomas characterized by cerebrospinal and/or systemic metastases. According to WHO 2004 classification, the diagnostic criteria for atypical adenomas consist of the following: excessive p53 immunoreactivity, elevated Ki67/MIB-1 proliferative index (> 3%), increased mitotic activity, and atypical morphological features (7).

O6-methylguanine-DNA methytransferase (MGMT) is a DNA repair protein that reverses alkylation

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at the O6 position of guanine by transferring the alkyl group to a sulphur group of cysteine within its sequence (8). The DNA-repair protein O6-alkylguanine DNA alkyl transferase (AGT) has a wide range of activities in normal tissues, and its evolutionary conservation indicates a fundamental role in cell physiology and the maintenance of the genome. Through the removal of alkylating lesions at the O6 of guanine, AGT protects against mutagenesis and malignant transformation (8). In human tumor cells, MGMT expression displays even greater variability than in normal tissue, but it is generally higher. MGMT activity is high in breast cancer and lung and renal carcinomas, with lower activity levels found in brain tumors and melanoma (9). The present study is the first of its kind in our country to assess the incidence of MGMT protein expression in pituitary adenoma. The primary objective of this study was to determine whether MGMT expression is associated with the invasiveness of pituitary tumors via immunohistochemistry. This study highlights the need to develop new biomarkers to facilitate the early detection of clinically aggressive, radiologically invasive pituitary adenomas.

MATERIAL AND METHODS

Tissue samples and tissue histopathological examination

We selected patients diagnosed with pituitary adenoma who underwent curative resection consecutively at the Istanbul Goztepe Education and Research Hospital, Turkey, between 2007 and 2012. Clinical, endocrinologic, radiologic and pathologic data were retrieved through hospital data resropectively. After reviewing the blocks, the patients in whom adequate tissues were available for MGMT expression analysis were included in the study, which resulted in a cohort of 40 pituitary adenoma patients. All cases diagnosed with pituitary adenoma through pathological evaluation are included in the study. Patients without clear clinical, endocrinologic and radiological data or cases without sufficient pathology material were excluded. Although 70 patients with pituitary adenoma had gone through surgery between these data, we could only include 40 of them in our study. Invasive features were described according to Hardy Classification by using preoperative MR findings radioanatomically (10) (Fig. 1). In Hardy's classification of pituitary adenomas, grades I and II are enclosed within the sella. Grades III and IV are invasive. Extrasellar classifications A, B, and C are increasing amounts of direct suprasellar adenomas. D is asymmetric extension, and E is lateral extension into the cavernous sinus. Non-functional pituitary adenoma (NFPA) was described as tumor which has no hormone secretion or clinically inactive hormone secretion. Information concerning sex, age at diagnosis, types of hormone secretion, invasive features, functional features according to clinical results of hormone secretion and recurrence were obtained retrospectively. The histopathological slides were reviewed by a neuropathologist (AS), and the diagnosis was confirmed. Formalin-fixed paraffin-

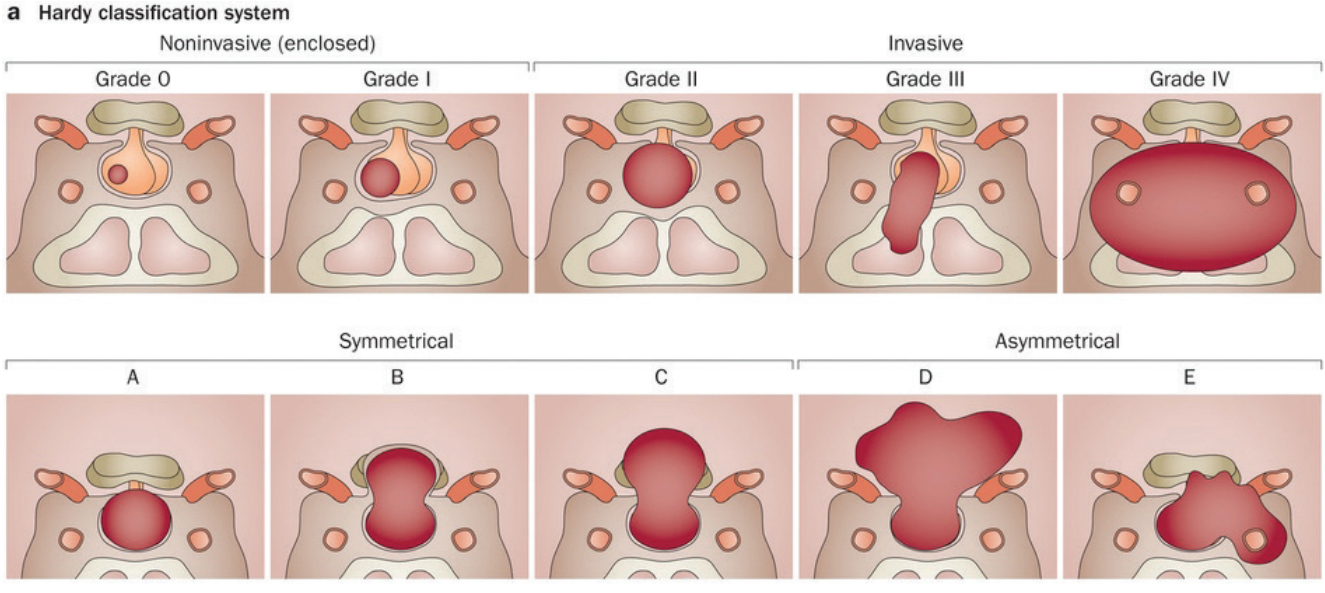

Figure 1. Hardy's classification of pituitary adenomas. Grades I and II are enclosed within the sella. Grades III and IV are invasive. Extrasellar classifications A, B, and C are increasing amounts of direct suprasellar adenomas. D is asymmetric extension, and E is lateral extension into the cavernous sinus. Adapted from Hardy J, Somma M.

embedded (FFPE) tissue specimens were stained with hematoxylin and eosin (HE), and then, pituitary tissue was examined under light microscope. Selected paraffin blocks that lack necrosis or hemorrhage have been chosen for histological examination. In addition to an immunoprofile battery that consisted of Ki-67 and p53, MGMT, Pan-CK and S-100 were evaluated.

