Increased protein kinase A type Iα regulatory subunit expression in parathyroid gland adenomas of patients with primary hyperparathyroidism

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Abstract. Protein kinase A (PKA) regulatory subunit type Iα (RIα) is a major regulatory subunit that functions as an inhibitor of PKA kinase activity. We have previously demonstrated that elevated RIα expression is associated with diffuse-to-nodular transformation of hyperplasia in parathyroid glands of renal hyperparathyroidism. The aim of the current study was to determine whether or not RIα expression is increased in adenomas of primary hyperparathyroidism (PHPT), because monoclonal proliferation has been demonstrated in both adenomas and nodular hyperplasia. Surgical specimens comprising 22 adenomas and 11 normal glands, obtained from 22 patients with PHPT, were analyzed. Western blot and immunohistochemical analyses were employed to evaluate RIα expression. PKA activities were determined in several adenomas highly expressing RIα. RIα expression was also separately evaluated in chief and oxyphilic cells using the “Allred score” system. Expression of proliferating cell nuclear antigen (PCNA), a proliferation marker, was also immunohistochemically examined.

Western blot analysis revealed that 5 out of 8 adenomas highly expressed RIα, compared with normal glands. PKA activity in adenomas was significantly less than in normal glands. Immunohistochemical analysis further demonstrated high expression of RIα in 20 out of 22 adenomas. In adenomas, the greater RIα expression and more PCNA positive cells were observed in both chief and oxyphilic cells. The present study suggested that high RIα expression could contribute to monoclonal proliferation of parathyroid cells by impairing the cAMP/PKA signaling pathway.

Key words: Parathyroid adenoma, Primary hyperparathyroidism, Protein kinase A regulatory subunit type Iα (PRKAR1α)
composed of two regulatory and two catalytic subunits. Binding of the tetramer with cAMP dissociates the enzyme, releasing a regulatory subunit dimer and two free active catalytic subunits. RIα is ubiquitously expressed, and is the main regulatory subunit of PKA in most cell types [19]. We previously reported that the PRKAR1A gene is frequently overexpressed in nodular hyperplasia cases of hyperparathyroidism associated with chronic renal failure, compared with diffuse hyperplasia cases [20]. Accordingly, PKA activity is significantly suppressed in nodular hyperplasia. In both nodular hyperplasias and PHPT adenomas, the affected cells have been reported to proliferate in a monoclonal fashion [1, 21]. In the present study, we investigate whether RIα protein expression is also altered in PHPT adenomas by measuring specimens obtained from sporadic PHPT patients.

**Materials and Methods**

**Case subjects and materials**

A total of 33 parathyroid glands were obtained from 22 patients with sporadic PHPT, including 11 pairs of adenomas and adenoma-associated normal glands. The patients underwent a parathyroidectomy procedure at Nagoya University Hospital or Fujita Health University Hospital between 2002 and 2006. Informed consent was obtained from patients for the use of tissue samples to investigate the expression of RIα protein. The investigation protocol was approved by the Ethics Committee, Fujita Health University School of Medicine. Usually one affected gland was resected, but in some cases an additional gland was also resected when it appeared to be enlarged. After operation, most of these glands were found to be normal by histological investigation, and used as a normal gland in this study. Table 1 summarizes the specimens used for this study. The patients did not have a familial history associated with PHPT. Most patients had a single gland disease, as demonstrated by the normal serum calcium level after excision of a histologically-proven parathyroid adenoma.

**Human tissue samples**

Fresh surgical specimens of 22 patients were

<table>
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<tr>
<th>Case No.</th>
<th>Weight of adenoma (mg)</th>
<th>Preoperative intact PTH (pg/mL)</th>
<th>PTH/weight ratio (pg/mL/mg)</th>
<th>Western blot analysis</th>
<th>PKA activities</th>
<th>Allred score of RIα staining</th>
<th>Composition of oxyphilic cells (%)</th>
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Mean 748.71 198.14 0.38 4.73 2.14 6.86 2.27 1.09 3.36 23.05 0.64
s.d. 599.07 130.63 0.22 0.46 0.89 0.99 1.85 0.94 2.58 35.42 1.57

n.p., not performed; n.a., not available
obtained from patients undergoing surgery. The materials, obtained immediately after the surgical procedure, were frozen in liquid nitrogen and stored at -80°C. Remaining tissues were fixed in 10% formaldehyde and embedded in paraffin.

**Western blot analysis**

Methodological procedures for sample preparation and Western blot analysis were performed as previously described [20]. Mouse monoclonal antibody specific to human RIA (BD Biosciences, Bedford, MA), and rabbit polyclonal antibody specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich, St. Louis, MO) served as primary antibodies. The secondary antibodies were goat polyclonal anti-mouse IgG and anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Sigma-Aldrich). Proteins were visualized by enhanced chemiluminescence reagents. Band densities were quantified using NIH image analysis software (version 1.62, National Institute of Health, Bethesda, MD).

