THE EXPRESSION OF STEM CELL MARKERS (CD133, NESTIN, OCT4, SOX2) IN INVASIVE PITUITARY ADENOMAS

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Abstract

Introduction. The pituitary gland serves as the center of the endocrine system. Stem cells are typically found in a specialized microenvironment of the tissue, called the niche, which regulates their maintenance, self-renewal, fate determination, and reaction to external influences. The aim of this study is to elucidate the role of stem cells in the initiation, invasion, and progression of pituitary adenomas.

Materials and methods. All specimens were collected between January 2007 and April 2015. Radiological classification (invasiveness) for all cases was performed according to the Wilson-Hardy classification system. Immunohistochemical staining was performed to all specimens for CD133, Oct4, Sox2 and nestin.

Results. The study included 48 patients. Of 48 patients, 17 (35.4%) were male and 31 (64.6%) were female. Mean age is 47.10 ± 14.14 (17–86 yrs.). According to the Wilson-Hardy classification system, 27 (56.3%) were non-invasive adenomas. There was no statistical significance between the expression of pituitary stem cell markers (CD133, OCT4, SOX2, nestin) and invasiveness.

Conclusion. All stem cell markers are stained extensively in pituitary adenomas, except for SOX2 which was stained weakly. However, there is no effect of stem cells on invasiveness of pituitary adenomas because we cannot find a difference of the staining level between invasive and non-invasive adenomas. Nestin was stained extensively in functional adenomas, especially for GH, PRL, and gonadotropin secreting adenomas. SOX2 was stained extensively for ACTH-secreting adenomas.

Keywords: pituitary adenoma, stem cell, CD133, OCT4, SOX2, Nestin, invasive.

INTRODUCTION

The pituitary gland serves as the center of the endocrine system. Hypothalamic and peripheral inputs

received by the pituitary gland lead to the release of various hypothalamic and peripheral inputs that, in turn, cause the release of hormones impacting downstream endocrine glands, thus regulating the core organism processes of growth, metabolism, procreation, and the ability to cope with immune challenges and stress. However, the question of how these hormonal cells are formed or renewed during postnatal life remains largely unsolved (1).

Various biomarkers have been found to be of predictive value regarding clinical and radiological criteria for the management of unexpected behaving pituitary tumors, though the first results of studies using these markers have been inconsistent. The published inconsistencies may be due to variations in the definition of invasion. In some studies, the presence or absence of tumor invasion is based on preoperative radiological and/or intraoperative surgical findings, though others define invasion through histological observation. Another factor in these inconsistencies may be the existence of a variety of pituitary subtypes, instead of a single or distinct pathological tumor type (2).

As tissues develop, the desired cell type matures due to the multipotent differentiation capacity of stem cells. These cells are typically found in a specialized microenvironment of the tissue, called the niche, which regulates their maintenance, self-renewal, fate determination, and reaction to external influences(1). Pituitary stem cells (PSC) express many stem cell-associated markers, such as sex determining region Y-box 2 (SOX2), nestin, octamer-binding transcription factor 4 (Oct4), and CD133 (prominin-1) (3). The pituitary stem cell niches form a three-dimensional cooperating network throughout the gland, in line with the other homotypic networks formed by the hormone cells in the pituitary (4, 5).

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The major cause of pituitary pathology is the development of a tumor. Pituitary adenomas, which are primary tumors that occur in the pituitary gland, are the third most common type of intracranial neoplasms. Approximately 10% of all intracranial neoplasms and 30–50% of all pituitary masses are pituitary adenomas (6). Invasive adenomas grow into adjacent structures, such as the sphenoid sinuses, bone, and cavernous sinus. MRI is the best tool for classifying invasion, and the Wilson-Hardy grading system is widely used (7).

The aim of this study is to elucidate the role of stem cells in the invasion of pituitary adenomas.

