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Expression of voltage gated potassium channel ether à go-go in pituitary adenomas of patients with acromegaly: A preliminary study

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Abstract

OBJECTIVE: To determine immunohistochemical expression of Eag1 in pituitary adenomas of patients with acromegaly and to assess the correlation between Eag1 expression with cavernous sinus invasion, tumoral Ki-67 labeling index (LI), age and gender of the patients.

METHODS: The paraffin embedded pituitary adenoma tissue sections of 28 patients with acromegaly who were diagnosed as monohormonal growth hormone (GH) secreting adenomas were immunostained for Eag1 using the avidin-biotin-peroxidase complex method. Eag1 immunoreactivity was scored according to the extensity of the cytoplasm and cell membrane immunoreactivity for Eag1 (score 1 = <10%, score 2 = 10–25%, score 3 = 25–50% and score 4 = >50% of the adenoma cells showed immunoreactivity for Eag1, respectively).

RESULTS: Overall, GH secreting pituitary adenomas displayed diverse levels of Eag1 immunoreactivity, however, 64% of the adenomas displayed a strong Eag1 immunoreactivity (score 3 and 4). Five of the tumors displayed Eag1 immunoreactivity score 1, 5 displayed score 2, 10 displayed score 3 and 8 displayed score 4, respectively. No correlation was found between Eag1 immunoreactivity with cavernous sinus invasion, Ki-67 LI, age and gender of the patients.

CONCLUSIONS: Our results suggest Eag1 is strongly expressed in the majority of GH secreting pituitary adenomas. However, we could not find any correlation between immunoreactivity of Eag1 with cavernous sinus invasion, Ki-67 LI, age and gender of the patients. Further studies with larger sample sizes are required to demonstrate the role of Eag1 on tumorigenesis, angiogenesis, invasion and response to the treatment in GH secreting pituitary adenomas.

ORIGINAL ARTICLE

Abbreviations:
GH - growth hormone
LI - labeling index
SSA - somatostatin analogs
MRI - magnetic resonance imaging
VEGF - vascular endothelial growth factor
HIF - hypoxia inducible factor

INTRODUCTION

Acromegaly is rare chronic disorder caused by excess production of growth hormone (GH) generally by a pituitary adenoma (Melmed, 2006). Several causative mechanisms have been suggested for tumorigenesis in these patients including abnormalities in tumor suppressor genes, oncogenes, growth factors and cell cycle regulators (Melmed, 2006), however, none of these mechanisms can fully explain the pathways involved in pituitary cell transformation, proliferation and tumorigenesis, particularly in sporadic GH secreting adenomas.

Recent studies focusing on the role of ion channels in cellular transformation and proliferation during tumorigenesis demonstrated the prominent role of K^+ channels in tumorigenesis (Asher *et al.* 2010). K^+ channel activity is required for G1 progression of the cell cycle and early-stage of cell proliferation (Wang, 2004). Moreover, these channels also control the activity of the cell cycle-regulating proteins (Wang, 2004). Different types of K^+ channels have been identified in tumor cells from diverse origin and among them the voltage gated K^+ channel ether à go-go (also known as Eag1, Kv10.1 or KCNH1) has extensively been studied for its implication in tumorigenesis (Wang, 2004). In recent studies, Eag1, a critical determinant of cell membrane potential, has been directly linked to cellular transformation and proliferation of various types of tumors (Wang, 2004; Pardo *et al.* 1999;Pardo 2004). Eag1 expression is extremely limited in normal tissues except for the specific areas of the brain (Pardo *et al.* 1999), but its overexpression has been determined in many different tumor cells including colon, breast, prostate, lung, hepatocellular and gliomas (Hemmerlein *et al.* 2006; Patt, 2004). Therefore, Eag1 is also thought to be a novel target for the treatment of various types of tumors (Pardo & Stühmer, 2008).

