Molecular and Cellular Endocrinology 247 (2006) 135–139

Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

HESX1 expression in human normal pituitaries and pituitary adenomas

G. Mantovani [a,*], C. Asteria [b], C. Pellegrini [c], S. Bosari [c], L. Alberti [d], S. Bondioni [a], E. Peverelli [a], Anna Spada [a], P. Beck-Peccoz [a]

[a] *Institute of Endocrine Sciences, Ospedale Maggiore IRCCS, University of Milan, Milan, Italy*
[b] *Policlinico MultiMedica, Sesto San Giovanni, Milan, Italy*
[c] *Pathology Unit, Department of Medicine, Surgery and Dentistry, University of Milan, A.O. San Paolo and Ospedale Maggiore IRCCS, Milan, Italy*
[d] *Istituto Auxologico Italiano IRCCS, Milan, Italy*

Received 1 June 2005; received in revised form 13 October 2005; accepted 9 December 2005

Abstract

Hesx1 is a paired-like homeobox gene first expressed during mouse embryogenesis in the anterior midline visceral endoderm. As gastrulation proceeds, Hesx1 is expressed in the ventral prosencephalon and, subsequently, at E9.0 appears in the ventral diencephalon and in the thickened layer of oral ectoderm that give rise to Rathke's pouch, the primordium of the anterior pituitary gland. Hesx1 continues to be expressed in the developing anterior pituitary until E11.5 when its transcripts disappear in a spatiotemporal sequence corresponding to progressive pituitary cell differentiation, becoming undetectable by E15.5. In the present study, we investigated whether HESX1 is expressed during adult life in human normal pituitaries and in different types of human pituitary adenomas. We analysed, using quantitative RT-PCR method, three normal pituitaries, seven GH-, two TSH-, two PRL-, one ACTH-secreting adenomas, and seven nonfunctioning pituitary tumors. HESX1 mRNA was found to be expressed in normal pituitaries and in all the pituitary tumors that we have analysed.

These results suggest that in humans HESX1 is not turned-off during the adult life as it occurs in mice. Thus, HESX1 in humans might play a role in the maintenance of the anterior pituitary cell types and function, as well as in the differentiation of pituitary adenomas, whose pathogenetic mechanisms remain to be further investigated. This is the first study on HESX1 expression in humans during adult life.

Keywords: HESX1; Homeobox gene; Transcription factor

1. Introduction

The development of the anterior pituitary gland is dependent upon a cascade of signalling molecules and developmental genes that function as transcription factors. Many of these genes are homeobox genes, which encodes a 60-amino-acid conserved DNA-binding domain, termed homeodomain, which mediates specific interaction between homeodomain proteins and DNA. Hesx1 (homeobox gene expressed in embryonic stem cells 1) is a member of the *paired-like* class of homeobox genes (Dattani et al., 1998), and is first expressed during mouse embryogenesis in a small patch of cells in the anterior midline visceral endoderm at the beginning of gastrulation. Subsequently, Hesx1 expression spreads to the adjacent ectoderm, then to the rostral neural folds, and then to the ventral diencephalon. At embryonic day 9.5 (E9.5) Hesx1 is also expressed in the oral ectoderm which give rise to Rathke's pouch and in the precursors of all pituitary cells types, but its expression declines after day 11.5 and is undetectable by E15.5, following the appearance of Pit-1 (pituitary transcription factor 1). Prop-1 (the prophet of Pit-1) is required for the repression of Hesx1 transcription, as there is abnormal persistence of Hesx1 expression until E17.5 in the Ames dwarf mouse, which has mutation in Prop-1 (Sornson et al., 1996). Since Hesx1 is a putative repressor gene with a repressor domain (Dattani et al., 1998), its role in pituitary development is thought to repress downstream target genes. In particular, it seems to be required for proper differentiation of the anterior pituitary, as well as for other part of the central nervous

* Corresponding author at: Institute of Endocrine Sciences, Pad. Granelli, Ospedale Maggiore IRCCS, via F. Sforza 35, 20122 Milano, Italy.
Tel.: +39 02 50320608; fax: +39 02 50320605.
E-mail address: giovanna.mantovani@unimi.it (G. Mantovani).

0303-7207/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2005.12.036

system. This role of Hesx1 was confirmed by targeted disruption of the gene in mice (Dattani et al., 1998). The homozygotes mutant animals present a reduction in prospective forebrain tissue, absence of developing optic vescicles, markedly decreased head size, craniofacial dysplasia and severe microftalmia. Histological analysis reveals in the mutant mice abnormalities of the septum pellucidum and corpus callosum, absence of the optic cups and the olfactory placodes and hypothalamic abnormalities and aberrant morphogenesis or absence of Rathke's pouch. These phenotypic features were reminiscent of the syndrome of septo-optic dysplasia (SOD) in humans. Recently, the homozygous and heterozygous mutations of HESX1 have been described in patients with SOD associated or not with combined pituitary hormone deficiency (Dattani et al., 1998; Thomas et al., 2001). Nowadays, no data are available concerning HESX1 gene expression in human pituitary during the adult life. Therefore, in this study we evaluated by RT-PCR method HESX1 expression after birth in normal human pituitary and in human pituitary adenomas in order to clarify whether this early transcription factor is differentially regulated in mice and in humans, and in this case the importance of its persistence.

2. Materials and methods

2.1. Tumors specimens

Informed consent and the approval of the Internal Ethic Committees were obtained before starting the genetic analysis.

