Acta Medica et Biologica
Vol. 51, No.1, 25-30, 2003

In situ Hybridization Analysis of Neuronatin mRNA in the Human Pituitary Gland and Pituitary Tumors

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Received December 12 2002, accepted January 24 2003

Summary. **Object: We have recently cloned cDNA of human neuronatin. Northern blot and RT-PCR analyses revealed that mRNA are expressed in the fetal brain, adult anterior pituitary gland, and pituitary adenomas, but they are hardly detected in the adult brain and brain tumors. To clarify the role of this gene, we investigated the cellular localization of neuronatin mRNA using *in situ* hybridization (ISH). Methods: ISH and immunohistochemistry (IHC) were performed on serial frozen sections of three normal pituitary glands and tumors (10 GH-omas, 5 PRL-omas, 2 ACTH-omas, 10 nonfunctioning adenomas, 3 craniopharyngiomas, and 1 Rathke's cleft cyst). In ISH, a radiolabelled oligonucleotide probe for neuronatin was used. In IHC, antibodies to GH, PRL, FSH-beta, LH-beta, TSH-beta, and ACTH were used. Results: 1) Neuronatin mRNA was detected in the anterior pituitary gland, but not in the posterior gland; 2) Neuronatin expression was also observed in various pituitary adenomas and epithelial cells of Rathke's cleft cyst, but hardly detected in craniopharyngiomas; 3) In pituitary adenomas, neuronatin expression was seen irrespective of hormone productivity of each cell; Discussion: 1) Neuronatin is thought to participate in the development, differentiation, or maintenance of the pituitary gland and pituitary adenomas, though it is not involved in hormone productivity; 2) Information about the distribution of neuronatin may facilitate an understanding of the origin of pituitary tumors, including craniopharyngiomas and Rathke's cleft cysts.**

Key words—neuronatin, *in situ* hybridization, immunohistochemistry, pituitary tumors.

INTRODUCTION

Neuronatin cDNA was first isolated from the neonatal rat brain[1]. It is selectively expressed during fetal development, but becomes repressed in adulthood[1,2]. The human neuronatin gene localizes to chromosome 20q 11.2-12[2], and spans 3973 bases. It contains three exons and two introns, and there are two alternative spliced forms, alpha and beta[3]. Our recent northen blot and RT-PCR analysis revealed that the anterior pituitary gland is the only place where the neuronatin mRNA is strongly expressed in adults. It is expressed in the fetal brain, adult anterior pituitary gland, and pituitary adenomas, but is hardly detected in the adult brain and brain tumors, such as gliomas or craniopharyngiomas[4-6].

This study clarified the role of neuronatin through an investigation of the cellular localization of neuronatin in the pituitary gland and pituitary tumors using *in situ* hybridization.

MATERIALS AND METHODS

Tissues

Three normal pituitary glands were collected at autopsy from adult patients with no evidence of any endocrinological disease. Specimens of pituitary adenomas were obtained at the time of transsphenoidal or transcranial surgery. The clinical diagnoses were 9 growth hormone (GH) -secreting

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Abbreviations—ACTH, adrenocorticotropic hormone; CR, craniopharyngioma; FSH, follicle stimulating hormone, GH, growth hormone, IHC, immunohistochemistry; ISH, *in situ* hybridization, LH, luteinizing hormone, RCC, Rathke's cleft cyst; TSH, thyroid stimulating hormone

adenomas, 5 prolactin (PRL) -secreting adenomas, 2 adrenocorticotropic hormone (ACTH) -secreting adenomas, 10 nonfunctioning adenomas, 3 craniopharyngiomas, and 1 Rathke's cleft cyst coexistent with nonfunctioning adenoma. Pieces of each tissue were immediately frozen in liquid nitrogen and stored at -80℃.