While the vast majority of GH secreting pituitary adenomas are slow growing benign tumors, some of these tumors may invade surrounding tissues such as cavernous sinuses (Melmed 2006). The high expression of nuclear antigen Ki-67 was reported to be positively correlated with cavernous sinus invasion, cure after surgery and response to somatostatin analogs (SSA), in GH secreting pituitary adenomas (Fusco *et al.* 2008), therefore, nowadays, its expression is routinely evaluated in most of the centers during histopathological evaluation of surgically resected pituitary adenomas. However, the relationship of Ki-67 labeling index (LI) with Eag1 expression in pituitary tumors including GH secreting adenomas, has also remained undefined and except for a study performed by Del Pliego *et al.* which included a limited number of diverse types of pituitary adenomas (Del Pliego *et al.* 2013), Eag1 expression has not been studied in pituitary adenomas, particularly GH secreting adenomas. Therefore, the aim of this study was to determine immunohistochemical expression of Eag1 in pituitary adenomas of patients with acromegaly and to assess the correlation between Eag1 expression with cavernous sinus invasion on magnetic resonance imaging (MRI), tumoral Ki-67 LI, age and gender of the patients.

MATERIALS AND METHODS

Patients

In this study, the paraffin embedded pituitary adenoma tissue sections of 28 patients with acromegaly who were diagnosed as monohormonal GH secreting adenomas according to the histopathological and immunohistochemical evaluations (Lloyd *et al.* 2004) and were operated at our center between 2003 and 2011 were eligible for immunohistochemical analysis. Specimens with multihormonal immunostaining besides GH, including prolactin, ACTH, FSH, LH and TSH, were not included in the study. Clinical and pituitary imaging data were obtained from our outpatient clinic computer records and from chart review. The clinical, biochemical and pituitary MRI results of 20 patients were available in our outpatient clinic database. All of the surgical specimens of these patients were obtained through first surgery, prior to medical or radiation therapy. The remaining 8 patients were just operated at our center, their surgical specimens were evaluated at our pathology clinic and the follow-up and imaging data of these patients were not available.

Immunohistochemistry

Immunohistochemistry for Eag1 was performed using the avidin-biotin-peroxidase complex method. Deparaffinaization of 3–5 μm cut formalin-fixed paraffin embedded tissue sections was performed through a series of xylene baths and rehydration was performed with a series of graded alcohol solutions. Specimens underwent heat induced antigen retrieval for 20 min in 1 M citrate buffer (pH 6) solution in a microwave. After blocking endogenous peroxidase activity with H_2O_2, sections were incubated overnight in a humidified chamber at 4 °C with a rabbit polyclonal Eag1 antibody (KCNH1 antibody, Novus Biologicals, NBP1-84935, Littleton, CO, USA) at 1/300 dilution. After washing the tissue samplaes in TBS for 10 min, the slides were counterstained with Meyer hematoxylin (Thermo Fisher Scientific, Waltham, USA) and coverslipped. Eag1 immunoexpression was evaluated on a multi-head light microscope (Olympus BX 50-53) by two observers (H.O and C.T) who were blinded for clinical data and

was defined semi-quantitatively by visual impression. Eag1 immunoreactivity was scored according to the extensity of the cytoplasm and cell membrane immunoreactivity (score 1 = <10%, score 2 = 10–25%, score 3 = 25–50% and score 4 = > 50% of the adenoma cells showed immunoreactivity for Eag1, respectively) (Del Pliego *et al.* 2013). Scores 3 and 4 immunoreactivity for Eag1 was considered as strong immunoreactivity. Human cerebral cortex tissue was used as positive control and invasive ductal carcinoma of the breast with negative immunoreactivity for Eag1 served as negative control. The age and gender of the patients, Ki-67 LI of the tumors and cavernous sinus invasion on MRI were assessed for correlation with Eag1 immunoreactivity. The Ki-67 LI results of the 18 patients were obtained from computer record of our previous study (Zuhur *et al.* 2011). These patient's tumor specimens had been included in our previous study and had been assessed for Ki-67 LI by the same pathologists that participated in the current study (Zuhur *et al.* 2011). The Ki-67 LI results of the remaining 2 patients were obtained from our pathology department records and had been determined just by one of our pathologists (C.T).

