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Estrogen-related receptor α in normal adrenal cortex and adrenocortical tumors: Involvement in development and oncogenesis

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ABSTRACT

Aims: The nuclear hormone receptor estrogen-related receptor α (ERRα) regulates the activation of mitochondrial genes in various human tissues, but its role in the adrenal gland and its disorders has not been defined. Therefore, we examined ERRα expression in both normal adrenal cortex (NAC) and adrenocortical tumor (ACT) in order to study the possible correlation of ERRα with adrenal development and tumor development.

Methods: Human adrenal specimens (non-pathological fetal $n = 7$; non-pathological post-birth $n = 40$; aldosterone producing adenoma (APA) $n = 11$; cortisol producing adenoma (CPA) $n = 11$; adrenocortical carcinoma (ACC) $n = 8$) were immunohistochemically examined in this study. NAC ($n = 13$) and ACT ($n = 28$) frozen tissue specimens were also available for studying ERRα mRNA levels.

Key findings: In fetal NAC tissues, ERRα labeling index (LI) in fetal zone (FZ) was significantly higher that that in neocortex (NC), and the differences among age groups for overall mean LI was statistically significant when analyzed according to individual cortical layers. ERRα LI was also significantly higher in ACC than in other types of ACT. ERRα mRNA was detected in NAC and all types of ACT.

Significance: Results of our present study suggest a possible role of ERRα in adrenal development and ACC.

1. Introduction

Nuclear receptors (NRs) play pivotal roles during the development of many vertebrates and also in regulation of physiological functions in adults (Chambon, 2005; Evans, 2005). The estrogen related receptor α (ERRα) belongs to the NR superfamily, a group of 48 structurally related, ligand-activated transcription factors (Alaynick, 2008; Giguere, 2008; Tremblay and Giguere, 2007). ERRα was originally cloned by DNA sequence homology to the estrogen receptor α (ERα, NR3A1) (Giguere et al., 1988), but subsequent ligand binding and reporter-gene transfection experiments demonstrated that estrogens did not regulate ERRα (Deblois and Giguere, 2011). Currently, ERRα is considered an orphan nuclear receptor (Deblois and Giguere, 2011; Giguere et al., 1988).

ERRα is expressed in several tissues requiring high energy demand, including the heart, skeletal muscle and the brain (Heard et al., 2000). Results of both *in vitro* and *in vivo* studies suggest that ERRα is required for the activation of mitochondrial genes as well as increased mitochondrial biogenesis (Schreiber et al., 2004; Villena et al., 2007). It has been reported that ERRα can activate reporters containing steroidogenesis factor 1 (SF-1) response elements as a result of transient transfection assays (Bonnelye et al., 1997). A possible role of ERRα in steroidogenesis with relation to SF-1 was subsequently demonstrated *in vitro* by Seely et al., who also demonstrated ERRα immunoreactivity in the three layers of the adult adrenal (Seely et al., 2005). However, ERRα expression in the adrenal gland, in which activation of mitochondrial genes play important roles, has not been examined in detail.

ERRα has been studied at the RNA or protein level in various cancer types, and high expression levels were reported to be

Abbreviations: NAC, normal adrenal cortex; ACT, adrenocortical tumors; APA, aldosterone producing adenoma; CPA, cortisol producing adenoma; ACC, adrenocortical carcinoma; NC, neocortex; FZ, fetal zone; ZG, zona glomerulosa; ZF, zona fasciculate; ZR, zona reticularis; LI, labeling index; y.o., years of age.

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associated with a poor prognosis in various human malignancies including colon, endometrium, ovary, prostate and breast cancers (Ariazi et al., 2002; Bianco et al., 2012; Cavallini et al., 2005; Fujimoto and Sato, 2009; Fujimura et al., 2007; Sun et al., 2005; Suzuki et al., 2004). ERRα has been previously reported to be present in H295R adrenocortical carcinoma cells (Seely et al., 2005) but it has not been studied at all in human adrenal gland disorders.

Therefore, in this study, we evaluated ERRα expression in both normal and neoplastic adrenocortical tissues in order to clarify its roles in adrenocortical development and tumorigenesis.

2. Materials and methods

2.1. Human adrenal samples

Research protocols were approved by the ethics committee at Tohoku University Graduate School of Medicine (Sendai, Japan) and the Fukushima Medical University (Fukushima, Japan).

For immunohistochemical analysis, specimens of non-pathological adrenal glands were obtained from autopsy files (fetal, $n = 7$; post-birth 4–80 y.o. age, $n = 40$) from Tohoku University Hospital (Sendai, Japan). Thirty cases of adrenocortical tumors (ATs) (11 aldosterone producing adenomas (APAs), 11 cortisol producing adenomas (CPAs), and 8 adrenocortical carcinomas (ACCs)) were retrieved from the surgical pathology files of Tohoku University Hospital.

