



Role of AGR2 Expression in Specimens from Pituitary Adenoma Tissue on Tumor Behavior

Hipofiz Adenom Doku Örneklerinde AGR2 Ekspresyonunun Tümör Davranışı Üzerine Etkisi

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Abstract

Objective: To investigate the anterior gradient 2 (AGR2) mRNA expression, which performs various cellular functions such as cell migration, differentiation, and proliferation in tissue specimens from pituitary adenomas. **Material and Methods:** A total of 44 pituitary adenoma specimens (20 female/24 male) from patients, and ten normal brain tissues (5 female/5 male) operated for epilepsy surgery (control group) were included in the study. Specimens were stored at -80 °C throughout the study. Molecular assessment of pituitary adenomas and control brain tissues was performed to quantify mRNA expression of AGR2 using real-time PCR. Distribution of AGR2 mRNA expression levels was evaluated according to pituitary adenoma subtypes, tumor aggressiveness, or invasiveness. **Results:** Expression levels of AGR2 mRNA in corticotroph (n=5) and somatotroph adenomas (n=14) were higher compared to control brain tissues (p=0.045 and p=0.005, respectively); however, expression levels of AGR2 mRNA in lactotroph (n=3), gonadotroph (n=14), and non-secretory adenomas (n=7) were similar to the control brain tissues. The thyrotroph adenoma (n=1) was not included in the analysis. Expression levels of AGR2 mRNA were similar in female and male patients. The expression levels of the AGR2 mRNA were higher in non-invasive tumors (n=20) than invasive tumors (n=24) (p=0.001), and in the non-aggressive tumors (n=27) than aggressive tumors (n=17) (p=0.018). A negative correlation between expression levels of AGR2 mRNA and Ki-67 labeling index (r=-0.328; p=0,029) was obtained. However, no correlation was found between the expression levels of AGR2 mRNA and age or maximum tumor diameter. **Conclusion:** AGR2 expression is inversely correlated to invasiveness and aggressiveness, independently from age and sex in pituitary adenomas.

Keywords: Anterior gradient 2; AGR2; pituitary adenoma; aggressiveness; invasiveness; somatotroph adenoma

Özet

Amaç: Migrasyon, farklılaşma ve proliferasyon gibi çeşitli hücresel görevleri olan anterior gradient 2 (AGR2) ekspresyonunun hipofiz adenom doku örneklerinde değerlendirilmesi. **Gereç ve Yöntemler:** Bu çalışmaya, 44 hastanın (20 kadın/24 erkek) hipofiz adenom doku örnekleri ve epilepsi cerrahisi uygulanan 10 hastanın (5 kadın/5 erkek) da normal beyin dokusu örnekleri kontrol grubu olarak kabul edilerek dâhil edilmiştir. Tüm doku örnekleri çalışılana kadar -80°C'de saklanmıştır. Dokularda AGR2 mRNA ekspresyonu real-time PCR kullanılarak değerlendirilmiştir. Hipofizer adenomunun subtipi, tümörün agresifliği ve invazivliğine göre AGR2 mRNA ekspresyonunun dağılımına bakılmıştır. **Bulgular:** AGR2 mRNA ekspresyonu kortikotrop adenomlarda (n=5) ve somatotrop adenomlarda (n=14), kontrol beyin dokusundan daha yüksekti (sırasıyla, p=0,045 ve p=0,005), bununla birlikte lactotrop adenomlarda (n=3), gonadotrop adenomlarda (n=14) ve non-sekretuar adenomlarda (n=7) kontrol beyin dokusuna göre benzer idi, tirotrop adenomlar ise (n=1) hesaplamlara dâhil edilmemiştir. AGR2 mRNA ekspresyonu kadın ve erkeklerde benzerdir. İnvaziv olmayanlarda (n=20), invaziv tümörlerden (n=24), agresif olmayanlarda (n=27) ise agresif tümörlerden (n=17) AGR2 mRNA ekspresyonu anlamlı derecede yüksekti (sırasıyla, p=0,001 ve p=0,018). AGR2 mRNA ekspresyonu ile Ki-67 labeling index arasında bir negatif korelasyon (r=-0,328; p=0,029) saptanırken yaş ve maksimum tümör boyutu arasında bir korelasyon saptanmadı. **Sonuç:** AGR2 ekspresyonu yaş ve cinsiyetten bağımsız olarak hipofiz adenomlarının agresifliği ve invazivliği ile ters orantılıdır.

