Expression of AGR2 in pituitary adenomas and its association with tumor aggressiveness

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Abstract. The aim of the present study was to study the expression of anterior gradient 2 (AGR2) to determine its clinical significance in pituitary adenoma (PA), and to evaluate the potential effect of AGR2 on the diagnosis, therapy or prognosis of PA. Immunohistochemistry was performed to detect the expression of AGR2 in 117 PA tissue samples. Western blotting was performed to confirm the expression profile of AGR2 in different subtypes of PAs. The data showed that in 117 different histological subtypes of PA, 51.3% exhibited AGR2-positive expression. Although AGR2 expression occurred more frequently in PAs secreting growth hormone (GH; 62.5%), adrenocorticotropic hormone (ACTH; 80.0%) and follicle-stimulating hormone (FSH; 75.0%),the positive expression rate of AGR2 showed no statistically significant differences between PA subtypes (P>0.05). The association between AGR2 expression and clinical parameters was analyzed using a χ^2 test, or Fisher's exact probability test when appropriate. The result showed that the aggressiveness of PA was significantly associated with AGR2 expression, and that in the majority of aggressive PAs, AGR2 was negative (P<0.001). In conclusion, the current study detected the expression of AGR2 in different subtypes of PAs for the first time. The data indicated that GH-, ACTH- and FSH-secreting PAs present marginally more frequently with AGR2 expression, howeverm the association was not significant (P>0.05). AGR2 may be a target for the study of PA aggressiveness, and a potential index for the diagnosis or prognosis of PAs.

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Introduction

Pituitary adenomas (PAs) are clinically relevant endocrine tumors that account for ~10% of all intracranial neoplasms. The majority of PAs are benign, however, 30-55% are locally invasive, and a number infiltrate the dura, bone and sinuses, and are thus considered to be highly aggressive (1,2). The surgical treatment of aggressive PAs is often incomplete, leading to the high recurrence rate (3). A potential treatment strategy for aggressive PAs is the targeting of tumor invasion and metastasis-associated genes.

In humans, anterior gradient 2 (AGR2) encodes the human homologue of a secreted protein that was first identified in *Xenopus laevis* (4). AGR2 has been reported to be overexpressed in several adenocarcinomas, including breast (5), colorectal (6), esophageal (7), lung (8), pancreatic (9) and prostate (10,11) carcinomas. ARG2 is considered to promote cell proliferation, cell survival and the metastasis of cancer cells. Salmans *et al* demonstrated that AGR2 is a marker of breast cancer metastasis and that its overexpression in estrogen receptor-positive breast cancer is associated with a poor prognosis, particularly in tumors that evade anti-hormone therapies (5). This indicates that AGR2 may participate in the process of cell metastasis in hormone-associated tumors. Since PAs are associated with multiple hormones in humans and as no studies have mentioned the role of AGR2 in PA, the present study investigated the expression profile of AGR2 in 117 PAs of different histological subtype by immunohistochemistry and western blotting.

Materials and methods

PA sample collection. A total of 117 PAs of different histological subtype were randomly selected from patients aged 17-69 year-old who underwent endoscopic or microscopic total resection between May 2013 and June 2014 in the Department of Neurosurgery, Jinling Hospital (School of Medicine, Nanjing University, Nanjing, Jiangsu, China). The study was approved by the ethics committee of Jinling Hospital and written informed consent was obtained from all patients. All resected PA tumor tissues were formalin-fixed and paraffin-embedded, and then pathologically diagnosed. The tissues consisted of 24 prolactin-secreting adenomas, 24 growth hormone (GH)-secreting adenomas, 5 adrenocorticotropic hormone