MATERIALS AND METHODS

Clinical specimens and classification

All specimens were collected from the Neurosurgery Department of the Medeniyet University Goztepe Education and Research Hospital between January 2007 and April 2015. Pituitary adenoma samples were taken from 48 patients (17 men and 31 women). Patients were studied after providing informed consent. WHO classification of pituitary adenomas was revised in 2017 (8). There are two critical changes to the classification for pituitary adenomas in this edition. The first one is that the term of atypical adenoma was completely eliminated. The second change is a more precise cell lineage-based classification of pituitary adenoma that is defined based on lineage-specific transcription factors and hormones produced. We divided samples histologically according to hormone secretion as null cell, corticotroph (adrenocorticotrophic hormone, ACTH), lactotroph (prolactin, PRL), somatotroph (growth hormone, GH), gonadotroph (follicle stimulating hormone, FSH, luteinizing hormone, LH) and plurihormonal. Tumors without any hormone secretion have been classified as null cell adenomas and if a tumor secretes more than one type of hormones it has been classified as pluripotent. We also classified our samples according to clinical presentation as FPA and NFPA. NFPAs consist of tumors that do not secrete hormone or tumors that do not cause any clinical effect even if the hormone secretion occurs. We divided our samples into two according to Wilson Hardy classification. Again using the same classification we also divided them as invasive-noninvasive, symmetrical-asymmetrical, suprasellar–parasellar. Invasiveness of pituitary adenomas was decided according to the radiological classification of Wilson-Hardy. Grades III (localized sellar destruction) and grade IV (diffuse destruction) are invasive. We made all classifications

with the guidance of an experienced pathologist. Immunohistochemical examination was made following classification and our colleagues who divided samples were unaware of immunohistochemical results. We used the diagram that shows Wilson Hardy classification made by Di Leva *et al.* (Fig. 1) (9).

Recurrence was defined as reappearance of tumors on MRI or CT in NFPA. In FPA, increase of hormone levels after initial remission was used to indicate recurrence. Recurrence incidence was the highest between 1 and 5 years after surgery (10). We followed up the patients 5 years after surgery. In patients with recurrence, reoperation was planned when clinical findings were apparent.

Immunohistochemical analysis

Surgical specimens were fixed with 4% paraformaldehyde; paraffin-embedded sections were mounted on glass slides, deparaffinized, and reacted with either a CD133 rabbit polyclonal antibody (Catalog no: ORB18124; Biorobot Limited, Cambridge, UK), a nestin rat monoclonal antibody (Clone no: 10C2; Gene Tex Inc, Irvine, CA, US), an OCT4 rabbit polyclonal antibody (Subclass: IgG; GeneTex Inc, Irvine, CA, US), or a SOX2 rabbit polyclonal antibody (Clone No: NT2D1; Gene Tex Inc; Irvine, CA, US) as the primary antibody (dilution 1:100).

Data interpretation

The immunohistochemical expressions of CD133, nestin, OCT4, and SOX2 were scored semi-quantitatively on all tumor tissue samples. Positive staining was classified as follows: 0 = no stained cells; 1 = up to 30% stained cells; 2 = from 31 to 60% stained cells; and 3 = from 61 to 100% stained cells. The staining intensity of CD133, nestin, OCT4, and SOX2 were graded as follows: 0 = negative; 1 = weak, 2 = moderate, and 3 = strong. The expression score was calculated by multiplying the proportion of stained cells by the grade of CD133, nestin, OCT4, and SOX2 staining intensity. Thus, the final expression score ranged from 0 to 3, with 0 indicating no expression, 1 indicating weak expression from 1 to 6, and 2 indicating the highest expression up to 6.

Statistical analysis

The IBM SPSS statistics 22.00 software package 2007 (Number Cruncher Statistical System, Kaysville, Utah, USA) was used for all analyses. For the evaluation of study data, we used definitive statistical methods (mean, standard deviation, median, frequency, ratio,

minimum, and maximum). For the comparison of two groups of qualitative data, we used the Mann-Whitney U test. For comparison of three or more groups, we used the Kruskal-Wallis test. In cases of $p < 0.01$ and $p < 0.05$, we considered the results as significant.