Preparation of oligonucleotide probe

Based on the human neuronatin alpha and beta sequences we reported, an antisense oligonucleotide probe (46 mer) was chemically synthesized with a DNA synthesizer (ABI, USA). The location of the probe along the cDNA sequence is shown in Fig. 1. The beta subunit was formed by the splicing of exon II. The sequence of the probe (5'-GCTTCTGCAGG-GAGTACCTGAACACCTGCCAGCAGCGGAGAT-3') corresponded to both flanking regions of the neuronatin-alpha specific insertion sequence.

For *in situ* hybridization, the oligonucleotide probes were 3' end labeled with [^{35}S] or [^{33}P] dATP (Amersham, UK) using terminal deoxynucleotidyl transferase (Takara, Japan), and purified by Sephadex G-25 Spin Columns (Boehringer, Germany). Under the present labeling conditions, about 15 [^{35}S] or [^{33}P] dATP were incorporated into each probe, as could be seen by electrophoresis on a sequencing gel.

Procedure for *in situ* hybridization

Tissues were embedded in an OCT compound (Sakura, Japan) at -15℃. Frozen sections, 16 μm thick, were made on a cryostat, attached to 0.01% poly-L-Lysin coated glass slides, and fixed in 4% paraformaldehyde / 0.1 M sodium phosphate buffered saline (PBS) (pH 7.2) for 30 min. The sections were washed with PBS containing 2 mg/ml glycine for 20 min and acetylated in 0.25% acetate / 0.1 M Tris-HCl (pH 8.0) for 10 min, followed by three washings with 2×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate) for 5 min each. The sections were then prehybridized for 3 hours at 37℃ in a prehybridization solution containing 50% formamide, 5×SSC, 50 mM sodium phosphate (pH 6.5), 2% Sarkosyl, 5×Denhardt's solution (0.1% Ficoll, 0.1% polyvinyl-pyrrolidone, 0.1% bovine serum albumin), 0.5 mg/ml heat-denatured herring sperm DNA, and 10 mM beta-mercapthanol, and hybridized for 24 hours at 37℃ in a solution containing 4 volumes of prehybridized solution, 1 volume of 50% dextran sulfate, and 1.0×10^5 cpm/μl of a radiolabeled oligonucleotide probe. The tissue sections were washed 3 times in 0.1×SSC / 0.1% Sarkosyl at 52℃ for 40 min, dehydrated, and autoradio-