**Immunohistochemistry**

Immunohistochemistry was performed using the universal immuno-enzyme polymer (UIP) method (ENVISION kit/HRP (DAB); Dako Japan, Tokyo, Japan). According to the manufacturer’s instructions, 3-µm thick, paraffin-embedded, tissue sections were mounted on silane-coated slides, followed by deparaffinization and rehydration. Antigen retrieval was achieved by heating the sections three times for 5 min with microwave radiation in 10 mM citrate buffer (pH 6.0). They were then treated with 1% hydrogen peroxide for 5 min to quench endogenous peroxidase activity. The sections were then incubated overnight at 4°C with mouse monoclonal antibody specific to human RIA (1:1000, BD Biosciences), or rabbit polyclonal antibody specific to human proliferating cell nuclear antigen (PCNA; 1:1000; FL-261, Santa Cruz, Santa Cruz, CA), followed by incubation for 60 min at room temperature with a reagent comprising peroxidase-labeled polymer conjugated to goat anti-rabbit IgG or anti-mouse IgG antibodies. A 3,3’-diaminobenzidine chromogen solution was then applied for 1 min. The sections were then counterstained with hematoxylin, then semi-quantitatively evaluated by light microscopy using the “Allred Score” system [22]. The score represented the estimated proportion of positively stained tumor cells (0 = none, 1 = less than 1/100, 2 = 1/100 to less than 1/10, 3 = 1/10 to less than 1/3, 4 = 1/3 to less than 2/3, and 5 = 2/3 or above). The staining intensities were averaged from the positive cells (0 = none, 1 = weak, 2 = intermediate, and 3 = strong). The sum of these scores served as the total score. A total score between 0 and 2 was considered “low,” between 3 and 5 “intermediate,” and between 6 and 8 “high.” A pathologist with no previous knowledge of the study scored the slides. The slides immunostained with anti-PCNA antibody were also quantitatively evaluated by counting cells displaying positive nuclear staining among 1000 cells detected in 5 randomly selected areas where there was a maximum number of PCNA positive cells.

**Determination of PKA activity**

PKA activity was measured in tissue lysates (30 µg protein) prepared from adenomas and normal glands using a PKA kinase activity assay kit (StressGen, Ann Arbor, MI). The assay was based on an enzyme-linked immunoabsorbent assay (ELISA) that utilized a specific synthetic peptide as a substrate for PKA and a polyclonal antibody specific to the phosphorylated form of the substrate. PKA activity specificity was ensured by measuring activities in the presence of a PKA inhibitor. PKA activities were determined in the presence and absence of 2 µM cAMP, and represented total and free PKA activity in tissues, respectively.

**Statistical analysis**

Statistical analysis was performed by Student’s t-test, and a P value < 0.05 was considered to be significant.

**Results**

**High expression of RIA in PHP adenomas**

RIA expression was evaluated by Western blot analysis, using 8 pairs of tissue lysates prepared from adenomas and normal glands. As shown in Fig. 1, a single, 49-kD, RIA band was detected. Five out of 8 adenomas (Case 1, 2, 4-6) exhibited increased RIA expression in adenomas, compared with the normal glands. However, Case 7 and 8 presented with decreased RIA expression. The lack of GAPDH expression in the normal gland of Case 3 was most likely due to protein degradation during preparation.

The functional significance of high RIA expression in adenomas was evaluated by measuring PKA activity...
in tissue lysates prepared from adenomas and normal glands (Case 1-6). Free PKA activity was measured in the absence of 2 µM cAMP, and was normalized by total PKA activity measured in the presence of 2 µM cAMP. In Case 7 and 8, since protein sample was not sufficient for PKA activity measurement after use for Western blot analysis, PKA activity measurement could not be performed. The individual values are displayed in Fig. 2A. As shown in Fig. 2B, The ratio of free to total PKA activity in adenomas was significantly less than in normal glands (0.24±0.18 vs. 0.63±0.18; Mean ± SD, p<0.05).

RIα expression was also immunohistochemically analyzed on 22 adenomas and 11 normal glands. Representative images are shown in Fig. 3. In the adenomas (Fig. 3A), most parenchymal cells were uniformly stained, which clearly indicated cytoplasmic distribution of RIα protein. In contrast, RIα was weakly stained in the normal gland (Fig. 3B). Immunostained slides were then semi-quantitatively evaluated using the “Allred Score” system. The individual scores are displayed in Fig. 3C. As shown in Fig. 3D, adenomas presented with a significantly higher average RIα staining score, compared with the normal glands (6.86±0.09 vs. 3.36±2.58; Mean ± SD, p<0.05). The proportion, intensity, and total scores of individual specimens are summarized in Table 1. Most adenomas (20 out of 22) exhibited a “high” score (Allred score 6-8), while only 2/11 normal glands exhibited a “high” score.

**Negative correlation between RIα staining scores and PKA activity**

A possible correlation between the RIα staining scores and Free/total PKA activity ratio (PKA activity)
PRKAR1α expression in parathyroid adenoma

from 6 adenomas and 6 normal glands was investigated. There was a significant negative correlation between R1α expression and PKA activity \( y = -0.055x + 0.704, R^2 = 0.346, p = 0.035 \), reflecting the role of R1α to suppress PKA activity.