Anahtar kelimeler: Anterior gradient 2; AGR2; hipofiz adenoma; agresiflik; invazivlik; somatotrof adenom

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Introduction

Pituitary adenomas are benign tumors. Although they are relatively rare among all intracranial tumors, these tumors have an increased risk of morbidity and mortality (1). Several complications caused by hyperfunctioning adenomas form one reason for the increased risk (2). Another reason is the compression symptoms caused by the mass effect of adenoma as up to 50% of pituitary adenomas exhibit invasive or aggressive behavior (3,4). The etiopathogenesis of pituitary adenomas has not been completely elucidated, and genetic mutations have been identified in less than 5% of all cases. There is no reliable biological marker to define the aggressive behavior of adenoma (5). Therefore, aggressive pituitary adenomas are rarely recognized in the early stages; this leads to therapeutic challenges in the subsequent period. Anterior gradient protein 2 (AGR2) is from family of protein disulphide isomerase gene (6). It plays a crucial role in providing endoplasmic reticulum homeostasis and participates in cellular functions, including cell migration, differentiation, and proliferation (6,7). It has been reported that AGR2 overexpression may be associated with the development of various malignancies such as breast, lung, ovary, prostate, colon, and pancreas; however, there are controversial data on this topic (8). There are limited studies about the role of AGR2 expression in pituitary adenomas (9,10).

In this study, the authors aimed to assess the importance of AGR2 expression in both identifying pituitary adenoma subtypes and tumor aggressiveness in tissue specimens from pituitary adenomas.

Material and Methods

The study was performed on adenoma specimens obtained from 44 patients (20 female, 24 male) who underwent pituitary surgery for two years. Patients with non-adenomatous sellar lesions in the histopathological examination performed after surgery were excluded. Normal brain tissues obtained from ten patients (5 female, 5 male) who underwent epilepsy surgery with normal pituitary imaging were included as a control group. All the tissue samples were formalin-fixed and embedded in paraffin blocks before being stored at -80° C until assays.

All procedures in this study complied with the 1964 Helsinki Declaration and were approved by the Ethics Committee of the Bakirkoy Dr. Sadi Konuk Training and Research Hospital. Written informed consent was obtained from all participants.

The AGR2 gene expression in tissue samples comprised 3-steps: RNA isolation, synthesis of complementary DNA (cDNA) from RNA by reverse transcription, and demonstration of gene expression with real-time polymerase chain reaction (RT-PCR).

RNA Isolation from Tissue Samples

RNA was isolated from frozen tissues of adenoma or the brain by TRIzol™ reagent. The process followed was: tissue samples were homogenized by adding 1 mL of TRISOL™ reagent to 50-100 mg of the tissue sample and incubated for 5 minutes. The supernatant was separated by centrifugation and transferred to an RNase-free micro-centrifuge tube (1.5 mL); 1 mL of GENEzol™ reagent was added to it for homogenization. The micro-centrifuge tube was disturbed, and the sample was re-centrifuged; the upper liquid phase was then transferred into another tube, followed by the RNA precipitation step. One volume of isopropanol was added to the liquid phase and mixed, which was incubated for 10 minutes and centrifuged to obtain an RNA pellet. After centrifugation, the supernatant was carefully removed and discarded. One mL of ethanol was added to wash the RNA pellet, and the sample was vortexed, then re-centrifuged, and the supernatant thus obtained was removed. The resultant RNA pellet was dried, and the dry pellet of RNA was re-suspended by RNase-free water. It was then incubated at 55-60°C for 15 minutes. The isolated RNA was stored at -80°C.

cDNA Synthesis from RNA

One µg of the total isolated RNA was utilized for cDNA synthesis. One microgram of total isolated RNA, 1 µL of random primer, and dH₂O were mixed to obtain a total volume of 10 µL. The obtained mixture was incubated for 5 minutes at 65°C before being placed on ice. Then, 5X reaction buffer, RNase inhibitor (0.5 µL), dNTP mix (2 µL), and reverse transcriptase (2 µL) were annexed to obtain 20 µL of the

total reaction volume. It was placed in a PCR device at 42°C (60 minutes) and 70°C (5 minutes).

