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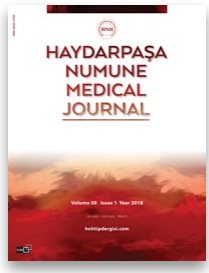


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ORIGINAL ARTICLE

# OX40 Gene Expression Level on Pathology Sections Obtained from Pituitary Adenomas Tissues

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## Abstract

**Introduction:** In this study, it was aimed to determine OX40 gene expression level on pathology sections obtained from PA tissues, and the level of circulating OX40L in peripheral blood samples of patients. Furthermore, it was aimed to evaluate the findings depending on the type, histopathological, and immunohistochemical features of the tumor.

**Methods:** The study was conducted with two groups (Study/Control). The study group (Group 1) consisted of patients (n=49) operated with the diagnosis of PA. The control group (Group 2) is the brain tissue samples obtained from patients (n=10) who underwent temporal lobectomy for the and treatment of epilepsy due to mesial temporal sclerosis.

**Results:** We found out that the tissue OX-40 gene expression levels of patients with PA did not differ from the gene expression levels of the control group. There was no statistically significant difference was found as a result of the comparison of pituitary ox40-tissue parameter measurement value with patient finding characteristics.

**Discussion and Conclusion:** PAs have altered cell behavior and epigenetic modifications that lead to various prognostic patterns and responses to treatment. As the disease progresses, up-and down-regulation of immune factors promotes the interaction between the immune system and tumor cells. Thus, it serves as a potential target for new diagnostic and therapeutic strategies.

**Keywords:** Gene expression; OX40; pituitary adenomas.

Pituitary adenomas (PA) are non-metastasizing neoplasms composed of adenohypophysial cells and accounting for approximately 10–15% of all intracranial neoplasms[1,2]. Despite the efforts for optimum surgical and medical treatment, PAs are associated with serious morbidity, worldwide[3].

Studies focused on medical treatment strategies are undertaken in recent years, to provide a brief understanding on the mechanisms of action and the structure and biology of the tumor tissue. Due to the advances in the field of molecular biology, the developmental stages of the cells that tumors originate, cell surface receptors and transcription

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factors have been studied in several reports.

Tumor cells have complex ecosystems many distinct cell types of the tumor microenvironment besides cancer cells[4]. The type and the intensity of immune response against the tumor cells provide important data regarding the behavior of the tumor, and targeted during the drug discovery process for the treatment of cancer to provide a favorale prognosis and improved survival.

OX40 is a tumor necrosis factor (TNF) receptor expressed primarily on activated T-cells and several other lymphoid and non-lymphoid cells[5]. It is a member of the TNFR superfamily (CD134) and play role in regulating T cell-mediated immune responses[6].

OX40 is expressed on the surface of tumor infiltrating lymphocytes of various tumors[7]. High expression of OX40 has been reported in glial tumors. As a consequence of the recent developments in monoclonal antibody technology, human IgG2 agonists specific for human OX40 are used in the treatment of various cancer types.

To the best of our knowledge, there is no study evaluating the relationship between OX40 gene expression and PA in the literature. Thus, in our study, we aimed to determine OX40 gene expression level on pathology sections obtained from PA tissues, and the level of circulating OX40L in peripheral blood samples of patients, and to evaluate the findings depending on the type, histopathological, and immunohistochemical features of the tumor.

## Materials and Methods

The study was carried out in accordance with the Declaration of Helsinki and approved by the Local Ethics Committee (2016/04). The study group (Group 1) consisted of patients (n=49) who were operated with the diagnosis of PA, whereas control group (Group 2) is the brain tissue samples obtained from patients (n=10) who underwent temporal lobectomy for the and treatment of epilepsy due to mesial temporal sclerosis. Informed consent was obtained from all patients for the data to be used in the study.

**Exclusion criteria were set as follows:** Patients with an unconfirmed histopathological diagnosis of PA, patients lost to follow-up, patients without the final results for both tissue OX40 expression and blood OX40L levels, patients diagnosed with additional malignity, renal failure, hyperproliferative disease and severe psoriasis in addition to PA, and patients who did not agree to participate in the study.

The tissue samples were stored in 1.5 mL dry polypropylene tubes (Eppendorf AG, Hamburg, Germany) at –80° until the day of analysis.