RESULTS

The study included 48 patients diagnosed with pituitary adenomas by histopathology between 2007 and 2015. Of the 48 patients, 17 (35.4%) were male and 31 (64.6%) were female with a mean age of 47.10 ± 14.14 (17–86 yrs.). According to the Wilson-Hardy classification system, 27 (56.3%) were non-invasive adenomas. Extension into the intrasellar area occurred in 9 (18.8%), the suprasellar area in 27 (56.3%), and the parasellar area in 12 (25%). Asymmetrical extension was found in 19 (39.6%) patients. Secreted hormone levels according to new WHO classification were measured as follows: null cell in 2 (4.2%), somatotroph (GH) in 10 (20.8%), corticotroph (ACTH) in 3 (6.3%), gonadotroph (GND) in 17 (35.4%), lactotroph (PRL) in 5 (10.4%), and plurihormonal in 11 (22.9%) (Table 1).

There were clinically non-functional pituitary adenomas (NFPA) in 25 (52.1%) of patients. The number of clinically functional adenomas (FPA) was 23 (47.9%). The recurrence rate was found to be 7 (14.6%). In terms of staining results, Ki-67 was found to be negative in 11 (22.9%), 0–1% in 18 (37.5%), 1–2% in 16 (33.3%), and 2–3% in 3 (6.3%). P53 was found to be negative in 23 (47.9%), 0–1% in 8 (16.7%), 1–2% in 12 (25%), and 2–3% in 53 (10.4%).

The expression of CD133 was not seen in 3 (6.6%) patients, was weak in 18 (39.6%), and extensive in 27 (53.8%) (Fig. 2). The expression of OCT4 was not seen in 2 (4.1%) patients, was weak in 8 (16.4%), and extensive in 38 (79.5%) (Fig. 2). The expression of SOX2 was not seen in 36 (77%) patients, was weak in 11 (22%), and extensive in 1 (2%) (Fig. 2). The expression of nestin was not seen in 4 (8.3%) patients, was weak in 7 (14.5%), and extensive in 37 (77.2%) (Fig. 2) (Table 1). In invasive pituitary adenomas CD133 was not stained in 1 (4.8%), was weak in 10 (47.6%), extensive in 10 (47.6%). OCT-4 was weak in 4 (19%), extensive in 17 (81%). SOX2 was not stained in 15 (71.4%), was weak in 6 (28.6%). Nestin was not stained in 2 (9.5%), was weak in 1 (4.8%), extensive in 18 (85.7%) (Table 1). Null cell adenomas were stained weakly for CD133 and SOX-2, extensively for OCT-4 and nestin. CD133 was not stained in gonadotropins (Table 2).

There was no statistical significance between

the expression of the various stem cells markers (CD133, OCT4, SOX2, nestin) and age, sex, invasiveness, extension, symmetry, ki-67, p53, and recurrence.

DISCUSSION

Most of our knowledge of the pituitary stem/progenitor cell field is probably due to Chen *et al.* (11). In previous studies, the presence of two subpopulations, based on protein expression levels, have been revealed: Stem Cell Antigen 1 (Sca1) high and non-Sca1 high. Expression profiling suggested that pituitary progenitors are found in the non-Sca1 high fraction, and that the Sca1 high population probably contains other progenitors, such as endothelial and mesenchymal progenitors and endothelial cells. In agreement with this result, only the non-Sca1 high fraction can form spheres that can give rise to all endocrine cell types found in anterior pituitary (AP) (12).

Similarly to other organs, the plasticity of the pituitary gland and its alterations during adult life may be secondary to the activity of adult stem cells present in the gland (2, 13). Stem cell markers, such as SOX2, nestin, and CD133, have been identified in subpopulations of cells in the adult pituitary gland of animal models (2, 13–17). In addition, ‘pituispheres’ have been generated *in vitro* in these models (15, 18, 19). These findings support a potential role for pituitary stem cells in adult pituitary plasticity.

In the following sections, the literature and herein reported results on the various stem cell markers are discussed in greater detail.

CD133

Membrane glycoprotein CD133 (Prominin-1) was initially identified as a cell surface antigen of hematopoietic stem cells. It has been used as a stem cell marker in various normal and neoplastic human tissues, including the brain, skin, prostate, pancreas, and kidney (13, 20–22).