Immunohistochemistry

Many methods and protocols have been applied for MGMT analysis but to date there is no consensus on which strategy should be primarily employed(11). Methylation-specific polymerase chain reaction (MSP) is the most used test but there are some methodological problems that limit the usefulness of this method (11). MGMT status can be also assessed by analyzing protein expression by immunohistochemistry (IHC). IHC is a reliable, available, easier to use, less expensive and faster method than MSP (11). Immunohistochemistry for hormone markers as prolactin, growth hormone (GH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid stimulating hormone (TSH) (BioGenex, San Ramón, CA) and adrenocorticotropic hormone (ACTH, DAKO, Carpinteria, Ca, at a 1:100 dilution) was performed as previously described (12).

In other sections, paraffin sections, 5 μm in thickness, were collected on silane-coated slides and subjected to immunohistochemistry using monoclonal antibodies: MIB-1 (Ki-67-specific monoclonal antibody; DAKO, Denmark, dilution – 1:50), p53 (ScyTek Lab Vision Co. Utah, USA / clone no: DO 7, dilution – 1:200), and MGMT (CO, USA; dilution – 1:50).

Immunohistochemistry for MGMT was performed on FFPE tissue using a mouse monoclonal antibody (Clone MT23.2, Novus Biological Inc. Littleton, CO, USA) at a 1/50 dilution. Slides were stained using the Ventana Benchmark X autostainer (Ventana medical system, AZ, USA), using a biotin-free detection system in accordance with the manufacturer's protocol. External positive and negative controls (tonsillar tissue with areas of known positive and negative staining) were included. In addition, endothelial cells and lymphocytes acted as internal positive controls. Slides were examined by a single observer (A.S.) who was blinded as to the clinical and molecular data including the hormone production status.

MGMT expression has been scored semi-quantitatively. In the studies about MGMT in literature, most of them used the semi-quantitative method (11). There were different scoring methods in literature. We chose the most commonly used method. As previously defined, the fraction of immunopositive tumor cells was evaluated according to the following score: −, no positive tumor cells; +, <10% positive tumor cells; ++, 10–50% positive tumor cells; +++, >50% positive tumor cells, regardless of intensity (13). The staining pattern was nuclear for all markers, and immunolabeling has been scored by a visual semi-quantitative method. The labelling index (LI) was calculated as the percentage positivity after counting 1,000 tumor cells. The criterion for a positive p53 immunoreaction was the presence of at least a group of immunostained cell nuclei.

Statistical Evaluation

All statistical analyses were performed using IBM SPSS Statistics 20® statistical software. All data were measured as nominal or ordinal. Non-parametric tests were used to analyze the relationship between variables. Spearman's Rho analysis was used for correlation analysis. In all tests, $p<0.05$ was considered to be statistically significant.

RESULTS

The present study included 40 patients with pituitary adenoma who underwent surgery at the neurosurgical center between 2007 and 2012. All of them were typical pituitary adenomas according to the 2004 WHO classification of pituitary adenomas. The subjects included 25 women and 15 men. The age of the patients ranged from 18 to 86 years (mean = 48.9 ± 14.5 yrs.), and 25 (63%) of 40 cases had invasive features, 15 (37%) of 40 cases had non-invasive features according to the Hardy classification. Of the pituitary adenomas, 17 (42%) were non-functioning pituitary adenomas (NFPAs), and 23 (58%) were functioning pituitary adenomas (FPAs). The FPAs secreted mixed hormones in five cases (22%), growth hormone (GH) in nine cases (39%), and PRL in nine cases (39%). The NFPAs consisted of silent gonadotroph cell adenoma in ten cases (59%), silent TSH cell adenoma in one case (6%), silent corticotroph cell adenoma in one case (6%), and null cell adenoma in five cases (29%) (Table 1). All patients have been treated by transsphenoidal route. Ki-67 index was found to be ≥1% in 11 (27%) and <1% in 29 (73%) of cases (Figs 2a-b). p53 was positive in 18 (45%) and negative in 22 (55%) of the adenomas. Of the 15 non-invasive pituitary adenomas, 14 (93%) were negative for p53 (Fig. 3a). Of the 25 invasive pituitary adenomas, 17 (68%) were positive for p53 (Fig. 3b). MGMT expression was found ≤50% in the case of 11 (27%) and >50% in the case of 29

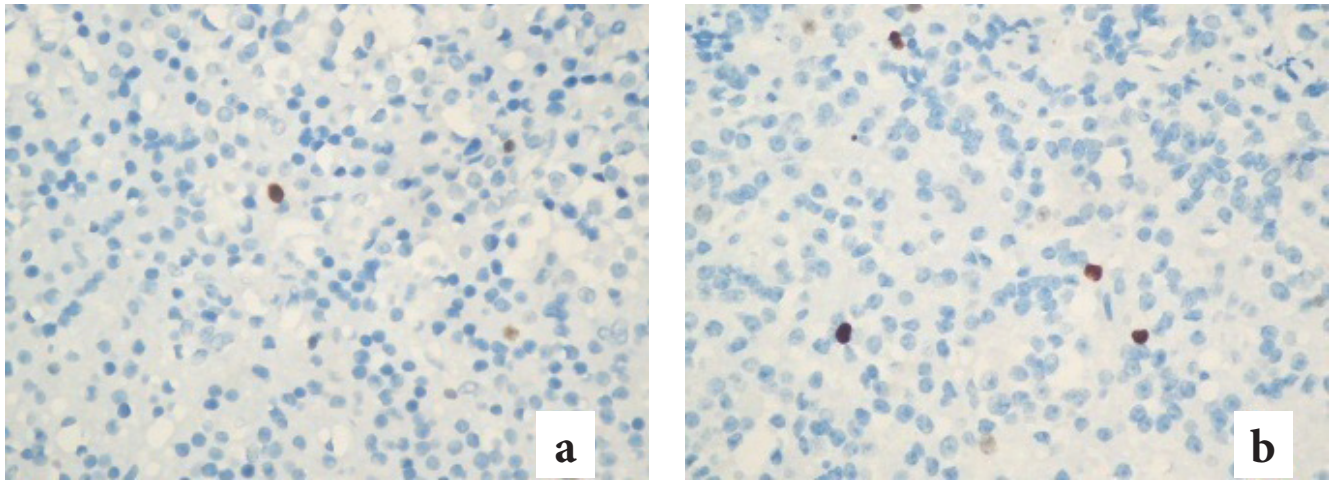

Figure 2. Immunohistochemical staining for Ki-67. Nuclear Ki-67, as a marker of cell division, is usually considered to determine the proliferation index in neoplasms. Ki-67 level was found to be <1 in 72.5% (**a**) and ≥1 in 27.5% (**b**) of cases.