For quantitative RT–PCR analysis, 28 cases of adrenocortical neoplasms (5 APAs, 6 CPAs, and 3 ACCs obtained from surgical procedures performed at Tohoku University Hospital and 6 APAs, 6 CPAs, and 2 ACCs obtained from Fukushima Medical University) as well as 13 samples of normal adrenal cortex (NAC), obtained from autopsies at Tohoku University Hospital, were available for RT–PCR analysis.

2.2. Immunohistochemistry

The specimens were fixed in 10% formalin for 24–48 h at room temperature and embedded in paraffin wax. Mouse monoclonal antibody for ERRα (2ZH5844H) was purchased from Perseus Proteomics Inc. (Tokyo, Japan). Antigen retrieval for immunostaining of ERRα was performed by heating the slides in an autoclave at 121 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilution of the primary antibody was 1:100. Immunostaining was performed employing the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan). The antigen–antibody complex was visualized with 3,3-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% H_2O_2), and counterstained with hematoxylin. For negative controls 0.01 M PBS instead of the primary antibody was incubated and no specific immunoreactivity was detected in these tissue sections, and for positive control we used a human heart sample (data not shown).

2.3. Evaluation of immunoreactivity

After completely reviewing the slides, relative immunoreactivity for ERRα in each zone of the adrenocortex as well as in tumor specimens was evaluated by labeling index (LI), carried out by examining high-power fields and counting 1000 cells. The LIs were independently and blindly evaluated by two of the authors (S.J.A.F. and X.G.H.) and the mean of these two values was used for analysis. The normal adrenals were further subclassified into the following age groups: fetal ($n = 7$), 4–10 y.o. ($n = 6$), 11–20 y.o. ($n = 10$), 21–40 y.o. ($n = 16$), and 41–80 y.o. ($n = 15$).

2.4. RNA isolation and quantitative RT–PCR (qPCR)

Specimens were snap-frozen for RNA isolation and stored at −80 °C until use. Total RNA was carefully extracted from the 28 specimens of adrenocortical neoplasms and 13 specimens of normal adrenocortex by using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) after cryostate disruption at −20 °C.

cDNA was produced with Quantitec reverse transcription kit (QIAGEN) and RT–PCR was performed with LightCycler FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland) in a LightCycler equipment (Roche). The primer sequences used in our study were: ERRα forward: 5′-CAC CAT CAG CTG GGC CAA GAG-3′ and reverse: 5′-GGT CAG ACA GCG ACA GCG ATG-3′ (Seely et al., 2005); RPL13A forward: 5′-CCT GGA GGA GAA GAG GAA AG-′3 and reverse 5′-TTG AGG ACC TCT GTG TAT TT-′3. The samples previously submitted to total RNA extraction and cDNA production were analyzed. The melting curve analysis was performed in order to verify amplification of the expected sequence. Negative control experiments did not contain cDNA substrate to check for the possibility of exogenous contaminant DNA, and no amplified products were detected under these conditions.

In all experiments, the relative mRNA expression was calculated as the ratio between the quantity of ERRα and house-keeping gene RPL13A cDNA transcripts, as previously reported (Nakamura et al., 2006; Shibahara et al., 2012).

2.5. Statistical analysis

The labeling index (LI) obtained by evaluation of immunoreactivity as the percentages of stained cell nuclei, and the ERRα/RPL13A relative mRNA values obtained from qPCR data, were submitted to statistical evaluation using Mann–Whitney or Kruskal–Wallis non-parametric tests. $P < 0.05$ was considered significant.

3. Results

3.1. Immunohistochemical analysis

Results for ERRα expression in fetal and post-birth NAC were summarized in Figs. 1 and 2, respectively. In post-birth NAC, ERRα immunoreactivity was detected in the nuclei of the zona glomerulosa (ZG), the zona fasciculata (ZF) and the zona reticularis (ZR) cells. In fetal adrenal gland, immunoreactivity was detected in the neocortex (NC), with fewer nuclei positive in the fetal zone (FZ). In post-birth NAC cases, the lowest LI was detected in the 4–10 y.o. age group and the highest in the 41–80 y.o. age group (Fig. 3A) in each zone. Statistical difference of LI among the three cortical layers was detected within the 11–20 y.o. age group ($P = 0.021$). The difference among age groups for overall mean LI was statistically significant as well as when analyzed according to different cortical layers ($P = 0.022$ for ZG, $P = 0.008$ for ZF and $P < 0.001$ for ZR, respectively). In fetal adrenal glands, ERRα LI in the NC was significantly higher (71.2 ± 4.99% (mean ± SEM)) than that in the FZ (24.9 ± 5.17%) ($P = 0.0017$) (Fig. 3B). In adrenal tumors, ERRα immunoreactivity was detected in the nuclei of APA, CPA and ACA (Fig. 4A). ERRα LI was significantly higher in ACCs (82.9 ± 2.3%) when compared to both APA (66.8 ± 3.4%) and CPA (60.1 ± 4.5%) ($P < 0.005$).

3.2. qPCR analysis

The qPCR results of ERRα mRNA levels were summarized in Fig. 4B.