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences for Windows, release 15.0.0 standard version; SPSS Inc, Chicago, Ill) software. Categorical data were evaluated using Spearman's correlation and Pearson's chi-square tests. A p-value <0.05 was considered statistically significant. An informed consent was obtained from all patients being followed-up in our center and the study was approved by the Sisli Etfal Training and Research Hospital's ethical board.

RESULTS

Twenty-eight patients with acromegaly [19 female, 9 male, ranging in age between 23–65 years old (mean age 44.1±11.2)] were included in this study, however, the follow-up data and information regarding treatments, imaging studies and Ki-67 LI were only available for 20 patients. According to MRI results performed before surgery, all of the tumors were macroadenomas (≥ 10 mm) and 9 (45%) of the tumors had invaded either right, left or both sides of the cavernous sinuses (Table 1). Overall, GH secreting adenomas displayed diverse levels of Eag1 immunoreactivity. However, 64% of the adenomas displayed a strong Eag1 immunoreactivity (score 3 and 4). In patients whose follow-up data were available, 4 of the tumors displayed Eag1 immunoreactivity score 1, 3 displayed score 2, 7 displayed

Tab. 1. Patient characteristics and immunohistochemistry results for Eag1 and Ki-67 LI.

Patient	Age	Gender	Tumor size	Cavernous sinus invasion	γ-knife RS	SSA	Pegvisomant	Ki-67 LI (%)	Eag1 immuno-reactivity score
1	30	f	MA	yes/R+L	yes	yes	yes	1	4+
2	38	f	MA	no	yes	yes	no	7	4+
3	44	m	MA	no	no	yes	no	1	1+
4	56	m	MA	yes/R	no	yes	no	8	2+
5	50	f	MA	yes/R	no	yes	yes	5	3+
6	41	f	MA	no	no	yes	no	8	3+
7	29	f	MA	yes/R+L	yes	yes	no	5	1+
8	46	f	MA	no	no	yes	no	1	3+
9	42	f	MA	yes/L	no	yes	no	3	2+
10	49	f	MA	yes/R	no	yes	no	5	1+
11	23	m	MA	no	no	yes	no	11	3+
12	65	m	MA	no	no	no	no	1	3+
13	36	f	MA	yes/L	no	yes	no	7	4+
14	31	m	MA	no	no	yes	no	1	1+
15	56	f	MA	no	no	no	no	1	3+
16	34	f	MA	yes/L	no	yes	no	3	4+
17	45	f	MA	yes/L	no	yes	no	3	2+
18	65	f	MA	no	no	yes	no	1	4+
19	27	f	MA	yes/R+L	no	yes	yes	5	3+
20	48	f	MA	no	no	no	no	1	4+

Abbreviations: f: female, m: male, LI: labelingindex, MA: macroadenoma,RS : radiosurgery, R: Right side, L: leftside
Tumor size andcavernous sinus invasion were determined by magnetic resonance imaging

score 3 and 6 displayed score 4, respectively (Figure 1). No relationship was found between Eag1 immunoreactivity with Ki-67 LI and cavernous sinus invasion (p=0.48 and p=0.23). In correlation analysis, no correlation was also found between Eag1 immunoreactivity with age, gender, cavernous sinus invasion and Ki-67 LI (rho=–0.002, p=0.99; rho=–0.02, p=0.99; rho=–0.13, p=0.56 and rho=0.12, p=0.61, respectively). In this study, a significant association was found between adenomas with ki-67 LI ≥3% and cavernous sinus invasion (p=0.020). In correlation analysis, a significant correlation was also found between adenomas with Ki-67 LI ≥3% and cavernous sinus invasion (rho=0.61, p=0.004).