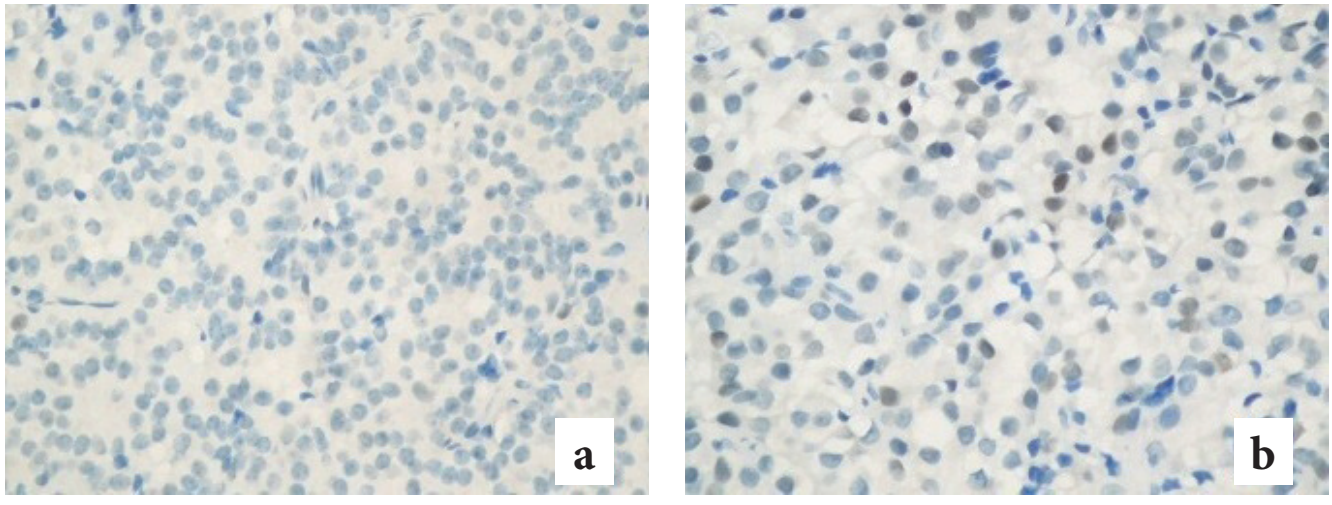

Figure 3. Immunohistochemical staining for p53. p53 plays an important role in cell proliferation, apoptosis, and genomic stability. Of the 15 non-invasive pituitary adenomas, 14 (93%) were negative for p53 (**a**). Of the 25 invasive pituitary adenomas, 17 (68%) were positive for p53 (**b**). Invasive adenomas had positive p53 expression.

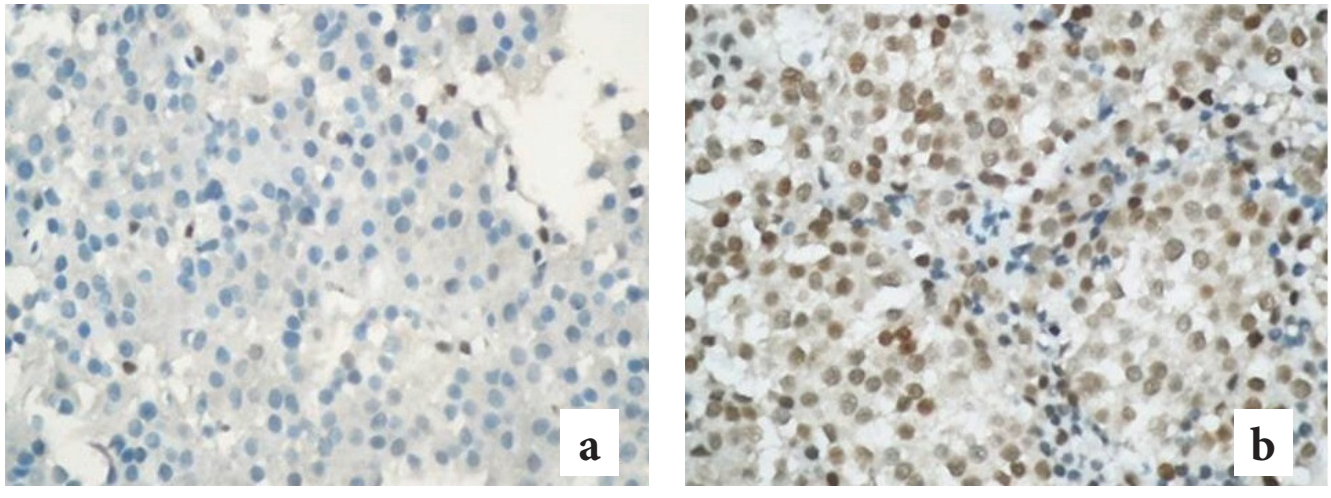

Figure 4. Immunohistochemical staining for MGMT. MGMT expression was found to be ≤50% (a) in 22.5% of patients but >50% (b) in 77.5% of pituitary adenomas. The rate of MGMT expression increases with the invasiveness of pituitary adenomas.