**Positive correlation between adenoma R1α staining scores and pre-operative serum levels of intact PTH**

The adenoma R1α staining scores and pre-operative serum levels of intact PTH, normalized by adenoma weights, were plotted to investigate a possible correlation between R1α expression and PTH production. As shown in Fig. 4, there was a significant positive correlation between the two parameters \( y = 0.120x - 0.446, R^2 = 0.282, p = 0.013 \), suggesting that R1α expression correlated with a hyperfunctional adenoma phenotype.
High RIα and PCNA expression in both chief and oxyphilic adenoma cells

According to the immunohistochemical analysis, many adenomas contained clusters of oxyphilic cells (Fig. 5A). The estimated composition of oxyphilic cells in each adenoma is displayed in Table 1. Adenomas from Cases 14 and 18 were determined to be “oxyphil adenomas,” which contained > 90% oxyphilic cells [23]. To determine the difference between RIα expression in chief and oxyphilic cells, the RIα staining scores were separately analyzed. The individual scores are depicted in Fig. 5B. As shown in Fig. 5C, the RIα expression in both chief and oxyphilic cell adenoma populations was significantly higher than in chief cells of normal glands (p < 0.05). PCNA immunohistochemistry staining was performed to identify proliferating cells. As shown in Fig. 6A, some chief and oxyphilic cell nuclei were PCNA-positive. PCNA-positive cells were separately counted in chief and oxyphilic cell populations and the individual values were depicted in Fig. 6B. As shown in Fig. 6C, the number of PCNA-positive cells in both chief and oxyphilic cell adenoma populations was significantly greater than in chief cells of normal glands (p < 0.05).

Discussion

The present study demonstrated elevated RIα expression in PHPT adenomas. Previous studies have reported elevated RIα expression in a variety of tumors, such as breast, renal, colorectal, and ovarian cancers, as well as retinoblastoma [24, 25], which correlated with a worse patient prognosis in these cancer patients. Elevated expression has also been reported in several human cancer cell lines [25]. Increased intracellular RIα levels were shown to promote G1 to S cell cycle transition [26, 27]. Furthermore, RIα expression inhibition by antisense oligodeoxynucleotide resulted in growth arrest and/or apoptosis of several cancer cell lines [28]. In addition, RIα antisense oligodeoxynucleotide inactivated the anti-apoptotic protein bcl-2 via PKA activation [24, 29, 30]. Apart from inhibition of the PKA catalytic subunit, RIα function was also reported [31]. Specifically, RIα is directly bound to cytochrome oxidase, and RIα inhibition results in cytochrome c release and apoptosis. These reports strongly suggest that elevated RIα expression could trigger cell cycle progression and apoptosis suppression.

![Fig. 5 Elevated RIα expression in chief and oxyphilic adenoma cells. Immunohistochemistry was performed on adenoma and normal gland tissue sections. RIα expression was visualized utilizing the UIP method. Sections were then counterstained with hematoxylin. A representative image of an adenoma, containing both chief and oxyphilic cells, is shown (Panel A). Scale bar, 200 µm. Semi-quantitative analysis using the “Allred Score” system was separately performed on chief and oxyphilic cell populations and the individual values were depicted in Panel B. As shown in Panel C, the number of PCNA-positive cells in both chief and oxyphilic cell adenoma populations was significantly greater than in chief cells of normal glands (p < 0.05).](image-url)
Paradoxically, inactivation of R1α results in the Carney Complex (CNC) [32, 33], which is a multiple neoplasia syndrome characterized by spotty skin pigmentation, cardiac and other myxomas, endocrine tumors, and schwannomas [34, 35]. Endocrine tumors are often multiple. It could be speculated that PKA activity is strictly controlled in normal cells and subtle dysregulation of PKA activity could, therefore, result in tumorigenesis [36, 37]. The oncogenic effects of altered R1α expression might depend upon the cell types. Although the cAMP/PKA pathway plays an important role in proliferation, differentiation, and oncogenic transformation of various cells, little is known about the role of this pathway in parathyroid cell proliferation. Further studies are required to elucidate these mechanisms.

In endocrine organs, in particular parathyroid glands, oxyphilic cells appear and increase with age. The oxyphilic cells in parathyroid glands are thought to be functionally inactive for PTH production [23]. However, it has been reported that, in hyperfunctional adenomas, oxyphilic cells synthesize and secrete PTH [38]. However, the molecular basis for this phenotype change remains obscure. Consistent with this report, the present study demonstrated that the preoperative serum levels of intact PTH are elevated in adenomas that consist primarily of oxyphilic cells (Table 1). Furthermore, in adenomas, oxyphilic cells, as well as chief cells, express high levels of R1α. In addition, many PCNA-positive cells were detected in chief and oxyphilic adenoma cell populations. These results strongly suggest that elevated R1α expression is involved in the tumorigenesis of oxyphilic cells, as well as chief cells.

In conclusion, while our previous results showed that R1α is also highly expressed in nodular hyperplasia of hyperparathyroidism associated with chronic renal failure [20], the present results suggest that dysregulated, elevated R1α expression could contribute to monoclonal proliferation of parathyroid cells by impairing the cAMP/PKA signaling pathway.

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References