AGR2 Gene Expression by RT-PCR

Applied Biosystems Step One Plus hardware was utilized for the RT-PCR analyses (Gene All SYBR Green Master Mix). Samples were assayed in triplicate, and reactions were generated using their genes and actin beta (ACTB) genes. The levels of gene expression were quantified with Applied Biosystems 7500 Fast Real-Time PCR System Software. AGR2 gene expression levels were described according to the threshold cycle (Ct), and ACTBs were utilized as reference genes that acted as an internal reference to normalize the RNA expression and were calculated as $2^{-\Delta\Delta CT}$.

Assessment of Parameters That Could Be Related to AGR2 mRNA Expression in Adenoma Tissues

According to pituitary adenoma subtypes, the distribution of AGR2 mRNA expression levels (corticotroph, somatotroph, lactotroph, gonadotroph, thyrotroph, and non-secretory adenomas) was compared. The correlations between AGR2 mRNA expression levels and age, maximum tumor diameter, and Ki-67 labeling index were assessed. Ki-67 labeling index was scored with the eye-balling method after the MIB1 antibody staining of adenomas tissues.

Adenoma size grading and cavernous sinus invasiveness were assessed according to Hardy and Knosp classifications, respectively (11,12), and the distribution of AGR2 mRNA expression levels according to Hardy and Knosp classification grade of adenomas. Pituitary tumor aggressiveness was determined according to the management of aggressive pituitary tumors and carcinomas in the European Society of Endocrinology Clinical Practice Guideline (4). According to this guideline, aggressive tumors were defined as rapidly growing with radiologically invasive tumors and/or clinically significant tumoral enlargement, although optimal standard treatments such as surgery, medical therapy, and radiotherapy. AGR2 mRNA expression levels were compared in invasive/noninvasive and aggressive/non-aggressive tumors.

SPSS 21.0 version was used for the statistical analyses. Descriptive statistical methods and quantitative data were used to assess the data. Frequency and percentage rates were used to define categorical variables; mean±standard deviation (SD) was used to describe numerical variables. The Chi-square test was utilized to compare categorical variables. Inter-variable relationships were assessed with Spearman's correlation analysis. Comparisons between qualitative data were made with Fisher's exact, Pearson's Chi-square, and Fisher-Freeman-Halton's tests. The statistical analyses of molecular calculations were performed by the One-way ANOVA and Student's T-test. p -value<0.05 was considered statistically significant.

Results

The mean age of 44 patients (20 female, 24 male) was 47 ± 15 (range, 20-71) years. Out of 44 pituitary adenoma tissues, there were 5 ACTH-positive corticotroph adenomas, 14 GH-positive somatotroph adenomas, 3 PRL-positive lactotroph adenomas, 14 LH-and/or FSH-positive gonadotroph adenomas, seven non-secretory adenomas with no immunoreactivity, and one TSH-positive thyrotroph adenoma according to immunohistochemical staining. While corticotroph, somatotroph, thyrotroph, and lactotroph adenomas exhibited hormone hyperfunction, there was no hyperfunction in gonadotroph adenomas. Expression levels of AGR2 mRNA in corticotroph and somatotroph adenomas were higher in comparison to the control brain tissues ($p=0.045$ and $p=0.005$, respectively); however, expression levels of AGR2 mRNA in lactotroph, gonadotroph, and non-secretory adenomas were similar to those in the control brain tissues ($p>0.05$) (Figure 1). Thyrotroph adenoma ($n=1$) was not included in the statistical analysis. Expression levels of AGR2 mRNA were similar in female and male patients.