### Pre-operative Evaluation

All patients were consulted and evaluated by the physicians of the endocrinology clinic in the pre-operative period.

Adenomas were subgrouped as non-functional and functional (hormone-secreting) adenomas (FA), whereas FA included patients with acromegaly (oversecreted GH), Cushing disease (oversecreted ACTH), prolactinoma, and TSH-secreting adenoma (TSHOMA).

A dynamic contrast-enhanced magnetic resonance imaging of the sellar region was performed before the operation. The patients were subdivided into five subclasses according to Knosp et al.[7] criteria based on the medial and lateral borders of the internal carotid artery.

"Knops classification: Grade 0, the adenoma has not crossed the medial intercarotid line; Grade 1, the adenoma has crossed the medial intercarotid line but not the median intercarotid line; Grade 2, the adenoma has crossed the median intercarotid line but not the lateral intercarotid line; Grade 3, the adenoma has crossed the lateral intercarotid line; Grade 4, the adenoma has surrounded the internal carotid by 360°

### Pathological Evaluation

Histopathological and immunohistochemical evaluations were performed by a single pathologist in the same center. Staining of the tissue sections was reported as GH, prolactin, ACTH, TSH, gonadotroph (Luteinizing hormone (LH), and follicle-stimulating hormone (FSH), pulihormonal, and staining negative according to the staining status of the tissues.

## Gene Expression Studies

### RNA Isolation from Tissue Samples

RNA isolation from frozen tissue samples was performed using Trizol Reagent. Tissues were homogenized by adding 1 mL of TRIzol™ Reagent to 50–100 mg of tissue sample. The homogenized sample was incubated at room temperature for 5 min, and transferred to a 1.5 mL clean microcentrifuge tube. The sample was centrifuged at 12–16,000 ×g for 10 min to remove insoluble particles. The supernatant was transferred to a clean 1.5 mL RNase-free microcentrifuge tube. 200 uL chloroform was added to 1 mL of GENEzol ™ Reagent which was used in homogenization. The microcentrifuge tube was shaken vigorously for 10 s. To separate the phases, the sample was centrifuged at 12–16,000 ×g for 15 min at 4°C. The RNA precipitation step was initiated

by transferring the upper aqueous phase to an unused 1.5 mL microcentrifuge tube. After adding 1 volume of isopropanol to the aqueous phase, the tube was mixed by inverting several times.

The sample was incubated at room temperature for 10 min. Then, centrifuged at 12–16,000 ×g for 10 min at 4°C to generate the RNA pellet. The supernatant is removed and discarded. To wash the RNA pellet, 1 mL of 70% ethanol was added and vortexed. The sample was centrifuged at 12–16,000 ×g for 5 min at 4°C, the supernatant was removed with a pipette. The RNA pellet was dried at room temperature for 5–10 min. 20–50 µL RNase-free water was added to resuspend the RNA pellet, and incubated at 55–60°C for 10–15 min. The isolated RNAs were kept in –80°C until the day of analysis.

### RNA Quantitation

Absorbances at 260 nm and 280 nm were measured for the determination of the amount and purity of the isolated RNAs. For this purpose, 200 µL dH2O as blank, 195 µL dH2O, and RNA were added to quartz cuvettes, and the amount of RNA was calculated with the formula as = A260×40×40 ng/µL, Purity = A260/A280 to evaluate the obtained absorbance values and purity.

### cDNA Synthesis with Real-time Polymerase Chain Reaction (RT-PCR)

1 µg of the isolated total RNA was used for cDNA synthesis. Briefly, 1 µg of total RNA, 1 µl of random primer and distilled water were mixed to make a total volume of 10 µL, and the mixture was incubated at 65°C for 5 min and then taken on ice. 5× Reaction buffer, 0.5 µL RNAase inhibitor, 2 µL dNTP mix, 2 µL reverse transcriptase were added to a final volume of 20 µL and located into the PCR device for 60 min at 42°C and 5 min at 70°C.

### PCR

All synthesized cDNAs were tested in PCR using primers for Human β-2 microglobulin gene. In a final concentration of 50 µL, 1 µL forward and reverse primers, 2 µL MgCl, 0.2 µL Taq polymerase, 5 µL of Taq polymerase buffer, and distilled water were mixed. After the reaction was completed, products were checked under UV light in 2% agarose gel electrophoresis. RNA isolation and cDNA synthesis were repeated for negative PCR results of cDNAs.