The sphere-forming population was found in primary brain tumor cells that expressed the NSC/precursor cell surface marker CD133. CD133+ cells constitute a subpopulation in brain tumors. Their frequency is as low as 1% or less in low-grade tumors, and it is as high as 30% in aggressive glioblastomas. Within one pathological type of brain tumor, the expression frequency of this marker could be extremely variable. At this time, it is not known for certain whether the CD133 fraction in a tumor has any prognostic significance (23).

Figure 1. Hardy classification system. Invasiveness is demonstrated on yellow line. Sella turcica tumors can be noninvasive (grade 0, intact with normal contour; grade I, intact with bulging floor; or grade II, intact, enlarged fossa) or invasive (grade III, localized sellar destruction; or grade IV, diffuse destruction). Symmetry is demonstrated on red line. Suprasellar tumors can be symmetrical (grade A, suprasellar cistern only; grade B, recess of the third ventricle; or grade C, whole anterior third ventricle) or asymmetrical (grade D, intracranial extradural; or grade E, extracranial extradural [cavernous sinus]). Elongation is demonstrated on blue line according to sella turcica. Pituitary tumors can be suprasellar (grade B, recess of the third ventricle; or grade C, whole anterior third ventricle) or parasellar (grade D, intracranial extradural; or grade E, extracranial extradural [cavernous sinus]).

Figure 2. a) Diffuse cytoplasmic expression of CD133 (original magnification x400), b) Diffuse cytoplasmic expression of nestin (original magnification x400), c) Diffuse nuclear expression of OCT-4 (original magnification x400), and d) Patchy nuclear expression of SOX-2 (original magnification x400) in pituitary adenoma cells.

Table 1. Patient and tumor characteristics, including histopathological staining results

		Invasion		<i>p</i>
		Non-Invasive (n:27) Count (%)	Invasive (n:21) Count (%)	
Age (Mean±SD) (yrs)		43.70±15.70	51.47±10.67	
Sex	Male	10(37%)	7(33.3%)	0.792
	Female	17(63%)	14(66.7%)	
Extension	Intrasellar	8(29.6%)	1(4.8%)	<0.001
	Suprasellar	19(70.4%)	8(38.1%)	
	Parasellar	-	12(57.1%)	
Symmetry	Symmetric	24(88.9%)	5(23.8%)	<0.001
	Asymmetric	3(11.1%)	16(76.2%)	
	None	9(33.3%)	2(9.5%)	
Ki67	0-1%	12(44.4%)	6(28.6%)	0.003
	1-2%	6(22.2%)	10(47.6%)	
	2-3%	0(0%)	3(14.3%)	
	None	18(66.7%)	5(23.8%)	
p53	0-1%	6(22.2%)	2(9.5%)	<0.001
	1-2%	3(11.1%)	9(42.9%)	
	2-3%	-	5(23.8%)	
	Null Cell	1(3.7%)	1(4.8%)	
Histology	ACTH	3(11.1%)	-	0.378
	GH	6(22.2%)	4(19%)	
	PRL	3(11.1%)	2(9.5%)	
	Gonadotropin	8(29.6%)	9(42.9%)	
	TSH	2 (7.4%)	2(7.4%)	
	Pluripotent	6(22.2%)	5(23.8%)	
NFPA		11(40.7%)	14(67.7%)	0.078
Recurrence		-	7(33.3%)	0.001
CD133	No Stained Cell	2(7.4%)	1(4.8%)	0.374
	Weak	8(29.6%)	10(47.6%)	
	Extensive	17(63%)	10(47.6%)	
Oct4	No Stained Cell	2(7.4%)	-	0.702
	Weak	4(14.8%)	4(19%)	
	Extensive	21(77.8%)	17(81%)	
Sox2	No Stained Cell	21(77.8%)	15(71.4%)	0.679
	Weak	5(18.5%)	6(28.6%)	
	Extensive	1(3.7%)	-	
Nestin	No Stained Cell	2(7.4%)	2(9.5%)	0.276
	Weak	6(22.2%)	1(4.8%)	
	Extensive	19(70.4%)	18(85.7%)	