Table 1. Patient characteristics and expression of Ki-67, p53, MGMT, S-100 and Pan-CK

Case	Sex	Age	Invasiveness	Hormone Secretion	NFPA	S100	p53	ki67	Pan-CK	MGMT >50%	Recurrence
1	M	77	Invasive	LH, FSH	Yes	(-)	(+)	<1%	(-)	(-)	Yes (1yrs)
2	M	49	Non-invasive	Null	Yes	(+)	(-)	<1%	(+)	(-)	No
3	F	64	Invasive	GH, PRL	No	(-)	(+)	<1%	(+)	(+)	No
4	F	25	Invasive	GH, PRL	No	(-)	(+)	≥1%	(+)	(+)	No
5	F	63	Non-invasive	LH, FSH	Yes	(+)	(-)	<1%	(-)	(-)	No
6	F	35	Invasive	ACTH	Yes	(-)	(-)	<1%	(+)	(+)	No
7	F	45	Non-invasive	Null	Yes	(+)	(-)	<1%	(+)	(-)	No
8	M	39	Non-invasive	LH, FSH	Yes	(-)	(-)	<1%	(-)	(+)	No
9	M	64	Invasive	LH, FSH	Yes	(-)	(-)	≥1%	(+)	(+)	No
10	F	56	Invasive	FSH	Yes	(-)	(+)	<1%	(-)	(+)	Yes (2 yrs)
11	F	41	Invasive	GH, PRL	No	(-)	(+)	<1%	(+)	(+)	No
12	F	53	Non-invasive	GH	No	(-)	(-)	<1%	(+)	(+)	No
13	F	51	Non-invasive	LH, FSH	Yes	(-)	(-)	<1%	(-)	(+)	No
14	F	53	Invasive	GH, PRL	No	(+)	(+)	<1%	(+)	(-)	No
15	F	50	Invasive	GH	No	(-)	(-)	≥1%	(+)	(+)	No
16	F	40	Invasive	GH, PRL	No	(-)	(+)	≥1%	(+)	(+)	No
17	M	58	Invasive	LH	Yes	(-)	(+)	<1%	(+)	(+)	No
18	M	43	Non-invasive	GH, ACTH	No	(+)	(-)	<1%	(+)	(+)	No
19	M	38	Invasive	PRL	No	(+)	(-)	≥1%	(+)	(+)	No
20	F	42	Invasive	GH	No	(+)	(-)	≥1%	(+)	(+)	No
21	F	18	Invasive	PRL	No	(-)	(+)	≥1%	(-)	(+)	No
22	M	61	Non-invasive	LH, FSH	Yes	(+)	(-)	<1%	(-)	(+)	No
23	F	67	Invasive	PRL, ACTH	No	(+)	(+)	<1%	(+)	(+)	No
24	F	47	Invasive	GH	No	(+)	(+)	<1%	(+)	(+)	No
25	F	86	Non-invasive	PRL, LH, FSH	No	(+)	(-)	<1%	(+)	(-)	No
26	F	45	Invasive	PRL	No	(+)	(+)	≥1%	(+)	(+)	No
27	F	39	Invasive	Null	Yes	(-)	(-)	≥1%	(-)	(+)	Yes (4 yrs)
28	M	48	Invasive	PRL	No	(-)	(+)	≥1%	(-)	(-)	No
29	F	53	Non-invasive	GH	No	(+)	(-)	<1%	(+)	(-)	No
30	F	50	Invasive	PRL	No	(+)	(+)	<1%	(+)	(+)	Yes (8 yrs)
31	M	41	Invasive	Null	Yes	(+)	(+)	<1%	(-)	(+)	No
32	F	34	Non-invasive	GH	No	(-)	(+)	<1%	(+)	(+)	No
33	M	31	Invasive	GH	No	(-)	(-)	≥1%	(+)	(-)	No
34	M	59	Non-invasive	Null	Yes	(+)	(-)	<1%	(+)	(-)	No
35	M	26	Non-invasive	GH	No	(-)	(-)	<1%	(+)	(+)	No
36	M	58	Invasive	LH, FSH	Yes	(-)	(-)	<1%	(+)	(+)	No
37	M	32	Invasive	GH, LH, TSH	No	(+)	(+)	<1%	(-)	(+)	No
38	F	70	Non-invasive	LH, FSH	Yes	(-)	(-)	<1%	(-)	(+)	No
39	F	67	Non-invasive	LH, FSH	Yes	(-)	(-)	<1%	(-)	(-)	No
40	F	39	Invasive	PRL	No	(-)	(+)	<1%	(+)	(+)	No

M: male, F: female, GH: growth hormone, FSH: follicular stimulating hormone, LH: luteinizing hormone, PRL: prolactin, TSH: thyroid stimulating hormone, Yrs: years.

(73%) (Figs 4a-b). Pan-CK was found negative 14 (35%) and positive 26 (65%) in the case of pituitary adenomas (Figs 5a-b). In addition, we found that S-100 was negative in 23 (58%) and positive in 17 (42%) (Figs 6a-b) (Table 2). Overall results of statistical analysis by Spearman's rho are shown in one table (Table 3).

There was a statistically significant relationship between the invasiveness of pituitary adenomas and Ki-67 index (p: 0.002) and p53 (p: 0.001) levels. All of the non-invasive adenomas had Ki-67 index less than 1%. Additionally, there was a statistically significant relationship between prolactinomas and the invasiveness of the adenomas (p: 0.007). To be more specific, 92% of prolactinomas (12/13) had invasive features. When we examined the relationship between FSH- and LH-secreting adenomas (gonodotropinomas) and the invasiveness of the adenomas, we found that gonodotropinomas were usually non-invasive. FPAs revealed invasive features more frequently than NFPAs. However, this result was not statistically significant (p: 0.080). There was a statistically significant relationship between invasiveness and MGMT expression (p: 0.042). Pituitary adenomas with MGMT expressions higher than 50% showed invasive features (Fig. 4) (Table 4).

