A Novel Invasive-Related Biomarker in Three Subtypes of Nonfunctioning Pituitary Adenomas

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■ OBJECTIVE: **To identify biomarkers key to invasiveness of the 3 subtypes of nonfunctioning pituitary adenomas (NFPAs) and provide a guidance for therapeutic decision making and identification of potential adjuvant drugs.**

■ METHODS: **Fifty NFPA tumor tissues obtained from transsphenoidal surgery were used in the study. Three invasive NFPAs and 4 noninvasive NFPAs were used for gene expression microarray analyses. In addition, there are 5 invasive NFPAs and 4 noninvasive NFPAs used for proteomic analyses. Invasive-related biomarkers were identified by bioinformatics analysis by integrating the transcriptomics and proteomics data sets. All 3 subtypes of NFPAs (null cell adenomas, oncocytomas, and gonadotroph adenomas) were used to validate differentially expressed candidate biomarkers by means of quantitative real-time reverse transcription polymerase chain reaction and Western blot. The level of EZR was downregulated in pituitary adenoma cell line GH3 to investigate the invasive effect of EZR on GH3 cells by using the RNA interference technique.**

■ RESULTS: **Eight genes involved in the invasion function were found by bioinformatics analysis, and the EZR gene was identified as a novel invasive-related biomarker in the 3 subtypes of NFPAs. The expression level of EZR was found higher in terms of invasiveness than the noninvasive ones of the 3 subtypes of NFPAs. Moreover, the knockdown of EZR inhibited the invasion of GH3 cells in vitro.**

■ CONCLUSIONS: **EZR is a novel biomarker in terms of invasion among the 3 subtypes of NFPAs, and it is a promising guide for therapeutic decision making as well.**

INTRODUCTION

Pituitary adenomas account for 10%–25% of all intracranial neoplasms,[1] and about one third of them are nonfunctioning pituitary adenomas (NFPAs) in clinical practice. Patients with NFPAs generally experience anterior pituitary hormonal deficits, visual loss, headaches, or less frequently, apoplexy, and so on. Pathologically, NFPAs are mostly classified as subtypes that include null cell adenomas, oncocytomas, gonadotrophic cell adenomas, silent corticotroph adenomas, and silent adenomas[2]; null cell adenomas, oncocytomas, and gonadotrophic cell adenomas make up 84.16% of NFPAs.[2,3] Although NFPAs appear benign histologically, most show characteristics of local invasion and spread into surrounding structures. Surgery is the priority treatment for this disease, and medication plays a minimal role. However, in the case of invasive local growth, surgical excision can be precluded, with significant neurologic morbidity. This situation presents a challenge in treatment of patients with invasive NFPAs, and invasion of NFPAs is an important prognostic factor for surgical outcome and recurrence.[4]

To understand the alteration of molecules and pathways deeply underlying the invasion, optimal therapies are investigated. Recent advances in molecular pathology have enabled a deeper

Key words
- Biomarkers of invasiveness
- Ezrin
- Gonadotroph adenomas
- Nonfunctioning pituitary adenomas
- Null cell adenomas
- Oncocytomas

Abbreviations and Acronyms
EZR: Ezrin
FDR: False discovery rate
IPA: Ingenuity Pathway Analysis
LC-MS/MS: Liquid chromatography tandem mass spectrometry
mRNA: messenger RNA
NFPA: Nonfunctioning pituitary adenoma
qRT-PCR: Quantitative real-time reverse transcription polymerase chain reaction

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Table 1. Clinical Characteristics of Patients with Nonfunctioning Pituitary Adenomas

	Null Cell Adenomas			Oncocytomas			Gonadotroph Adenomas		
Clinical Data	**Noninvasive**	**Invasive**	***P* Value**	**Noninvasive**	**Invasive**	***P* Value**	**Noninvasive**	**Invasive**	***P* Value**
Age (years)	44.40 ± 6.54	48.56 ± 12.24	0.499	52.00 ± 9.67	56.89 ± 4.70	0.22	43.80 ± 0.03	48.00 ± 4.70	0.495
Gender (male/female)	3/2	5/4	0.872	10/4	5/1	0.573	3/5	1/7	0.248
Headache	2 (40.0)	4 (44.4)	0.872	5 (35.7)	3 (50.0)	0.550	3 (37.5)	4 (50.0)	0.614
Visual deficits	3 (60.0)	6 (66.7)	0.803	8 (57.1)	4 (66.7)	0.690	4 (50.0)	5 (62.5)	0.614
Visual field defects	2 (40.0)	4 (44.4)	0.872	5 (35.7)	3 (50.0)	0.550	3 (37.5)	4 (50.0)	0.614
Cystic change	1 (20.0)	3 (33.3)	0.597	3 (21.4)	2 (33.3)	0.573	2 (25.0)	3 (37.5)	0.590
Recurrence	1 (20.0)	3 (33.3)	0.597	2 (14.3)	2 (33.3)	0.329	1 (12.5)	2 (25.0)	0.522