Table 2. Distribution of pituitary stem cells staining features according to histology of the adenomas

		Histology					
		Null cell Count	ACTH Count	GH Count	PRL Count	Gonadotropin Count	Pluripotent Count
CD133	no stained cell	0	0	0	0	2	1
	weak	2	1	4	1	7	3
	extensive	0	2	6	4	8	7
OCT-4	no stained cell	0	0	0	0	0	2
	weak	0	1	1	0	3	3
	extensive	2	2	9	5	14	6
SOX-2	no stained cell	1	1	9	4	12	9
	weak	1	1	1	1	5	2
	extensive	0	1	0	0	0	0
Nestin	no stained cell	0	0	0	0	3	1
	weak	0	2	0	0	4	1
	extensive	2	1	10	5	10	9

Tang H *et al.* developed a comprehensive digestion method and defined a culture system. Using this method and system, they reported that meningioma sphere cells were expressing the stem cell marker, CD133, but not nestin (22). It has been reported that CD133 has prognostic significance in gliomas and it is an important stem cell factor for glioma progression (24-26).

Yunoue S *et al.* reported that the expression of CD133 in pituitary adenomas was related to immature endothelial progenitor cells that possibly play a role in the neovascularization of pituitary adenomas (16). According to the study results by Chen L *et al.*, a subpopulation of stem/progenitor-like cells are present in pituitary adenomas, and it is possible that these cells are responsible for benign tumor formation and maintenance. They reported that CD133 expression was significantly increased in invasive pituitary adenomas in comparison to non-invasive pituitary adenomas (27). Orciani M *et al.* evaluated three GH adenomas and three NFPAs. At the end of their study, they demonstrated that pituitary tumors contained a stem cell population that expressed CD133 and could generate cell types characteristic of mesenchymal stem cells (28).

Our study, containing a sufficient number of invasive and non-invasive pituitary adenomas, revealed that pituitary adenomas show a significant CD133 expression. Thus, we can conclude that there is no relation between invasiveness and CD133 expression. Similarly, we were unable to detect any effect of CD133 on recurring adenomas. The weakness in our study is that all adenomas are of the typical subtype, according to the WHO classification, because atypical and pituitary carcinomas are very rare, and therefore, not represented here. Undoubtedly, it might be predicted that ongoing studies with larger patient samples that include atypical and pituitary carcinomas would shed light on possible roles of stem cells in tumor genesis.

Nestin

Nestin is expressed in proliferating progenitor cells of developing and regenerating tissues. It is an intermediate filament protein that belongs to the 6th class of intermediate filaments and is considered as a marker for multi-potential neuroepithelial stem cells. Also, nestin is mostly expressed in tumors that arise from immature cells, such as primitive neuroectodermal tumors, medulloblastomas, pediatric rhabdomyosarcomas, melanomas, testicular stromal tumors, and gastrointestinal tumors (GISTs) (13, 29). In the study of glioblastoma multiforme (GBM), it was

reported that the membrane nestin signal was a valid prognostic factor (30). In terms of pituitary tumors, nestin is found in the Sca1 high population, and this is in agreement with its heterogeneous expression in non-hormonal cells within the pituitary (15, 31).

In our study, nestin staining was positive in both functional and non-functional adenomas. Although the stain was significantly more extensive in functional adenomas, the most extensive staining was observed for GH-secreting, PRL-secreting, and gonadotropin-secreting adenomas.

OCT4

OCT4, also known as OCT3 and POU5F1, is a 86POU (Pit-1, Oct-1 and Oct-2, unc-86)-domain octamer-binding transcription factor (32). OCT4 expression is confined to the pluripotent cells of embryos and embryonic stem cells (ESCs)(33), and it plays a role in the self-renewal of normal stem cells and the maintenance of pluripotency in ESCs. The aberrant expression of OCT4 in pluripotent cells causes differentiation and a progressive loss of potency (13, 32, 34). OCT4 was reported as a highly specific and sensitive immunohistochemical marker for primary intracranial germinomas (35).