Table 2. Distribution of different markers and features in pituitary adenomas

		n	%
Invasiveness	Non-invasive	15	37
	Invasive	25	63
P53	Negative (-)	18	45
	Positive (+)	22	55
Ki-67	<1%	11	73
	≥1%	29	27
MGMT	≤50%	11	27
	>50%	29	73
Pan-CK	(-)	14	35
	(+)	26	65
S-100	(-)	23	58
	(+)	17	42
NFPA	(-)	23	58
	(+)	17	42

There was a statistically significant correlation between Ki-67 index and age (p: 0.017). With increasing age, Ki-67 index became higher than 1%. We found a statistically insignificant correlation between Ki-67 index and PRL- and LH-secreting adenomas (p: 0.075 and 0.077, respectively). The Ki-67 index of prolactinomas was mostly over 1%, but the Ki-67 index of other adenomas was mostly lower than 1%. When we examined pituitary adenomas, we found that Ki-67 index was below 1% in NFPAs and above 1% in FPAs. However, this result was not statistically significant (p:0.057). P53 was positive in 45% and negative in 55% of pituitary adenomas. There was a statistically significant relationship between p53 and the histologic features of pituitary adenomas (p: 0.029). P53 levels

Table 3. Overall results of Spearman's regression analysis of differen markers and features of pituitary adenomas

			Invasiveness	GH	PRL	ACTH	GND	NFPA	S100	p53	ki67	PANCK	MGMT
Spearman's rho	**Invasiveness**	r	1.000	.067	**.427****	.025	-.234	-.274	-.170	**.597****	**.477****	.124	**.325***
		Sig. (2-tailed)		.683	**.006**	.881	.146	.087	.295	**.000**	**.002**	.446	**.041**
	GH	r	.067	1.000	.014	-.025	**-.427***	**-.666****	-.039	.130	.101	**.427****	.046
		Sig. (2-tailed)	.683		.933	.881	**.006**	**.000**	.810	.425	.534	**.006**	.776
	PRL	r	**.427****	.014	1.000	.005	**-.368***	**-.597****	.051	**.553****	.290	.254	-.010
		Sig. (2-tailed)	**.006**	.933		.975	**.020**	**.000**	.753	**.000**	.070	.114	.953
	GND	r	-.282	**-.394***	**-.338***	-.186	1.000	**.541****	-.121	-.263	-.281	**-.478****	-.039
		Sig. (2-tailed)	.078	**.012**	**.033**	.249		**.000**	.456	.101	.079	**.002**	.810
	Histology	r	.130	.130	**.428****	.139	**.349***	-.186	-.092	**.323***	-.114	-.088	.085
		Sig. (2-tailed)	.422	.422	**.006**	.393	**.027**	.250	.573	**.042**	.483	.591	.602
	NFPA	r	-.274	**-.666****	**-.597****	-.053	.142	1.000	-.125	**-.371***	-.303	**-.483****	-.021
		Sig. (2-tailed)	.087	**.000**	**.000**	.746	.382		.441	**.018**	.057	**.002**	.897

GH: Growth hormone, PRL: prolactin, ACTH: adrenocorticotropic hormone, GND: gonodotropine, NFPA: non-functional pituitary adenoma *. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).

Table 4. Correlation between invasiveness and different markers, histopathological features of pituitary adenoma

		Invasive (n=25) n (%)	Non-invasive (n=15) n (%)	*r*	*p*
Ki-67	<1%	14 (56%)	15 (100%)	***0.477***	***0.002****
	≥1%	11 (44%)	0 (0%)		
P53	(-)	8 (32%)	14 (93%)	***0.597***	***0.001****
	(+)	17 (68%)	1 (7%)		
PRL		12 (48%)	1 (6,7%)	***0.427***	***0.007****
FSH		4 (16%)	7 (46,7%)	***-0.332***	***0.042****
LH		5 (20%)	7 (46,7%)	***-0.282***	***0.078***
MGMT	≤50%	3 (12%)	6 (40%)	***0.325***	***0.042****
	>50%	22 (88%)	9 (60%)		
NFPA		8 (32%)	9 (60%)	***-0.274***	***0.080***

Spearman's rho *Fisher's Exact Test Pearson Ki-kare Test* **p<0.05* ***p<0.01*

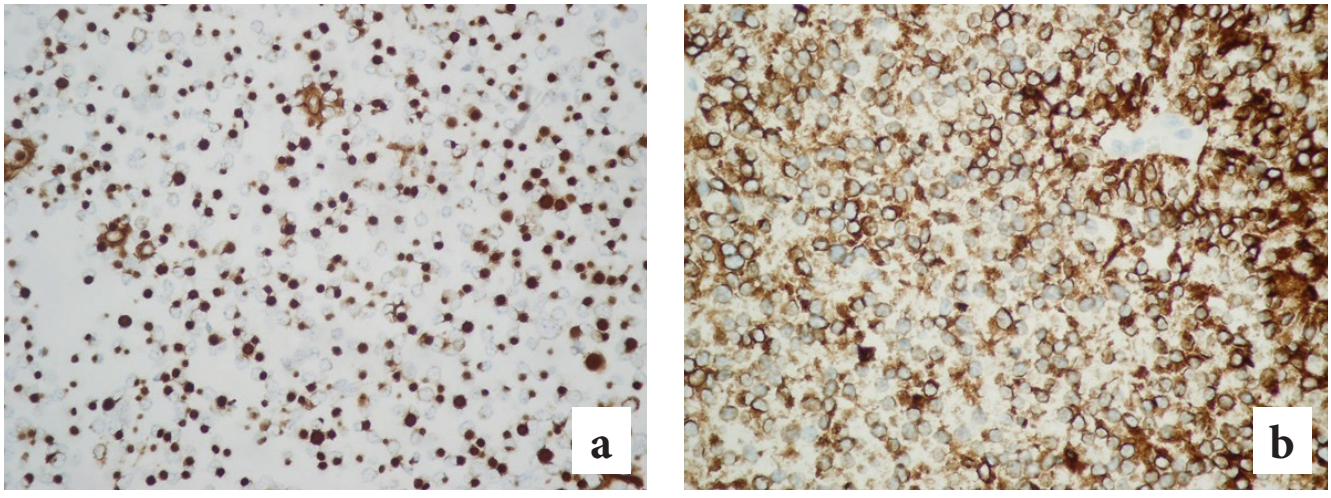

Figure 5. Immunohistochemical staining for Pan-CK. The pan-cytokeratin (Pan-CK), or cytokeratin, test has become the cornerstone of evaluating evidence of epithelial differentiation. Pan-CK was found to be negative in 32.5% (**a**) and positive in 67.5% (**b**) of cases. Pan-CK was positive in GH-secreting adenomas but negative in FSH- and LH-secreting adenomas.