### RT-PCR

After obtaining cDNA, PCR was performed under optimized conditions for OX40 and human β-2 microglobulin genes, using 7500 Fast Real-Time PCR System (Applied Biosystem, CA, USA). The primers for OX40 and human β-2 microglobulin were purchased from TAQMAN (ThermoScientific). Five µl Master mix, 1 µL cDNA, 0.5 µL forward and reverse primers of 100 ng/µL, 3 µL distilled H2O were added to make a final volume of 10 µL. Each cDNA was replicated in triplicates and the human β-2-microglobulin gene was used for normalization.

### Evaluation of Results

RT-PCR results were evaluated according to the Livak method, as each Ct values obtained for OX40 was subtracted from the average of the human β-2 microglobulin gene and the power of the 2 base value was taken. The results obtained from the replication of OX40 in the PA group samples were divided by the results of the control group.

### Statistical Analysis

In the data analysis process, the mean and standard deviation were used while making the statistics of the continuous data in the scales. Frequency and percentage values were used to define categorical variables and minimum/maximum values. Since the data did not show a normal distribution, Mann–Whitney U test statistics were used to compare the means of two groups of OX40 marker parameters, and the Kruskal–Wallis test statistics were used to compare the means of more than two groups. The statistical significance level was taken as $p<0.05$. Statistical Package for the Social Sciences 23.0 package program was used to evaluate the data.

## Results

The demographic data, pre-operative radiological, and endocrinological evaluation findings, and post-operative pathological diagnoses of 49 patients included in the study are summarized in Table 1.

The tissue OX-40 gene expression levels of patients with PA (20.489±71.506) did not differ from the gene expression levels of the control group (1±0) ($p>0.05$) (Table 2).

We did not find a statistically significant difference of OX40 expression while we subclassified the PA patients in terms of gender, pre-operative hormone profile, degree of invasion (Knops classification), margin of resection, and histopathological hormone staining panels ($p>0.05$) (Table 3).

**Table 1.** The pituitary OX40 marker descriptive statistics

| n=59 | n | % |
|---|---|---|
| Group | | |
| Patient | 49 | 83.1 |
| Control | 10 | 16.9 |
| Gender (Patient Group) | | |
| Female | 22 | 44.9 |
| Male | 27 | 55.1 |
| Gender (Control Group) | | |
| Male | 5 | 50 |
| Female | 5 | 50 |
| Patient | | |
| Knosp | | |
| 0 | 5 | 10.2 |
| 1 | 10 | 20.4 |
| 2 | 9 | 18.4 |
| 3 | 12 | 24.5 |
| 4 | 13 | 26.5 |
| Aggressiveness | | |
| Invasive (Knops 3,4) | 25 | 51.03 |
| Non-invasive (Knops 0,1,2) | 24 | 48.97 |
| Hormone Panel Staining | | |
| Negative(Null Cell) | 9 | 18.4 |
| ACTH | 6 | 12.2 |
| GH | 9 | 18.4 |
| Gonadotrope(FSH-LH) | 14 | 28.6 |
| Plurihormonal | 6 | 12.2 |
| Prolactin | 4 | 8.2 |
| TSH | 1 | 2.0 |
| Pre-operative Hormone Profile | | |
| Non-functional | 24 | 49 |
| Cushing disease | 5 | 10.2 |
| Acromegaly | 14 | 28.6 |
| Prolactinoma | 5 | 10.2 |
| TSH-secreting adenoma (TSHOMA) | 1 | 2 |
| **Patient** | **$\bar{X}$ ±SS** | **Minimum-Maximum** |
| Age | 46.57±15.07 | 20-71 |
| OX40 Tissue | 20.489±71.506 | 0.01-470.84 |
| Control | | |
| Age | 45.1±12.1 | 32-62 |
| OX40 Tissue | 1±0 | 1-1 |

**Table 2.** The comparison of pituitary OX40 tissue measurement value

| OX40 Tissue | Number (n) | Mean±SD | Mean Rank | Z | P |
|---|---|---|---|---|---|
| Patient | 49 | 20.489±71.506 | 31.12 | -1.114 | 0.265 |
| Control | 10 | 1±0 | 24.50 | | |

SD: Standard Deviation.