In 8 patients whose follow-up data were not available, all of the tumors were also macroadenomas and 2 tumors displayed Eag1 immunoreactivity score 4, 3 displayed score 3, 2 displayed score 2, and 1 displayed score 1, respectively). Patient characteristics, Eag1 immunoreactivity scores and tumoral Ki-67 LI of 20 patients with available follow-up data are presented in Table 1.

DISCUSSION

GH secreting pituitary adenomas develop from monoclonal expansion of somatotroph cells (Melmed, 2006). However, the mechanisms underlying tumorigenesis in these tumors are not fully understood. As in other tumors of epithelial origin, tumorigenesis in GH secreting pituitary adenomas also evolve through a multistep process of genetic and biomolecular changes (Melmed, 2006). The identification of biomolecular pathways involved in tumoral transformation, progression and invasion of these tumors could provide ground for development of new diagnostic and therapeutic methods.

Recent studies support the involvement of Eag1 in tumoral transformation, differentiation, proliferation and invasion of diverse types of tumors. Although the exact role of K^+ channels in tumorigenesis has not been fully understood, it was suggested that the overexpression of K^+ channels on cell membrane increases the influx of Ca^{2+} into the cells and results in increased transition of cells through G1/S phase of cell cycle. Moreover, K^+ channels also act through activation of mitogen activated protein kinase (MAPK) and Ca^{2+} calmodulin pathways to activate cell proliferation (Asher *et al.* 2010). In addition, Eag channel expression was shown to be associated with re-organisation of the cytoskeleton and extracellular matrix, thereby influencing adhesion, proliferation and metastasis of the tumors (Toral *et al.* 2007). Evidence also suggests the involvement of K^+ channels in angiogenesis of the tumors. In a study of Downie *et al.* Eag1 expressing tumors exhibited increased angiogenesis and promoted vascular endothelial growth factor (VEGF) in vitro by increased hypoxia inducible factor-1 (HIF-1) activity (Downie *et al.* 2008). The authors suggested that Eag1 contributes to tumorigenesis independent of its canonical action as an ion channel (Downie *et al.* 2008).

In normal adult anterior pituitary tissues, Eag1 expression, observed by RT-PCR and immunohistochemistry is very low (Hemmerlein *et al.* 2006). Del Pliego *et al.* analyzed Eag1 immunoexpression in normal anterior pituitary tissues and in a few invasive and non-invasive pituitary adenomas of diverse origin (Del Pliego *et al.* 2013). In their study, normal pituitary tissue showed Eag1 expression only in a few cells. However, various degrees of Eag1 were expressed in all the tumors analyzed. On the other hand, they could not find a correlation between Eag1 expression and specific