The mean adenoma diameter was 28 ± 16 (range, 4-75) mm. The distribution of adenomas according to Hardy and Knosp classification is Hardy 0 ($n=3$), Hardy 1 ($n=2$), Hardy 2 ($n=12$), Hardy 3 ($n=9$), Hardy 4 ($n=18$) and Knosp 0 ($n=4$), Knosp 1

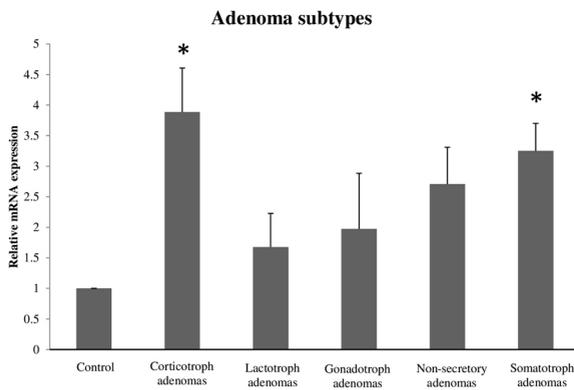


Figure 1. Distribution of AGR2 mRNA expression levels according to pituitary adenoma subtypes. The expression levels of AGR2 mRNA in corticotroph (n=5) and somatotroph adenomas (n=14) were higher compared to the control brain tissues ($p=0.045$ and $p=0.005$, respectively); however, the expression levels of AGR2 mRNA in lactotroph (n=3), gonadotroph (n=14), and non-secretory adenomas (n=7) were similar to the control brain tissues ($p>0.05$). Thyrotroph adenoma (n=1) has not been included in the statistical analysis.

(n=10), Knosp 2 (n=8), Knosp 3 (n=10), and Knosp 4 (n=12). AGR2 mRNA expression levels, according to Hardy and Knosp classification grade of adenomas have been shown in Figure 2.

AGR2 mRNA expression levels were higher in non-invasive tumors (n=20) than invasive tumors (n=24) ($p=0.001$). AGR2 mRNA expression levels were higher in non-aggressive tumors (n=27) ($p=0.018$) than aggressive tumors (n=17). A comparison of AGR2 mRNA expression levels according to invasiveness and aggressiveness of adenoma is presented in Figure 3.

A negative correlation was also found between the Ki-67 labeling index and AGR2 mRNA expression levels ($r=-0.328$; $p=0,029$). However, no correlation was observed between AGR2 mRNA expression levels and age or maximum tumor diameter.

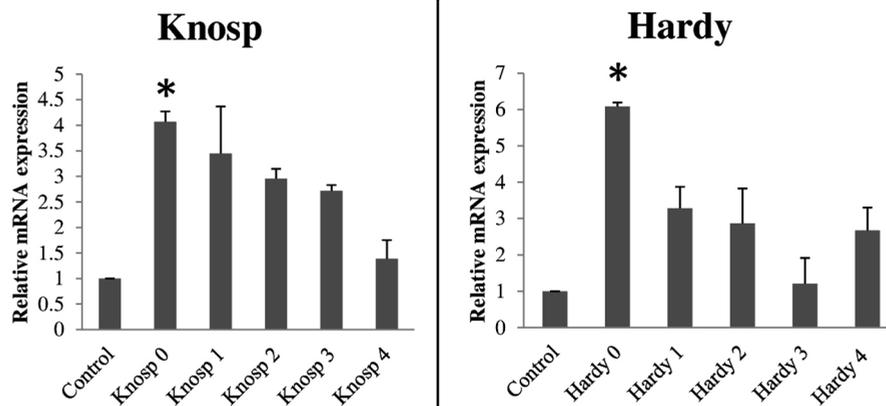


Figure 2. Distribution of AGR2 mRNA expression levels according to Hardy and Knosp classification grade of adenomas.