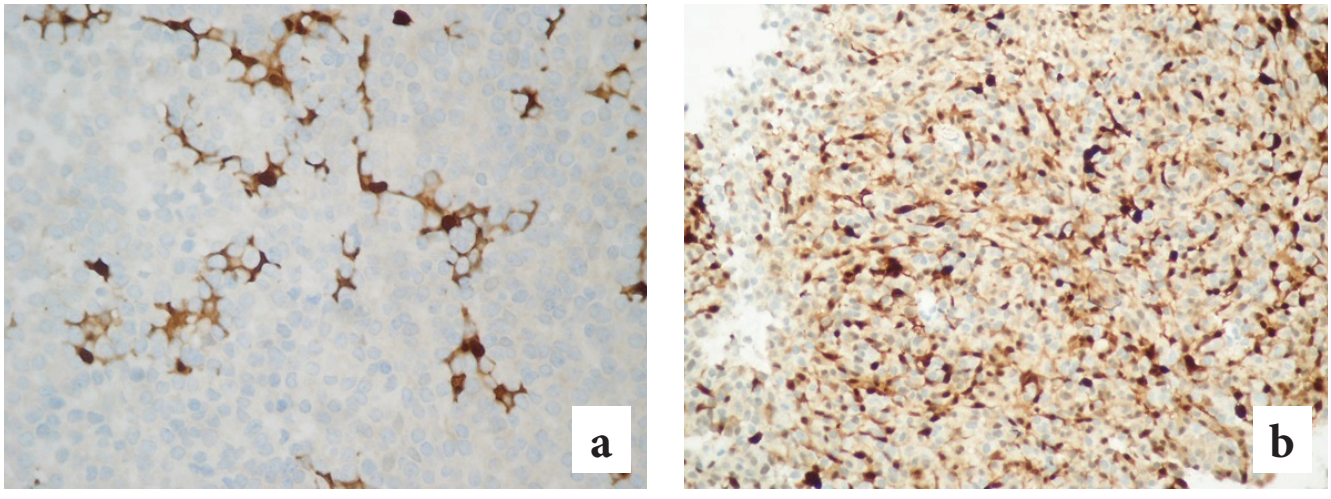

Figure 6. Immunohistochemical staining for S-100. S-100 was found to be negative in 57.5% (**a**) and positive in 42.5% (**b**) of cases.

Table 5. Correlation the expression of Ki-67 index and p53 with histopathological features of pituitary adenoma

	Ki-67 (≥1%) n (%)	***r***	***p***	**P53 (+) n (%)**	***r***	***p***
PRL	6 (55%)	***0.290***	***0.075***	11 (61%)	***0.553***	***0.001*****
FSH	1 (9%)	***-0.254***	***>0.05***	2 (11%)	***-0.332***	***0.038****
LH	1 (9%)	***-0.281***	***0.077***	3 (17%)	***-0.263***	***0.093***
GH	5 (45%)	***0.101***	***>0.05***	8 (44%)	***0.130***	***>0.05***
NFPA	2 (18%)	***-0.303***	***0.057***	4 (22%)	***-0.371***	***0.020****

Spearman's rho *Fisher's Exact Test* *Pearson Ki-kare Test* **p<0,05 **p<0,01*

were especially high in PRLs (p: 0.001) and negative in FSH-secreting adenomas (p: 0.038). Similarly, p53 was usually negative in LH-secreting adenomas but this negativity was not statistically significant (p:0.093). In addition, p53 was negative in NFPAs and positive in FPAs. This result was statistically significant (p: 0.020) (Table 5).

Pan-CK was found to be negative in 32% and positive in 68% of cases (Figs 5a-b). There was a statistically significant relationship between Pan-CK and the histologic features of pituitary adenomas (p: 0.006). Histologically, there was a close correlation between Pan-CK and GH- (p: 0.008), LH- (p: 0.003) and FSH- (p: 0.001) secreting adenomas. Pan-CK was positive in GH-secreting adenomas but negative in FSH- and LH-secreting adenomas. Via immunohistochemistry,

MGMT expression was found to be ≤ 50 % in 22% of patients but > 50 % in 78% of pituitary adenomas. There was a statistically significant correlation between MGMT expression and invasiveness (p: 0.047). Invasive pituitary adenomas revealed strong staining by MGMT (Table 6).

S-100 was found to be negative in 58% and positive in 42% of cases. We did not detect any relationship between S-100 and any features of the pituitary adenomas.

There were recurrences in 4 of 40 typical pituitary adenomas. We detected the recurrence rate as 10% in our study. There was no statistical relationship between recurrence and Ki-67, p53, MGMT, Pan-CK (p>0.05).

DISCUSSION

Definition of invasiveness and aggressiveness

Pituitary adenomas can be classified according to their pathologically as typical, atypical and carcinoma, radiologically as invasive and non-invasive, clinically behavior as aggressive and nonaggressive. The term 'aggressive' has been used synonymously with invasive or atypical when evaluating pituitary tumors. It is a clinical terminology. Some biomarkers as FGFR4, MMP, PTTG, Ki-67, p53, and deletions in chromosome 11 can be used to decide management of aggressive pituitary adenomas (14). However, there are no specific definitions.

Invasiveness is a radiological definition (3). Invasive adenomas are considered tumors with proven growth to adjacent structures, such as the cavernous sinuses, bone, and sphenoid sinus. Invasive adenomas show pathologic or radiological signs of invasion to the cavernous or sphenoid sinuses, bone, or nasal mucosa (3). Suprasellar extension is not considered a criterion of invasiveness. Invasion can be detected radiologically with preoperative MRI, during surgery, or with histologic demonstration of tumor spread to the dura, bone, or nasal mucosa (15). MRI is the most practical common approach to classifying invasion and the Hardy classification is used commonly for extension and invasion of pituitary adenomas and also the Knosp grading system is widely used for involvement of cavernous sinus by pituitary macroadenoma (3). Dural invasion detected by microscopic examination is common, therefore it is not regarded as a consistent indicator of aggressive tumor behavior (15). The overall rate of invasion into the cavernous sinus is 35% and macroadenomas tend to invade more frequently than microadenomas (2, 15).

Invasiveness and Ki-67

The rate of invasive pituitary adenoma, which is described as the invasion of surrounding structures, was reported as 25-55% in the literature (15). The grading of invasiveness was described by Hardy, and in his original classification of pituitary adenomas, grades 3 and 4 are described as invasive (4). The description of aggressiveness is different from that of invasiveness. Aggressive pituitary adenomas as a clinical terminology can be microadenomas or macroadenomas, invasive or non-invasive (3).