**Table 3.** The comparison of pituitary OX40-tissue parameter measurement value

| OX40-TISSUE* | Number (n) | Mean±SD | Mean Rank | Z/X²** | P |
|---|---|---|---|---|---|
| Gender | | | | | |
| Female | 22 | 36.25±105.09 | 25.50 | -0.221 | 0.825 |
| Male | 27 | 7.64±11.68 | 24.59 | | |
| Aggressiveness | | | | | |
| Invasive | 24 | 27.73±95.13 | 25.42 | -0.200 | 0.841 |
| Noninvasive | 25 | 13.53±38.06 | 24.60 | | |
| Hormone panel staining | | | | | |
| Null | 9 | 23.13±62.14 | 23.78 | 1.648 | 0.949 |
| Acth | 6 | 10.60±13.75 | 28 | | |
| Gh | 9 | 3.66±4.93 | 21.33 | | |
| Gonadotrop (FSH, LH) | 14 | 10.44±14.21 | 26.64 | | |
| Plurihor | 6 | 85.75±189.15 | 28 | | |
| Prl | 4 | 9.37±16.51 | 22.75 | | |
| Tsh | 1 | 0.95±– | 19 | | |
| Preop hormone profile | | | | | |
| Non-Functional (NFA) | 24 | 14.81±38.78 | 25.29 | 1.299 | 0.862 |
| Cushing Disease | 5 | 12.51±14.46 | 29.60 | | |
| Acromegaly | 14 | 36.53±125.07 | 22.43 | | |
| Prolactinoma | 5 | 14.68±18.59 | 27.40 | | |
| TSH-secreting adenoma (TSHOMA) | 1 | 0.95±– | 19 | | |
| Knosp | | | | | |
| 0 | 5 | 101.53±207.02 | 24 | 0.667 | 0.955 |
| 1 | 10 | 7.97±12.88 | 25.70 | | |
| 2 | 9 | 8.69±10.36 | 25.89 | | |
| 3 | 12 | 20.32±53.74 | 26.83 | | |
| 4 | 13 | 6.98±12.38 | 22.54 | | |

*Mann–Whitney U test was used to compare the means of two groups, and the Kruskal–Wallis test was used for comparisons of more than two groups, **Z value was used in Mann–Whitney U test and X2 value was used in Kruskal–Wallis test.

## Discussion

The biological behavior of PAs is subject of interest due to the distinct differences regarding their invasion capacity, growth pattern, and hormonal activities. Furthermore, they may hypersecrete hormones or cause mass effects.[8] Thus, novel research focus on epigenetic variations, examining the modifications that affect the gene expression at any level without changing the DNA sequence in order to understand the marked differences between tumors.

Epigenetics have been used in classification studies in an attempt to further subclassify the tumors and identify the prognostic factors[9-14]. Recently, the relationship between methylation of the promoter region in glioblastoma treatment and the implementation of DNA methylation-based classifications in meningiomas provided clinical convenience.

In our study, we studied the gene expression level of OX40, a member of the TNF receptor superfamily, expressed on activated cells of the immune system. Ox40 and its ligand OX40L are well-studied modulators of the immune cells, playing a pivotal role in the interplay between cytokine production and T-cell differentiation[15]. The altered expression profile of OX40 and OX40L has been demonstrated in several studies aiming to understand the tumor behavior. In an attempt to evaluate the gene expression level of OX40 in PA, we conducted a RT-PCR based study and compared the PA cells to the brain tissue samples of patients who underwent temporal lobectomy as the control group. We did not find an increased expression of the OX40 gene on the tumor tissue, and OX40 expression studies in PA patients did not yield a different outcome while we subgrouped the patients according to gender, pre-operative hormone profile, degree of invasion, degree of postoperative remission, the margin of resection, and histopathological hormone staining patterns.

In its new PA classification guide published in 2017, the World Health Organization classifies PAs into subgroups according to altered expression of transcription factors and hormone secretion pattern, associating the aggressive behavior of the adenoma, with transcription factors and mutations[16]. Although transcription factors might be determined with immunohistochemical studies, gene-level studies would provide additional evidence regarding the tumor behavior, and determining new biomarkers and therapeutic targets are needed.