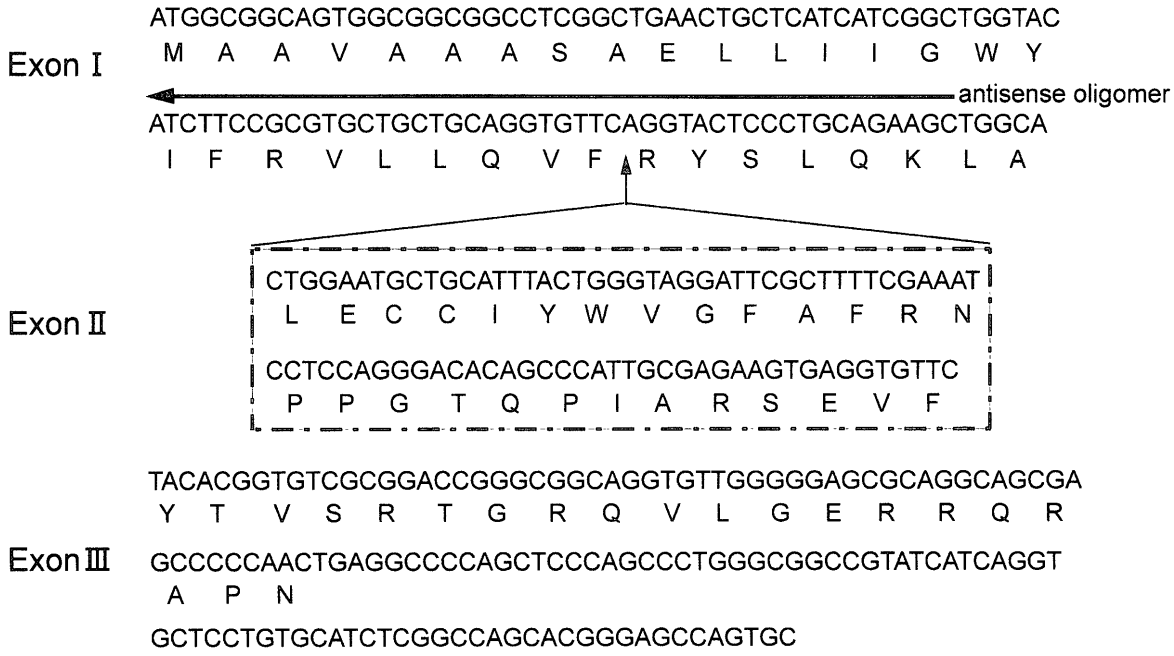

Fig. 1. Structure of human neuronatin cDNA and *in situ* hybridization probes. Neuronatin has two kinds of subunits, alpha and beta. The beta-subunit is formed by the splicing of exon II.

graphed using NTB2 nuclear track emulsion (Kodak, USA) for 1-2 months at 4℃. After development, the sections were counterstained lightly with pyronin-methylgreen.

Immunohistochemistry

For characterization of the cellular hormonal contents, IHC (immunohistochemistry) for anterior pituitary hormones was performed on serial sections of these ISH specimens. The primary antibodies for human GH, PRL, ACTH, luteinizing hormone (LH) -beta, follicle stimulating hormone (FSH) -beta and thyroid stimulating hormone (TSH) -beta (diluted properly for DAKO PAP KIT® systems) were obtained from DAKO (Carpinteria, Calif.). The standard avidin-biotin peroxydase complex (ABC) method was applied, and antibody binding was visualized using 3, 3' diaminobenzidine (DAB): H_2O_2.

RESULTS

Examination of normal pituitary gland

Fig.2 shows the expression of neuronatin in the normal pituitary gland. Strong neuronatin signals were detected in the anterior lobe, but virtually not in the posterior lobe. Neuronatin mRNA was detected in almost all the cells of the anterior pituitary gland irrespective of hormone productivity. (Fig. 3)

Examination of neoplastic human tissues

Table 1 shows the expression of neuronatin in neoplastic human tissues. The intensity of neuronatin signals showed considerable variety among cases, although neuronatin mRNA was observed in various pituitary adenomas, whether they were functioning or not. Neuronatin signals were not detected in the cases of craniopharyngioma (CR).

Fig. 4 shows the expression of neuronatin in two cases of GH-producing adenoma. In some GH-producing adenomas, GH cells were observed sparse-

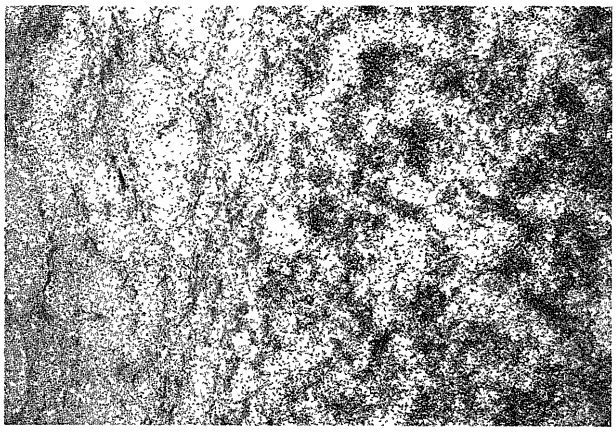

Fig. 2. Expression of neuronatin in a normal pituitary gland. (ISH; ×100). Posterior lobe (*Left side*). Anterior lobe (*Right side*).