Nuclear Ki-67, as a marker of cell division, is usually considered to determine the proliferation index in neoplasms. Ki-67 has been studied in pituitary adenomas. Salehi *et al.* reported Ki-67 index varies from 1% to 23% (16). Thapar *et al.* indicated a threshold of 3% for Ki-67 to distinguish invasive adenomas from non-invasive ones with 97% specificity and 73% sensitivity, and this was associated with positive and negative predictive values of 96% and 80%, respectively (17). According to several studies, the Ki-67 index was not significantly different in the subgroup of adenomas with invasion of the cavernous

Table 6. Correlation between MGMT, Pan-CK and different markers, histopathological features of pituitary adenoma

		MGMT (>50%) n (%)	*r*	*p*	Pan-CK (+) n (%)	*r*	*p*
Ki-67	<1%	22 (71%)	***0.064***	***>0.05***	19 (70%)	***0.069***	***>0.05***
	≥1%	9 (29%)			8 (40%)		
P53	(-)	15 (48%)	***0.247***	***>0.05***	15 (56%)	***-0.016***	***>0.05***
	(+)	16 (52%)			12 (44%)		
PRL		10 (32%)	***-0.010***	***>0.05***	11 (41%)	***0.254***	***>0.05***
FSH		8 (26%)	***-0.070***	***>0.05***	3 (11%)	***-0.529***	***0.002****
LH		9 (29%)	***-0.039***	***>0.05***	4 (15%)	***-0.478***	***0.004****
GH		12 (39%)	***0.046***	***>0.05***	14 (52%)	***0.427***	***0.007****
NFPA		8 (32%)	***-0.021***	***>0.05***	7 (26%)	***-0.483***	***0.003****

*Spearman's rho Fisher's Exact Test Pearson Ki-kare Test *p<0.05 **p<0.01*

sinus as compared with groups those with other types of invasiveness. Nevertheless, the ability of the Ki-67 index to predict tumour invasiveness is somewhat controversial because others have found no difference in Ki-67 index between invasive and non-invasive pituitary adenomas(16).

In the present study, Ki-67 index was found to be below 3%, except for one case. However, there was a close relationship between the invasiveness of pituitary adenomas and Ki-67 index (p: 0.003). All cases involved in this study are typical adenomas according to the WHO criteria. Surprisingly, our cases had no aggressive clinical features, such as high recurrence rate or metastasis. To conclude this study, a cut-off value of 3% for aggressiveness is not applicable as a criterion for determining invasiveness in pituitary adenomas. Previously, it has been documented that not all invasive tumours have aggressive behaviour. There is no general agreement about the relationship of Ki-67 index and invasiveness and recurrence of pituitary adenoma (16). In our study, we detected the rate of recurrence as 10%. There was no statistical relationship between recurrence and invasiveness, Ki-67 index of pituitary adenoma. According to WHO classification, atypical are considered as higher mitotic activity, a Ki-67 index greater than 3%, and/or extensive p53 immunoreactivity (6). The present study indicates that pituitary adenomas with Ki-67 indices between 1 and 3% can be described as invasive but non-aggressive pituitary adenomas. They are treatable surgically and have low recurrence rate.

Invasiveness and p53

It seems that p53 plays an important role in cell proliferation, apoptosis, and genomic stability. Also, p53 expression has been associated with aggressive tumour behaviour in pituitary adenomas (18). Thapar *et al.* demonstrated that non-invasive and invasive adenomas and pituitary carcinomas revealed p53 expression in 0%, 15.2%, and 100% of cases, respectively (18). Again, similar to Ki-67 index, several authors did not find any significant correlation between invasiveness and p53 expression (16). Frequently, the positivity or negativity of p53 is taken into account, with >10 strongly positive nuclei per ten HPFs (18).

In our study, all invasive adenomas had positive p53 expression. We detected a correlation between the invasion of surrounding structures and p53 expression, but we did not detect any correlation between p53 levels and recurrence. These results suggest that p53 alone is not an independent prognostic factor to determine aggressive behaviour like recurrence in pituitary tumours but can be useful for determining invasiveness.

Invasiveness and prolactinomas

Prolactinomas are adenomas that secrete prolactin hormone and have a high density of secretory granules. They are seen typically in females, but they are more aggressive in males than in females. They usually generate invasive adenomas (19). To our knowledge, there are no studies investigating the relationship between invasiveness and histopathologic subtypes of pituitary adenomas in the literature.

Typically, GH-secreting, ACTH-secreting, and prolactin-secreting adenomas had more aggressive features (19). Shimon I *et al.* found "hst" protein in prolactinomas but not in normal pituitary tissue. This finding is supported by previous studies on rats. The activation of "hst" protein is responsible for prolactinoma pathogenesis, causes increased cell proliferation, and generates invasive features (20).

In the present study, we compared invasiveness and the histological features of pituitary adenomas and then found that most of the invasive pituitary adenomas were prolactin-secreting adenomas (p: 0.007). Also, p53 was highly positive in prolactinomas (p: 0.001). As a result of these findings, we reported that prolactinomas have more invasive features and can be more aggressive than other adenomas. The treatment and close follow-up of prolactinomas are crucial.

Invasiveness and NFPA

The definition of non-functioning pituitary adenomas (NFPAs) is clinical in essence, indicating the absence of symptoms or signs secondary to hormonal hypersecretion by the tumour. They typically cause symptoms because of increasing size and pressure effect on the normal pituitary gland and on structures near the pituitary such as the optic nerves and chiasm. NFPAs consist of several histological subtypes, including null cell adenoma (NCA), silent gonadotroph cell adenoma (SGA), silent corticotroph adenoma (SCA), and other silent adenomas (OSA) (i.e., GH-, TSH-, and prolactin-secreting adenomas). NFPAs most commonly originate from gonadotroph cells and can secrete low amounts of gonadotropins. In terms of the distribution of NFPAs, 64% are silent gonadotroph adenomas, 18% are null cell adenomas, 12% are silent corticotroph adenomas, 4% are silent Subtype 3 adenomas, and 1% are other types of adenomas with an aggressive biological behaviour in young ages (21).