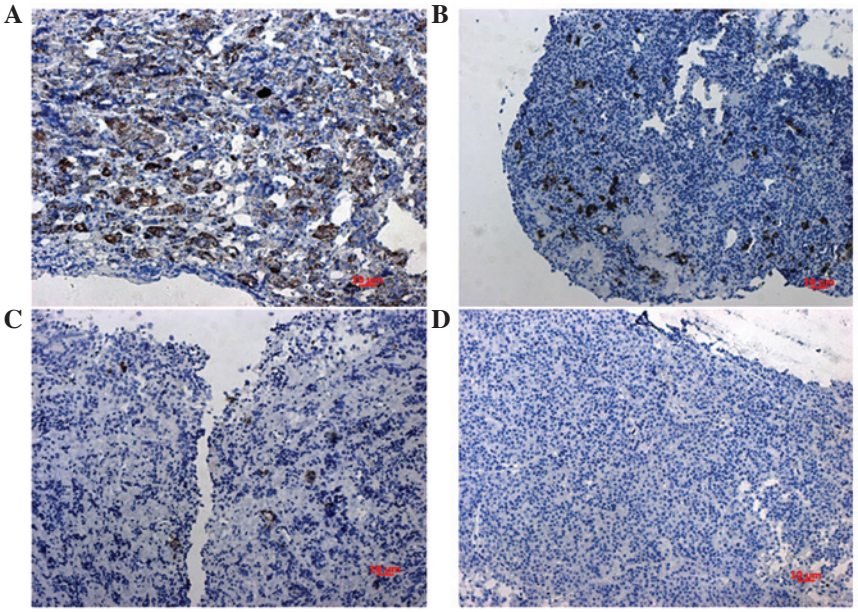

Figure 1. Expression of AGR2 in pituitary adenomas. (A) High expression of AGR2 (+++). (B) Intermediate expression of AGR2 (++). (C) Low expression of AGR2 (+). (D) AGR2-negative. Bar, 10 μm. AGR2, anterior gradient 2.

(ACTH)-secreting adenomas, 8 follicle-stimulating hormone (FSH)-secreting adenomas and 56 non-functioning adenomas.

Immunohistochemical staining. A streptavidin-peroxidase method was used for immunostaining, as previously described (12). Briefly, slides were deparaffinized with xylene three times (for 5-10 min each), dehydrated three times in a gradient series of ethanol (100, 95 and 75%), and rinsed with phosphate-buffered saline (PBS). Each slide was treated with 3% H_2O_2 for 15 min to quench endogenous peroxidase activity. Non-specific binding was blocked by treating the slides with normal goat serum for 20 min. The slides were first incubated with rabbit anti-human monoclonal anti-AGR2 (#13062; 1:500; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C, and then rinsed twice with PBS. The slides were then incubated with secondary antibody (goat anti-rabbit horseradish peroxidase-conjugated IgG; #BS13278; 1:5,000; Beyotime Institute of Biotechnology, Shanghai, China) for 15 min at 37˚C, followed by treatment with streptavidin-peroxidase reagent for 15 min, and were rinsed twice with PBS. The slides were visualized with 3,3'-diaminobenzidine for 3 min, counterstained with hematoxylin and mounted for microscopy.

Evaluation of staining. The slides were independently evaluated by two investigators under a light microscope (TE200; Nikon, Tokyo, Japan). As described previously (12), the staining intensity was scored as follows: 0, negative; 1, weak; 2, medium; and 3, strong. The extent of staining was scored as follows: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%, according to the percentages of the positive staining area in relation to the whole carcinoma area. The sum of the intensity and extent scores was used as the final staining score (range, 0-7). Tumors with a final staining score of >2 were considered to be positive.

Western blotting. For western blot analysis, the lysates were separated by SDS-PAGE followed by being transferred to an Immobilon-P Transfer membrane (Millipore Corporation, Bedford, MA, USA). The membranes were probed with the anti-AGR2 and rabbit anti-mouse polyclonal GAPDH (#AP0063; 1:5,000; Bioworld Technology, Inc., St. Louis Park, MN, USA) primary antibodies, followed by incubation with secondary antibody. Proteins were visualized with chemiluminescence luminol reagents (Beyotime Institute of Biotechnology, Shanghai, China).