Values are number (%) except where indicated otherwise.

insight into numerous genetic alterations underlying the development of NFPAs. For example, biomarkers are involved in the invasion of pituitary adenomas, including proliferation markers, growth factors, and their receptors, as well as factors related to angiogenesis or cell adhesion.[5] However, the genetic factors associated with invasiveness of the 3 subtypes of NFPAs (null cell adenomas, oncocytomas, and gonadotroph adenomas) remain unclear and few biomarkers have been identified with invasion of specific subtypes of NFPAs.[6] This study focused on gene expression microarrays and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, so that biomarkers that play a key role in the invasive behavior of specific subtypes of NFPAs are identified, to guide therapeutic decision making and identify promising adjuvant drugs. Furthermore, differentially expressed candidate biomarkers were validated by means of quantitative real-time polymerase chain reaction (qRT-PCR), Western blot, and RNA interference techniques in vitro.

METHODS

Patients

Fifty NFPA tumor tissues were obtained from patients undergoing transsphenoidal surgery at Beijing Tiantan Hospital. Those fresh samples were frozen at −80°C in isopentane and stored in liquid nitrogen. Clinical and pathologic data are shown in Table 1, including the age, gender, clinical symptoms, pathologic findings, preoperative imaging, and postoperative studies. These NFPAs were divided into 3 groups: 14 null cell adenomas, 20 oncocytomas, and 16 gonadotroph adenomas. Three invasive and 4 noninvasive NFPAs were used for gene expression microarray analyses and 5 invasive and 4 noninvasive NFPAs were used for proteomic analyses as well. All 50 NFPAs were used to validate differentially expressed candidate biomarkers.

The NFPAs classified as types based on ultrastructural and immunohistochemical characteristics are highly heterogeneous. Three subtypes of NFPA (null cell adenomas, oncocytomas and gonadotroph adenomas) were selected for further research. Tumor invasiveness was defined by 3 aspects: preoperative magnetic resonance imaging, intraoperative records, and pathology slides. The tumors were considered as invasive NFPAs if they were consistent with 1 of the following criteria: 1) grade 4 on the Knosp grading system; 2) grade 2/3 on the Knosp grading system combined with any type of extrasellar extension (eroding bone tissue of sellar floor/clivus or invading into the unilateral/bilateral cavernous sinus) on intraoperative records and/or with dural invasion on the pathology slides.[7] In this study, there were 9 invasive and 5 noninvasive null cell adenomas, 6 invasive and 14 noninvasive oncocytomas, and 8 invasive and 8 noninvasive gonadotroph adenomas.

This study was approved by the research ethics committee of Tiantan Hospital (KY2013-015-02). Informed consent was obtained from all enrolled participants, and the study is fully compliant with all provisions in the Helsinki Declaration.

Total RNA Extraction and Microarray Hybridization

Total RNA was extracted and purified using the mir-Vana miRNA Isolation Kit (catalog no. 1561 [Ambion, Austin, Texas, USA]) following the manufacturer's instructions and analyzed with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). Samples in use were limited to those with no degradation (2100 RIN ≥7.0 and 28S/18S ≥0.7), which were used to generate labeled targets. Total RNA was amplified and labeled using the Low Input Quick Amp Labeling Kit and One-Color (catalog no. 5190-2305 [Agilent Technologies]). Labeled complementary RNA was purified with the RNeasy Mini Kit (catalog no. 74106 [Qiagen, Hilden, Germany]). Each slide was hybridized with 1.65 μg of Cy3-labeled complementary RNA using the Gene Expression Hybridization Kit (catalog no. 5188-5242 [Agilent Technologies]) in a hybridization oven (catalog no. G2545A [Agilent Technologies]). After 17 hours of hybridization, the slides were washed in staining dishes (catalog no. 121 [Thermo Shandon, Waltham, Massachusetts, USA]) using the Gene Expression Wash Buffer Kit (catalog no. 5188-5327 [Agilent Technologies]). The slides were scanned with an Agilent Microarray Scanner (catalog no. G2565CA [Agilent Technologies]) using the default settings (dye channel, green; scan resolution, 5 μm; photomultiplier tube, 100%; 10%; 16 bit). Data were extracted with the Feature Extraction software 10.7 (Agilent Technologies). Raw data