An increased OCT4 fraction was thought to be related to tumor malignancy, but this seemed to be without independent prognostic influence in glioblastomas (36). In the literature, OCT4 has been identified in gliomas and glioma cell lines by immunohistochemistry (IHC) and real-time PCR (RT-PCR). The increase in OCT4 expression with tumor grade was reported in a study conducted using 41 astrocytic and oligodendroglial gliomas assessed by pathologist-based scoring (33).

In this study, we report extensive staining of OCT4 in pituitary adenomas. However, we did not detect any correlation between OCT4 and invasiveness or recurrence.

SOX2

SOX2 is a member of the sex-determining region of the Y chromosome-related high-mobility group box (SOX) family of transcription factors (37). SOX2 plays a role in the development of other tissues, especially in the pituitary, forebrain and eye (38), and it was also found to be critical in the development of the hypothalamo-pituitary axis (39, 40).

It has been reported that SOX2 is deregulated in several human cancers (41, 42) including glioblastoma, where SOX2 is overexpressed due

to several mechanisms such as amplification and promoter hypomethylation (43, 44). Elevated SOX2 levels correlate positively with the malignancy grade of brain tumors, and thus, in brain tumor patients, a higher stem cell gene expression profile correlates with a poor clinical outcome (44).

So far, in the literature, no significant change was detected in SOX2 expression between different pituitary tumors or in comparison with the normal pituitary samples. Alternatively, SOX2 expression was highly variable among the adamantinomatous craniopharyngioma (ACP) samples. In immunohistochemistry analysis, the same SOX2 variability was observed at the protein level (45, 46).

In a study of canine corticotroph pituitary adenomas, different SOX2 expression levels could not be identified between the studied subgroups, and SOX2 immunopositivity did not correlate to clinical parameters. Also, the expression of SOX2 mRNA was detected as significantly lower in adenoma samples in comparison to normal pituitary tissue (47).

Stem cells, or undifferentiated cells, have the capacity to commit or generate daughter cells of three main progenitor lineages, which are characterized by the respective expression of these transcription factors: pituitary-specific transcription factor 1 (PIT1) (POU1F1), t-box transcription factor (TPIT) (TBX19), and Steroidogenic factor-1 (SF1) (NR5A1). Expression of these factors is necessary for hormone-secreting cells in the pituitary, where PIT1-positive cells differentiate into GH-expressing somatotrophs, PRL-expressing lactotrophs, and TSH-expressing thyrotrophs (PIT1-cell lineage); TPIT-positive cells differentiate into ACTH-expressing adrenocorticotrophs and melanotrophs; and finally, SF1-positive cells differentiate into LH- and FSH-expressing gonadotrophs (48). SOX2-expressing cells give rise to all committed progenitor cell types (PIT1, TPIT, and SF1), so they, therefore, give rise to all hormone secreting cells types of the anterior lobe (GH, PRL, TSH, ACTH, and LH/FSH). A limitation of the assay used in the study is that it does not distinguish among multipotent populations of SOX2-expressing cells, and there is the possibility that several oligopotent or unipotent populations of SOX2-expressing cells exist (48).

In our study we detected that SOX2 immunoexpression was either weak or absent, with extensive staining in only one sample. SOX2 does not have any effect on invasion or recurrence of adenomas. Although it was significant that only ACTH-secreting adenomas were immunostained extensively, more accurate and elucidatory results could be obtained

in studies with greater numbers of ACTH-secreting adenoma samples.

In conclusion, we examined stem cells from pituitary tissues and the expression of various stem cell markers in pituitary adenomas. We concluded that all stem cell markers are stained extensively in pituitary adenomas, except for SOX2 which was stained weakly. However, there is no effect of stem cells on invasiveness of pituitary adenomas because we cannot find a difference of staining level between invasive and non-invasive pituitary adenomas. Nestin was stained extensively in functional adenomas, especially for GH-secreting, PRL-secreting, and gonadotropin-secreting adenomas. SOX2 was stained extensively for ACTH-secreting adenomas.

Conflict of interest

The authors declare that they have no conflict of interest.

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