Statistical analysis. Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The positive expression rate of AGR2 in the different subtypes of PA was compared using χ^2 tests. The association between the expression and clinical parameters was analyzed using a χ^2 test, or Fisher's exact probability test when appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of AGR2 in PA tissues. The location of AGR2 in the nuclei was considered for scoring (Fig. 1A-D). The positive expression of AGR2 was detected in 60 tissues. The proportions of negative (score of ≤2) or positive (score of >2) expression in the different subtypes of PAs are shown in Table I. In total, 51.3% of all PAs exhibited AGR2 positive expression. The positive expression rate of AGR2 showed no significant differences in the PA subtypes ($\chi^2=6.537$; $P=0.162$, $P<0.05$), thus, the expression of AGR2 cannot be considered as discrepant in the different subtypes of PAs. The western blotting data supported and confirmed the immunohistochemistry results, including high and negative expression of AGR2 in the different subtypes of PA (Fig. 2A).

Association of AGR2 expression with clinical features of PAs. In the 117 cases, 65 patients were male and 52 were female, with 60 patients >50 years old. The tumors were defined as

Table I. Expression profile of AGR2 in different subtypes of PA.

PA subtypes	No. of patients	AGR2		
		Negative, n	Positive, n	Positive rate, %
PRL	24	14	10	41.7
GH	24	9	15	62.5
ACTH	5	1	4	80.0
FSH	8	2	6	75.0
NF	56	31	25	44.6
Total	117	57	60	51.3

AGR2, anterior gradient 2; PA, pituitary adenoma; PRL, prolactin-secreting; GH, growth hormone-secreting; ACTH, adenocorticotropic hormone-secreting; FSH, follicle-stimulating hormone-secreting; NF, non-functioning.

Table II. Association of AGR2 expression with clinicopathological characteristics from patients with pituitary adenomas.

Parameters	No. of patients	AGR2, n		P-value
		-	+	
Cases	117	57	60	
Gender				0.901
Male	65	32	33	
Female	52	25	27	
Age, years				0.323
≤50	67	30	37	
>50	60	27	23	
Aggressive				<0.001
Yes	70	46	24	
No	47	11	36	
Recurrence				0.482
Yes	12	7	5	
No	105	50	55	
Tumor size, mm				0.170
≤10	13	4	9	
>10	104	53	51	

AGR2, anterior gradient 2.

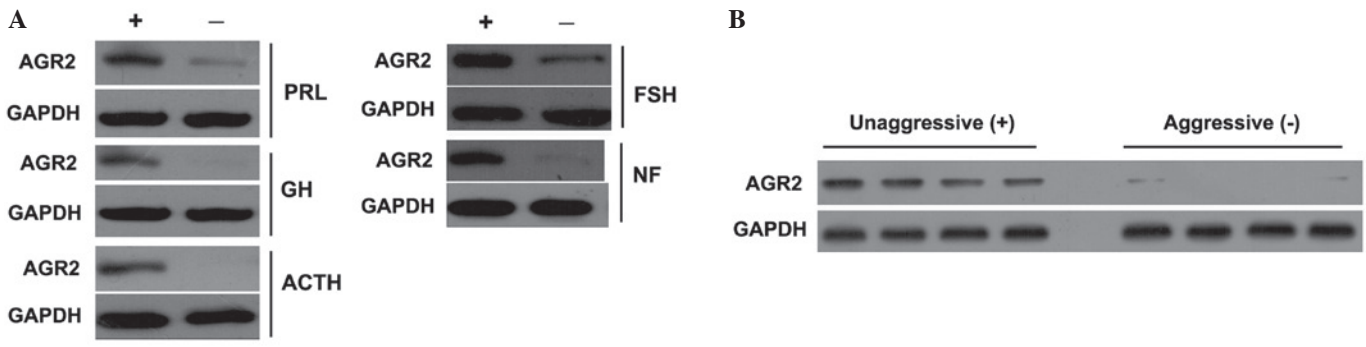

Figure 2. (A) The expression of AGR2 in different PA subtypes, as detected by western blotting. (B) The expression of AGR2 in aggressive or unaggressive PAs. GAPDH served as a loading control. AGR2, anterior gradient 2; PRL, prolactin-secreting PA; GH, growth hormone-secreting PA; ACTH, adrenocorticotropic hormone-secreting PA; FSH, follicle-stimulating hormone-secreting PA; NF, non-functioning PA.