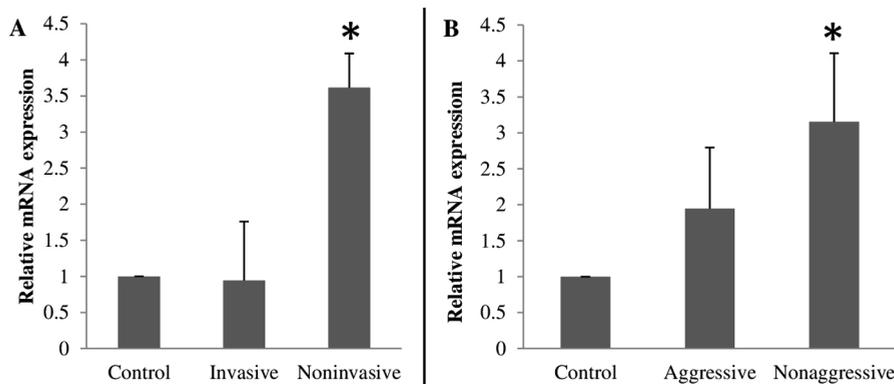


Figure 3. Comparison of AGR2 mRNA expression levels according to invasiveness and aggressiveness of adenomas. A. The expression levels of the AGR2 mRNA were higher in the noninvasive tumors (n=20) than invasive tumors (n=24) ($p=0.001$). B. The expression levels of AGR2 mRNA were higher in non-aggressive tumors (n=27) ($p=0.018$) than the aggressive tumors (n=17).

Discussion

In this study, we demonstrated the negative relationship between tumor aggressiveness and AGR2 expression in pituitary adenomas. Previous studies have proved overexpression of AGR2 in various malignancies such as breast, lung, ovary, prostate, colon, and pancreas cancers and suggested that it might be associated with poor prognosis and metastasis or resistance to chemotherapy (8). The reason is that AGR2 plays an important role in the homeostasis of the endoplasmic reticulum (6,7) and may gain pro-oncogenic function in the microenvironment of the tumor via secretion from the extracellular matrix (13). It may also play a role in inflammation, immunogenicity, angiogenesis, and thus remodeling of the extracellular matrix in tumor niche microenvironment (14). In vivo and in vitro investigations showed that it is involved in the tumor niche signaling or may also be present in cancer stem cells niche and chemoresistance niche (14). For all these reasons, AGR2 expression levels were found to be related to poor prognosis in malignancies. However, we found a negative association between AGR2 expression and aggressiveness in pituitary adenomas but these tumors are not malignant. Similar results were reported in two studies assessing AGR2 expression in pituitary adenomas (9,10). In the first study, AGR2 expression was assessed in pituitary adenoma tissues by immunohistochemical staining, and AGR2 staining was found to be negative in the majority of aggressive adenomas (9). In the same group's subsequent study, the authors assessed the use of serum AGR2 levels in the differentiation between adenomatous and non-adenomatous lesions of the pituitary gland. The same study showed that serum AGR2 levels were higher in controls than in patients with non-adenomatous pituitary lesions and that serum AGR2 levels were lower in larger tumors, although the difference could not reach a statistical significance (10).

The present study results, together with those from the above-mentioned studies, support the negative correlation between AGR2 expression and aggressiveness in pituitary adenomas. The present study also directly presented AGR2 gene expression in

adenoma tissues; thus, results regarding gene expression, protein synthesis, and serum levels have been shown consistently in pituitary adenomas.

It is currently challenging to draw definitive conclusions on the reason for decreased AGR2 expression in pituitary adenomas, unlike other malignancies. However, literature reports studies stating that AGR2 expression was associated with lower tumor grade or proliferative index, more prolonged overall survival, and better prognosis in colorectal cancers, non-small cell lung carcinoma, and breast cancer, which are in agreement with the findings of the present study (15-17). These contradictory data may be due to different mutations of AGR2 that affect functions of AGR2 protein (14).