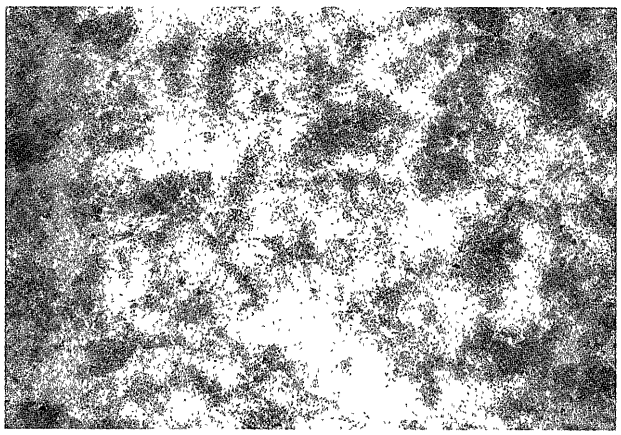

Fig. 3. Expression of neuronatin in the anterior lobe of a normal pituitary gland. (ISH; ×200).

Table 1. Expression of neuronatin in neoplastic human tissues

	S	D	W	N	No.
Nonfunctioning pituitary adenoma	2	6	2	0	10
GH-producing pituitary adenoma	2	7	0	0	9
PRL-producing pituitary adenoma	1	2	2	0	5
ACTH-producing pituitary adenoma	1	1	0	0	2
Craniopharyngioma	0	0	0	3	3
Rathke's cleft cyst (epithelium)	0	1	0	0	1

S, strong; D, distinct; W, weak; N, negative.