follows: 70 aggressive and 47 non-aggressive PAs (according to Knosp's classification) (13); 12 recurrent and 105 primary PAs; and 13 microadenoma (diameter, ≤10 mm) and 104 macroadenoma (diameter, >10 mm). The associations between clinical variables and AGR2 expression are showed in Table II. Only the aggressiveness of PA was found to be associated with AGR2 expression (P=0.0003, P<0.001). The majority of aggressive PAs were negative for AGR2 expression. The western blotting data also supported this result (Fig. 2B).

Discussion

AGR2 is well studied in malignant tumors, and to the best of our knowledge, it has never been mentioned with regard to PAs. In the present study, the expression profile was detected in 117 PA tissues of different histological subtypes for the first time. Aberrant AGR2 expression has been reported in primary breast, lung and prostate carcinomas (14-17). AGR2 overexpression in breast epithelial cell lines results in the development of metastases in an animal model (18). However, overexpression is not the only manner in which AGR2 contributes to tumor development (19). The loss of AGR2 expression has also been demonstrated to be associated with the dysplasia-to-carcinoma sequence in colonic polyps (20). The present study data demonstrated that in 117 different histological subtypes of PA, 51.3% exhibited AGR2-positive expression. The positive expression rate of AGR2 showed no significant differences in the PA subtypes, although its expression occurred more frequently in PAs secreting GH (62.5%), ACTH (80.0%) and FSH (75.0%). Next, the association between AGR2 expression and clinical parameters was analyzed. Notably, the result showed that the aggressiveness of PA was associated with AGR2 expression, and that in the majority of aggressive PAs, AGR2 expression was negative. This suggested that AGR2-negative expression may be an indication for PA aggressiveness.

The AGR2 protein contains a canonical cleavable N-terminal signal peptide that targets it to the secretory pathway (21). The secreted proteins metastasis-associated GPI-anchored C4.4A protein and the extracellular domain of α-dystroglycan, have been reported to directly interact with AGR2, indicating potential mechanisms for AGR2 in the promotion of tumor metastasis via the regulation of receptor adhesion and the interaction with the extracellular matrix (17,22). Thus, AGR2 interacts with the cell surface in order to modulate adhesion and promote tumor cell dissemination. However, by contrast, Riener *et al* (23) demonstrated that the loss of AGR2 expression occurred in colorectal cancer cell lines and tissue samples, which was significantly associated with a higher tumor grade and metastasis. Thus, the study suggested that AGR2 was an independent prognostic factor in primary colorectal carcinoma. These results may partly support our suggestion that AGR2-negative expression may be an indication for PA aggressiveness. Numerous PAs present with aggressive characteristics, although PA is considered as a benign tumor. Aggressive PAs are usually hard to totally resect and show a tendency to recur, even after initially successful treatment (24). Post-operative radiotherapy is always recommended to treat residual tumors and to prevent recurrence (25). Identifying the aggressiveness of pituitary tumors is important for the selection of appropriate treatment and prognostic evaluation (26). The present study results showing that AGR2 is negative in the majority of aggressive PAs indicated that the loss of AGR2 expression may also associate with aggressiveness, similar to the dysplasia-to-carcinoma sequence in colonic polyps (20). To clarify the role of AGR2 in PA aggressiveness or maybe canceration, further cellular *in vitro* and animal *in vivo* experiments are necessary.

In conclusion, in the present study, 117 cases of PA of different subtypes were detected. PAs secreting GH, ACTH and FSH were found to present with more frequent AGR2 expression; however, this association was not statistically significant. The aggressiveness of PA is associated with AGR2 expression, and in the majority of aggressive PAs, AGR2 expression was negative. AGR2 may be a target for the study of PAs aggressiveness, and a potential index for the diagnosis or prognosis of PAs.

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