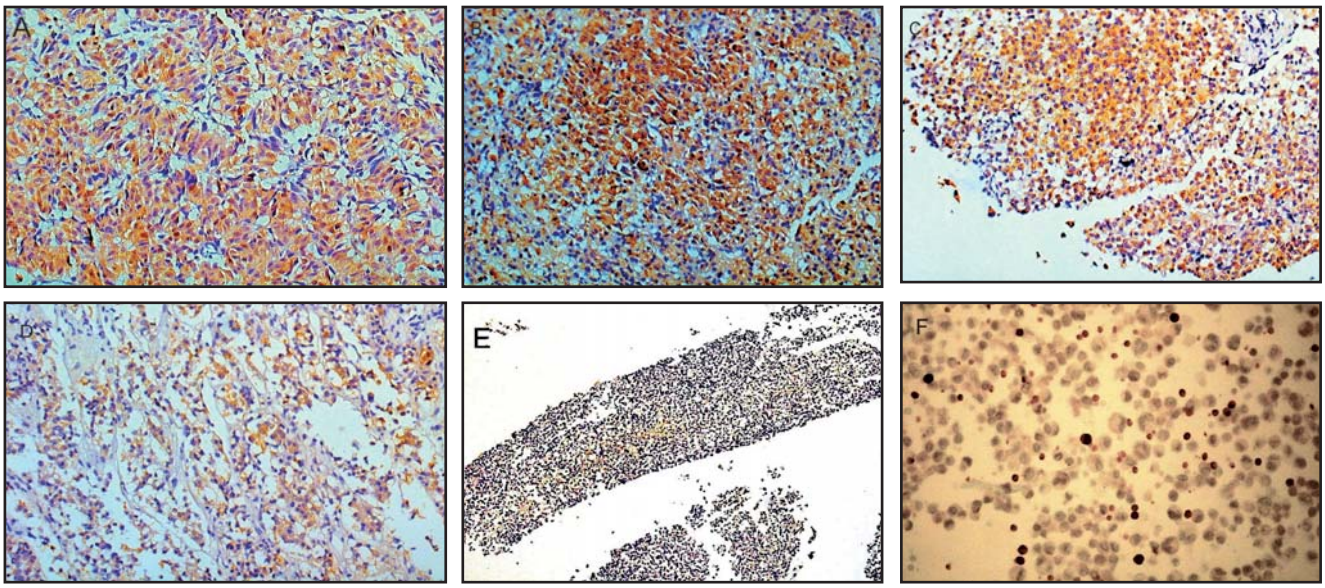

Fig. 1. Representative images of Eag1 immunoreactivity in GH secreting pituitary adenomas. **A:** score 4, **B:** Score 3, **C:** Score 2, **D:** score 1 and **E:** score 1 Eag1 immunoreactivity. **F:** Ki-67 LI (11%) of the case displayed in Figure 1B (case 11 in Table 1). (A and F ×400; B, C, D ×200, and E ×40).

characteristics of the pituitary adenomas. Our study results are in line with what reported by Del pliego *et al.* and suggest a strong expression of Eag1 in the majority of GH secreting pituitary adenomas. As indicated in other studies performed in different human tumor tissues, our study also indicate that Eag1 channels may play a role in tumorigenesis of GH secreting pituitary adenomas.

The identification of Ki-67 LI, a nuclear antigen associated with growth potential of the tumors, correlates best with invasiveness and prognosis in pituitary adenomas (Fusco *et al.* 2008; Zuhur *et al.* 2011) and is a prognostic indicator of pituitary adenomas in the recent WHO classification of pituitary tumors where pituitary adenomas with Ki-67 LI more than 3% are classified as atypical adenomas (Lloyd *et al.* 2004). In concordance with previous reports (Fusco *et al.* 2008; Chacko *et al.* 2010) we also found a significant association between Ki-67 LI and cavernous sinus invasion. As presented in Table 1, most of the tumors with cavernous sinus invasion on MRI displayed Ki-67 LI ≥3%. However, no correlation was found between Eag1 immunoreactivity either with Ki-67 LI or cavernous sinus invasion on MRI. As presented in Table 1, 3 patients had been cured at first surgery, according to the criteria used for the cure of acromegaly (Giustina *et al.* 2010). Ki-67 LI was < 3% in all these 3 tumors, however, Eag1 was strongly expressed in all these 3 tumors. On the other hand, the biochemical control was not achieved in 3 patients with surgery and SSA alone. Therefore, pegvisomant was added to their treatment regimens. All these 3 patients showed cavernous sinus invasion on MRI, Ki-67 LI was <3% in 1 and >3% in 2 of their tumors. However, Eag1 was also strongly expressed in all these 3 tumors as well (Cases 1, 5 and 19 in Table 1). Although the number of patients who were cured at first surgery and those who did not achieve biochemical control by surgery and medical therapy were limited for comparison, results of the present study indicate that Eag1 channels may not play a role in response to the treatment of GH secreting pituitary adenomas.