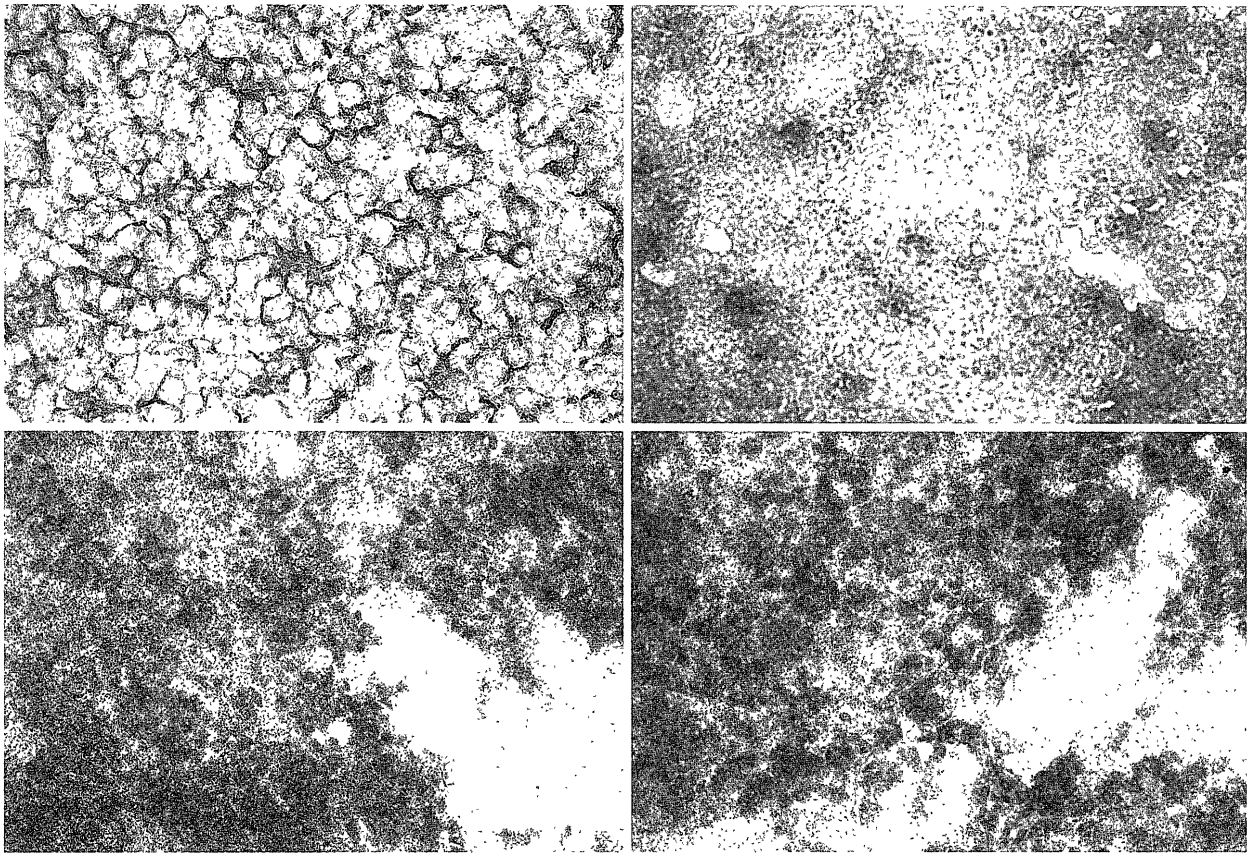

Fig. 4. Expression of neuronatin in two cases of a GH-producing adenoma. Case 1 (*Left*). Case 2 (*Right*). IHC using an anti-GH antibody ((*Upper*) Case 1: ×400, Case 2: ×100). Expression of neuronatin (*Lower*), ISH (×200).

ly among the silent cells as in Case 2, although most of the cases had dense GH cells as in Case 1. Neuronatin signals were distributed equally in both cases irrespective of the density of GH-producing cells.

There was one case of a nonfunctioning pituitary adenoma including a Rathke's cleft cyst (RCC). Here, neuronatin signals were detected not only in the adenoma cells, but also in the lining cells of RCC (Fig. 5).

DISCUSSION

This study investigated the expression of neuronatin mRNA in each individual cell using ISH.

Neuronatin mRNA was observed in the normal pituitary gland anterior lobe, as we previously showed using Northern blot analysis, but was hardly detected in the posterior lobe. In pituitary adenomas, the intensity of neuronatin mRNA showed considerable variety among cases, although it was detected in various adenomas irrespective of their hormone productivity. In a tumor containing GH-producing cells and non-GH cells, signals of neuronatin mRNA were distributed equally on the same specimen. These results suggest that the expression of neuronatin is not related to the hormone productivity of each individual cell.

Ever since the cloning of Pit-1 in 1988[7], a variety of transcriptional factors related to pituitary development has been discovered in rapid succession in the field of molecular pituitary research.

Pit-1 is well known as the transcriptional factor regulating GH, TSH, and PRL secretion. The differentiation of somatotrope, lactotrope, and thyrotrope is also dependent on Pit-1 expression. Human Pit-1 mRNA first appears at 6 weeks of gestation, preceding the onset of hormonal immunoreactivity. It is first detected in both the anterior and posterior pituitary, but after 12 weeks it is clearly localized in cells that contain GH, PRL, and TSH[8]. In the same way, many homeobox genes such as Rpx,