T-cell-mediated responses are the primary mechanism of action of the immune system against tumor cells[17]. Two distinct signaling pathways must be activated for the initiation of T-cell response: the interaction of T-cell antigen receptors and antigenic peptide/MHC Class I or II complexes, and the costimulators on APCs and their counter receptors on T-cells. Hence, without costimulation, exposure of T-cells to antigen may cause unresponsiveness or anergy. Therefore, enhancing costimulation response through the members of the tumor necrosis factor receptor superfamily has been an attractive focal point to augment weak immune responses during progressive tumor growth.

Recent whole transcriptome sequencing (RNAseq) on conventional null cell PAs suggested the involvement of proteins secreted by the tumor tissue and involved in the suppression of T-lymphocyte activity, through downregulation of genes, acting in antigen presentation by the adenoma to cytotoxic T-cells. Novel studies reported the domination of T-cells in the immune microenvironment of all subtypes of PAs, alongside an upregulated expression of programmed cell death protein 1/programmed cell death 1 ligand 2 (PD1/PD-L2).

OX40L and PD-L2 are well-known regulators of cytokine production in the immune response against various types of antigens[18]. In our study, we aimed to show the OX-40L expression, which is expressed in glioblastomas, in PAs in an attempt to pave the way for its therapeutic implications, as a possible alternative to current methods and invasive surgical interventions. However, we did not observe an increased expression pattern of OX40, and its correlation with the hormone staining patterns, remission status, and the extent of resected tumor tissue.

In their study on patients with advanced colorectal cancer, Sawada et al.[19] reported that increased blood levels of soluble OX40, which was determined with an immunoassay study, were correlated with a reduced survival time in patients. Thus, circulating levels of OX40 and its ligand in patients with PAs would yield additional data regarding the T-cell mediated immune response against tumor cells. However, our study did not involve either an mRNA expression pattern, or the protein level measurement of the OX40 and its ligand.

On the other hand, OX40 expression might arise in a certain stage of the immune response and cell culture studies with PA tumor cells of differing metabolism, differentiation, and proliferation patterns are required for a better understanding of the role of OX40 and its ligand in cytokine production from CD4 T-cells. Furthermore, immunohistochemical staining studies with other costimulators of the immune response in PA cells of different phases of the cell cycle would provide a well-documented understanding of the role of OX40 in PA tumorigenesis.

However, until the near future, the OX-40 receptor-ligand costimulation system has received relatively little attention to exploiting its ability to increase antitumor immunity since the previous conception suggested receptor expression to be limited to primary CD4 T-cells. In the inflammatory region, T cells expressing OX-40R appear to be cells that recognize the autoantigen and play a role in the experimental autoimmune encephalomyelitis pathogenesis. Shibahara et al.[20] demonstrated for the first time that glioblastoma cells express OX40L, which activates OX-40 signaling, and enhances antitumor adaptive immunity and that higher expression of OX40L is significantly associated with long-term progression-free survival. They also showed that hypoxia increased CD-40L expression in glioblastoma cells. The core part of the tumor tissue is known to be the most hypoxic tumor part, and cell sampling from more exterior portions of the tumor might be the reason underlying the lack of increased expression in our cases.

PAs are heterogeneous tumors, with varying cell behavior and epigenetic modifications, leading to diverse prognostic patterns and responses to treatment. Down and upregulation of immune factors during the progression of the disease promote the interplay between the immune system and the tumor cells, thereby serving as a potential target for novel diagnostic and therapeutic strategies. However, further research combining epigenetics, genetics, cell culture, and immunohistochemical studies is needed to identify immune pathways involved in the malignant transformation, progression, and prognosis of PAs.

**Ethics Committee Approval:** The study was carried out in accordance with the Declaration of Helsinki and approved by the Local Ethics Committee (2016/04).

**Peer-review:** Externally peer-reviewed.

**Authorship Contributions:** Concept: B.E.; Design: M.K., O.B.; Supervision: Ö.G., O.T.; Fundings: B.Ç., O.H.; Materials: O.H.; Data Collection or Processing: D.G.G., B.Ç.; Analysis or Interpretation: M.E., U.A.; Literature Search: O.B.; Writing: B.E.; Critical Review: Ö.G.

**Conflict of Interest:** None declared.

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