Controversial results were reported in studies evaluating the association between Eag1 expression with invasiveness and metastasis in solid and hematologic malignancies. Eag1 expression was correlated with tumor size, lymph node metastasis and shorter overall survival in patients with head and neck, colorectal and acute myeloid leukemia (AML) patients, respectively (Menéndez *et al.* 2010; Ding *et al.* 2007; Agarwal *et al.* 2010). Eag1 expression was also associated with poor prognosis in patients with AML, colon and ovarian cancers (Ding *et al.* 2007; Agarwal *et al.* 2010; Asher *et al.* 2010). Moreover, the transfection of Eag1 in tumor tissues induced a high proliferation rate and an epithelial-mesenchymal transition, which are features of aggressive phenotype (Pardo *et al.* 1999; Hemmerlein *et al.* 2006). However, in a recent study conducted by Lai *et al.* negative correlation was found between expression of Eag1 with tumor size and lymph node status in patients with breast carcinoma. (Lai *et al.* 2010). Although the majority of studies evaluating the role of Eag1 in tumorigenesis and invasion of diverse types of tumors were performed in malignant tumors, most of the GH secreting pituitary adenomas are benign tumors and only a few malignant cases were reported up to date (Hirohata *et al.* 2014). However, in the present study, we also could not find an association between Ki-67 LI, an indicator of proliferative activity in pituitary adenomas (Fusco *et al.* 2008), and cavernous sinus invasion on MRI with Eag1 immunoreactivity. So, further studies are required to determine the relationship between Eag1 channels and invasiveness of the pituitary adenomas, including GH secreting adenomas.

The management of acromegaly is complex and includes many treatment options. Generally, surgery is the first treatment option for patients with acromegaly, medical therapy including SSA and pegvisomant and radiotherapy are the treatment options in patients whose disease is not controlled by surgery alone. However, disease control is difficult in some patients due to the irresponsiveness of the tumor to the currently available drugs (Chinezu *et al.* 2014). Therefore, the development of new drugs is crucial. Eag1 has been suggested as a novel target for therapeutic intervention (Gómez-Varela *et al.* 2007; Pardo & Stühmer 2008). A few number of non-selective K^+ channel inhibitors such as imipramine and an antihistamine astemizol that inhibit Eag1 channels, were shown to decrease cell proliferation in tumor cells (García-Ferreiro *et al.* 2004; Gavrilova-Ruch *et al.* 2002). On the other hand, because they inhibit other K^+ channels of the superfamily as well and may cause serious cardiac dysrhythmias (Pardo & Stühmer 2008), researchers have focused on design of specific anti Eag1 antibodies. Gomez-Valera *et al.* designed a monoclonal antibody and showed that it can selectively inhibit ion flow through Eag1 channels in intact cells with no cardiac side effects (Gomez-Valera *et al.* 2007). Recently, tumor cell-selective apoptosis induction through targeting of Eag1 by a bifunctional TRAIL antibody was demonstrated in prostate cancer cells (Hartung *et al.* 2011). Although to date, neither non-selective nor selective K^+ channel inhibitors have been tested for the treatment of any human tumor, the design of aforementioned antibodies against Eag1 shows promise for the future use in the treatment of diverse types of tumors.

The main limitation of the present study was the relatively small number of patients with available follow-up data. Therefore, further studies with large sample sizes should be carried out to assess the relationship between Eag1 expression and invasiveness, recurrence and response to the treatment in GH secreting pituitary adenoms.

In conclusion, a strong immunoreactivity for Eag1 was demonstrated for the first time, in the majority of GH secreting pituitary adenomas. Our results indicate

that Eag1 channels may play a role in tumorigenesis of these tumors, however, we could not find any correlation between Eag1 immunoexpression with cavernous sinus invasion, tumoral Ki-67 LI, age and gender of the patients. Functional studies with larger sample sizes should be carried out to demonstrate the role of Eag1 channels on tumorigenesis, angiogenesis, invasion, recurrence after surgery and response to the treatment in GH secreting pituitary adenomas.

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