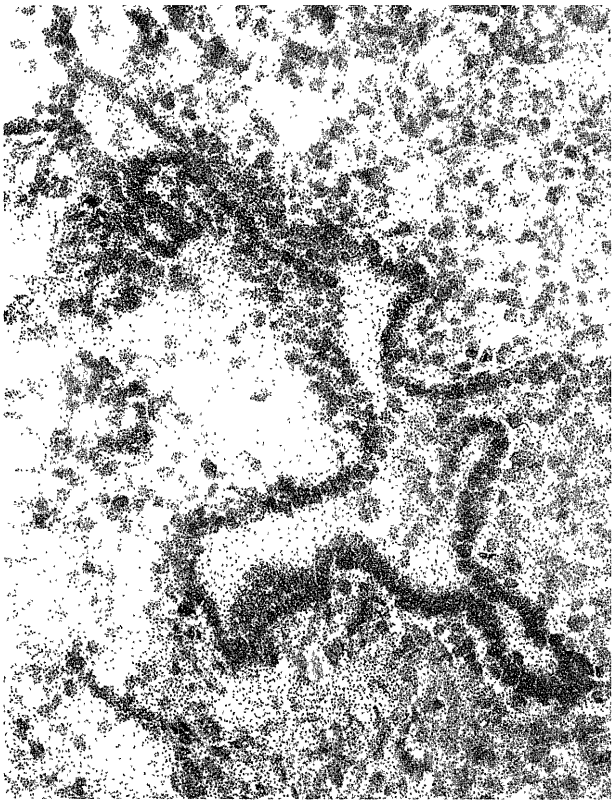

Fig. 5. Expression of neuronatin in a case of nonfunctioning pituitary adenoma including a Rathke's cleft cyst (ISH, ×200).

Pit-x1, Pit-x2, Prop-1, Lhx-3, Lhx-4 and others have been discovered. The timing of expression, role in pituitary development and cell differentiation, or cofactors of these genes have been gradually clarified, and the importance of these genes has been demonstrated[9].

Neuronatin may also serve in the development and differentiation of the pituitary gland. This gene codes for novel proteins of 54, 55, and 81 amino acids, and the deduced proteins are thought to be membrane bound, suggesting a possibility that the gene might function as a protein ligand, cofactor, or small cell adhesion molecule[1]. Recent studies on rodents show that neuronatin is most strongly expressed in the neonatal brain during mid to late stages of gestation[10,11]. Neuronatin mRNA appears slightly later than Rpx, the earliest known marker for the pituitary primordium[12], and earlier than Pit-1 or other homeobox genes related to the differentiation of anterior pituitary cell types. Moreover, as we have shown, it is not involved in the hormone productivity of each anterior lobe cell. This suggests that neuronatin works at a more primitive stage of adenohypophysial development, before hormonal differentiation of the anterior pituitary cells. In adults, it localizes to anterior pituitary cells and may participate in the maintenance of pituitary function as a part of the intracellular transmission system. The probe used in this study appears to have specifically detected both neuronatin alpha and beta pan, although it will still be necessary to examine the respective expressions of these two alternatively spliced forms for a better understanding of the role of this gene.

One interesting finding here was that the neuronatin signals were positive in the epithelial cells of RCC, but could be hardly detected in CRs. The pathogeneses of RCC and CR remain controversial, and they are based on some differing hypotheses. As to the origin of RCC: 1) remnants of Rathke's pouch; 2) the neuroepithelium; or 3) metaplasia of anterior pituitary cells are candidates. There are also two hypotheses as to the origin of CR, namely 1) ectopic embryonal remnants of the craniopharyngial duct, and 2) metaplastic squamous epithelial cells in the adenohypophysis[13]. RCC and CR sometimes overlap[14], so their similarities and differences have been discussed[15] based on findings from immunohistochemical studies[16] or electron-microscopic examination[17]. There is a possibility of neuronatin being used as a marker to determine the origin of pituitary tumors, including sellar cystic lesions.

CONCLUSION

1) Neuronatin is thought to participate in the development, differentiation, or maintenance of the anterior pituitary gland and pituitary adenomas, although it is not involved in hormone productivity.
2) Information about the distribution of neuronatin may facilitate an understanding of the origin of pituitary tumors, including Rathke's cleft cysts and craniopharyngiomas.

Acknowledgments. We are grateful to Dr. Toshiro Kumanishi and Dr. Hiroshi Usui of the Department of Molecular Neuropathology in our Institute for many helpful suggestions. We also wish to thank Mr. Tomio Ichikawa and Mr. Kazuo Kobayashi for their skillful technical assistance.

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