Human normal pituitaries ($n = 3$) from subjects without endocrinological diseases were obtained from autopsy cases. The post-mortem interval was within 6 h. Tissues of the pituitary adenomas ($n = 19$) were obtained at the time of trans-sphenoidal surgery and they were frozen in liquid nitrogen immediately after surgical resection and stored at −80 °C until analysis. Tumor types were determined on the basis of clinical and biochemical findings (baseline hormones and dynamic testing) before surgery, and on the basis of morphological and immunohistochemical data.

Moreover, we evaluated HESX1 gene expression in normal brain tissue as negative control.

2.2. RT-PCR

Total RNA extraction was performed by the single-step method (TRIzol reagent kit, Life Technologies). DNAase was used to prevent genomic DNA contaminations.

Random primed cDNAs were synthesized from 2 μg of total RNA with M-MLV Reverse Transcriptase (Promega, USA). RT reaction was performed at 37 °C for 60 min in a final volume of 30 μL. PCR was performed in 50 μl final reaction volume containing 3 μL (180 ng) RT reaction product as template, 1× PCR buffer, 1.5 $MgCl_2$, 0.2 mmol/L of dNTP mix, 10 pmol of each sense and antisense primer for HESX1, and 2.5 U amplitaq gold DNA polymerase (Perkin-Elmer). The oligonucleotide primers for the amplification of HESX1 were synthesized on the basis of published sequence (forward 5′-GAT GGT AAC TTA TGT CTA CAT CTC-3′ and reverse 5′-GGA TTC TGT CTT CCT CTA GAT TCA-3′) and generate a product of 295 bp. This primer set was designed to span one putative intron to allow distinction of genomic contamination.

HGPRT (hypoxanthine guanine phosphoribosyltransferase) mRNA (97 bp) was used as internal control.

Programmed temperature cycling was performed with the following cycle profile: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s. After the last cycle, the elongation step was done at 72 °C for 5 min.

The amplified RT-PCR products were run on agarose gel (3%) electrophoresis, stained by ethidium bromide and visualized under UV light.

2.3. Real-time RT-PCR

HESX1 mRNA levels were measured by real-time quantitative RT-PCR based on TaqMan methodology, using ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR data analysis was performed with geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/) (Vandesompele et al., 2002). Using this approach we selected the following three internal control genes: hydroxymethylbilane synthase (HMBS), TATA box binding protein (TBP), and ubiquitin C (UBC). For the quantification we used a ready-to-use assay (Assay-on-Demand™ Gene Expression Products), purchased from Applied Biosystems. The assay identification numbers are: Hs00609297_m1 (HMBS), Hs00427620_m1 (TBP), Hs00824723_m1 (UBC) and Hs00172696_m1 (HESX1).

Total RNA (500 ng) was reverse transcribed, in a final volume of 100 μl, using high-capacity cDNA Archieve Kit (Applied Biosystems), as described by the manufacturer. Amplification reactions were performed with TaqMan Universal PCR master mix (Applied Biosystems), using 5 μl of cDNA in a final volume of 25 μl. Primers and fluorescent probes were added to the reaction mixture according to the manufacture's directions. All reactions were performed in duplicate. The thermal cycling conditions included 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.4. Western blot analysis

Twenty micrograms of nuclear proteins were extracted from frozen tissue. In order to obtain the nuclei, the tissues were homogenate in hypotonic buffer (10 mM Tris pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$) supplemented with a cocktail of proteinase inhibitors, (Amersham Biosciences, UK) and washed three times for 15′ at 3300 × g at 4 °C in the same buffer. Successively, the nuclei were lysed in TENN buffer (50 mM Tris pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.5% NP40 and proteinase inhibitors) and centrifugated at 14000 × g for 10′ at 4 °C. Proteins in nuclear lysates were quantified using the Pirce BCA assay protein. The nuclear proteins were resolved by 12% SDS-PAGE and transferred on nitrocellulose membrane (Transfer Blot, BioRad). HESX1 protein was detected using a goat monoclonal antibody raised against the N-terminal region (N-17) of the HESX1 protein (Santa Cruz Biotechnology), and then with a secondary anti-gaot antibody (SIGMA, Saint Louis, MS, USA) conjugated to horseradish peroxidase. The membranes were finally treated with chemiluminescent substrate and enhancer (LumiGLO, New England, Biolabs, Beverly, MA, USA).

2.5. Sequence analysis

In order to confirm the band of 295 bp corresponds to HESX1 cDNA, it was purified from agarose gel and sequenced by automated method (PE Applied Biosystems, ABI PRISM 310 DNA Sequencer, Perkin-Elmer) using specific primers.

3. Results

The clinical profiles of each patient with pituitary tumor are represented in Table 1. The patients consisted of 12 males and 7 females with age range from 22 to 65 years. Among the pituitary tumors studied, 7 (37%) were nonfunctioning pituitary tumors (NFPAs) and 12 (63%) were secreting adenomas: seven GH-secreting adenoams (GH-omas), two prolactinomas (PRL-omas), two TSH-secreting adenomas (TSH-omas), and one ACTH-secreting tumor (ACTH-oma). There were 15 macroadenomas (79%) and 4 (21%) microadenomas. Invasion of cavernus and sphenoid sinuses, as well as of the optic chiasm, was seen in nine patients, including all the NFPAs, one GH-oma and one PRL-oma.