The effects of the underlying mechanism of AGR2 in pituitary adenoma are unclear. The AGR2 expression is induced by several stimulations such as sex hormones, endoplasmic reticulum stress, and hypoxia (6,18,19). AGR2 mRNA was significantly upregulated in response to estradiol treatment (20). Zhang et al. (21) have reported that AGR2 mRNA is androgen inducible, depending on dose and time. The literature has also shown that estrogens and androgens increase AGR2 expression in breast and prostate cancers, respectively (22,23). Presumably, the decreased AGR2 expression in invasive pituitary adenomas may be due to hypogonadism, which is more likely to be present; however, this opinion is hypothetical. It was also shown that AGR expression is regulated in glioblastoma, an intracranial malignant tumor, by hypoxia-induced factor-1 (HIF-1), which is related to the control of growth angiogenesis in the tumor (24). HIF-1 alpha expression is shown to be present in pituitary adenomas (25); thus, undiscovered mechanisms may determine the relationship between AGR2 expression and adenoma's aggressiveness through this pathway. Further studies investigating both HIF-1 alpha and AGR2 expression will clarify this issue.

In this study, AGR2 expression was also evaluated according to adenoma subtypes. Although the number of patients was low in this comparison, AGR2 expression was higher in corticotroph and somatotroph adenomas. However, AGR2 expression was

similar in the other adenomas compared to brain tissues. A similar result was observed in another study, and AGR2 expression was found more frequently in corticotroph and somatotroph adenomas (9). In the other study by the same researchers, the highest serum AGR2 level was observed in somatotrophic adenomas. Although the present study results are similar to those on AGR2 expression in somatotroph and corticotroph adenomas, more extensive studies are needed to clarify the exact mechanisms affecting AGR2 expression in these adenomas.

The study's major limitation is the low sample size; the study period was restricted to two years to prevent tissue samples' reliability. The number of pituitary adenoma surgeries was much higher during the study period, but some were lesions with mimicking adenoma or adenoma tissues that could not be obtained in a few microadenomas. In addition, some tissue samples were excluded due to technical reasons. However, despite the limited number of samples evaluated, finding a relationship between AGR2 expression and tumor aggressiveness will shed light on further studies. In particular, a comparison of hormone secretion patterns and AGR2 expression in a larger sample will help establish a treatment plan for hyperfunctioning adenomas. Another limitation was the failure to obtain normal pituitary tissues for comparison due to ethical reasons. Future studies with tissues from cadavers will reveal more definitive results. The strength of this study lies in the establishment of a negative correlation between adenoma aggressiveness and AGR2 expression, which was similar to that observed in the two previous studies (9,10). This would play a significant role in determining the prognosis and individualized treatment in pituitary adenomas.

In conclusion, AGR2 expression is inversely correlated to aggressiveness and invasiveness in pituitary adenomas. Moreover, AGR2 expression is associated with the adenoma subtype; AGR2 expression is increased, especially in somatotroph adenomas. However, more comprehensive and extensive studies are required to understand the exact mechanism or pathway of action of AGR2.

Compliance with ethical standards

Ethical Approval

All procedures in this study complied with the 1964 Helsinki Declaration and were approved by the Bakırköy Dr. Sadi Konuk Training and Research Hospital Ethics Committee.

Informed Consent

Informed consent was obtained from all participants included in the study.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: Sema Çiftçi Doğanşen, Ayla Solmaz Avcıkurt; Design: Sema Çiftçi Doğanşen, Ömür Günaldı; Control/Supervision: Sema Çiftçi Doğanşen, Meral Mert, Osman Tanrıverdi; Data Collection and/or Processing: Sema Çiftçi Doğanşen, Barış Çolluoğlu, İlhan Yılmaz; Analysis and/or Interpretation: Ayla Solmaz Avcıkurt, Sema Çiftçi Doğanşen; Literature Review: Sema Çiftçi Doğanşen; Writing the Article: Sema Çiftçi Doğanşen; Critical Review: Sema Çiftçi Doğanşen, Meral Mert; References and Fundings: Meral Mert, Ömür Günaldı; Materials: Ayla Solmaz Avcıkurt, Ömür Günaldı.

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