In our study, 59% of NPDAs were silent

gonadotroph cell adenomas, 29% were null cell adenomas, and 12% were other silent adenomas.

In general, NFPAs showed low Ki-67 index, but invasive NFPAs had higher Ki-67 index; however, this difference was not significant. For Ki-67 index, a significantly higher rate of staining was found in the invasive adenomas in the null cell adenomas and oncocytomas, particularly the adenomas expressing strong p53 levels (22). Also, p53 positivity was restricted to invasive adenomas but was found in only 20% of all invasive adenomas. These data confirm that p53 and Ki-67 immunohistology is useful in evaluating the aggressive behaviour of clinically silent pituitary adenomas. Nevertheless, negative results do not exclude clinically invasive behaviour (22).

In our study, it was shown that functional pituitary adenomas predominantly have invasive features and non-functional pituitary adenomas have non-invasive features. However, there was no statistical significance between NFPA and the invasiveness of the adenomas (p: 0.080). We have found a close relationship between invasiveness and the Ki-67 index of the adenomas. Invasive pituitary adenomas had peak Ki-67 index. Upon the detection of the Ki-67 levels of the NFPAs, it was found that the Ki-67 index was less than 1%. These two results indicate that majority of NFPAs showed non-invasive features. It is evident that further studies including more pathological data are needed for a better understanding of this specific issue. Additionally, the present study revealed that NFPAs are negative for p53, while FPAs are positive for p53, and FPAs have more aggressive features than NFPAs: MGMT, p53 (aggressiveness), PRL and S-100.

Immunohistochemistry is an attractive technique for determining the MGMT expression levels of tumour tissue. However, there is no consensus about the clear-cut percentage of nuclear MGMT expression (23). As previously defined, the fraction of immunopositive tumour cells was evaluated according to the following score: −, no positive tumour cells; +, <10% positive tumour cells; ++, 10–50% positive tumour cells; +++, >50% positive tumour cells, regardless of intensity (13, 24, 25). There are several studies about MGMT expression in various tumour types. These studies are especially focused on glioblastoma multiforme and pituitary adenomas. Rodriguez *et al.* showed that there was no significant correlation between MGMT expression and methylation, and no significant survival difference was observed between patients whose tumours were negative *versus* those whose tumours were positive for MGMT protein, as ascertained by immunohistochemistry (25). Also, in a study concerning craniopharyngiomas, Zuhur *et al.* suggest that a high proportion of adamantinomatous craniopharyngioma express a low level of MGMT (26). In studies of pituitary adenomas, the expression of MGMT has no associations with the aggressiveness or relapse of pituitary adenomas (27, 28).

In the present study, the rate of MGMT expression increases the invasiveness of pituitary adenomas. MGMT expression has a role in invading the surrounding structures of pituitary adenomas. It is known that all invasive adenomas are not aggressive tumours. In our study there are not any pituitary adenoma cases who used temozolamide. McCormack *et al.* indicate that tumours with low MGMT expression demonstrate clinical and radiologic responsiveness to temozolomide therapy, while those with high MGMT expressivity were associated with resistance to temozolomide. Thus, MGMT immunostaining appears to have promise in predicting the response to temozolomide therapy in patients with aggressive pituitary adenomas and carcinomas. Temozolomide is the first chemotherapeutic agent to show substantial activity in the management of aggressive pituitary tumors (23, 29).

Pan-CK

The pan-cytokeratin (Pan-CK), or cytokeratin, test has become the cornerstone of evaluating evidence of epithelial differentiation, and it is probably the most commonly used IHC test in almost every general or subspecialty practice in pathology and cytology (30). Cytokeratins (CKs) are intermediate filaments (IFs) that are specific to normal and neoplastic epithelial cell differentiation (31). They play an important role as structural and functional proteins of the cytoskeleton. The immunohistochemical examination of IF proteins reveals characteristic expression patterns in diverse normal and cancer cell types (32, 33).

A study of ectopic sphenoid sinus pituitary adenoma (ESPA) indicated that a pan-cytokeratin test was positive in 79% of cases (34). Studies of the relationship between pituitary adenoma and Pan-CK are focused on GH-secreting adenoma (acromegaly). Mori *et al.* examined the pathological features of acromegaly. This study indicates that subclassification based on CK distribution will help to identify clinically aggressive monohormonal GH adenomas but not plurihormonal adenomas (35). In somatotroph adenomas, cases with oncocytic change showed higher percentages of Ki-67 (P = 0.05), but no correlation with extrasellar extension

or cytokeratin staining (dot pattern *versus* perinuclear) was found (36). In GH-secreting pituitary adenomas, in terms of MGMT expression, there was no correlation between the Ki-67 and CK distribution patterns and MGMT immunoreactivity (37).

In the present study, there was a statistically significant difference between Pan-CK and histopathological subtypes of pituitary adenomas. Most frequently, GH-secreting adenomas are Pan-CK-positive (93%). NFPAs, LH and FSH secreting adenomas have negative expression for Pan-CK. The present study indicated that Pan-CK cannot be used as an indicator of invasiveness and aggressiveness in pituitary adenomas. We need larger studies about Pan-CK on invasiveness and aggressiveness of pituitary adenomas for more comprehensive results.

In conclusion, Ki-67 index and p53 in lower expression level can be used for evaluating invasiveness but not for recurrence in pituitary adenomas. Functional pituitary adenomas have usually invasive features. Especially prolactinomas are the most common invasive adenomas. MGMT expression can be a useful immunohistochemical indicator for invasiveness of the adenomas. However, Pan-CK cannot be used for invasive or aggressive features of the adenomas. Hardy classification as radiological evaluation is neither reliable nor sufficient for the prediction of recurrence. It should be kept in mind that invasiveness and aggressiveness are different notions. For understanding aggressiveness of the adenomas we needed larger studies including atypical adenomas and pituitary carcinomas. Both radiologic data and biomarkers, i.e., Ki-67, p53, MGMT, and, maybe, Pan-CK, can be utilized in proposing an unproblematic and comprehensible prognosis and also classification of pituitary adenomas.

Conflict of interest

The authors declare that they have no conflict of interest.

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