CASR Expression Differs Between Secondary Hyperparathyroidism and Primary Hyperparathyroidism

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Abstract

OBJECTIVE: Calcium-sensitive receptor (CASR) play an important role in the pathogenesis and progression of secondary parathyroidism (SHPT). The purpose of this study is to study the protein and gene expression characteristics of CASR in SHPT.

Methods: Immunohistochemistry and real-time PCR were used to detect the expression of CASR protein and genes in SHPT tissues, and compared with the expression of CASR in primary hyperparathyroidism (PHPT).

Results: CASR protein was down-regulated in SHPT and PHPT compared with normal parathyroid tissue; 2.42±0.5 vs 3.2±0.62, P <0.05; 1.8±0.83 vs 3.2±0.62, P <0.05; meanwhile SHPT tissue was higher than PHPT in the expression of CASR protein; 2.42±0.5 vs 1.8±0.83, P <0.05; CASR expression in SHPT tissue was higher than PHPT in gene expression; 0.29±0.23 vs 0.01±0.12, P <0.05.

Conclusion: The expression of CASR protein and gene in SHPT is higher than that of PHPT. This feature provides a theoretical basis and ideas for studying the mechanism of CASR down-regulation.

Introduction

Secondary hyperparathyroidism is a common comorbidity of chronic kidney disease (CKD). It occurs early in the progression of renal insufficiency and is an adaptation mechanism of the body that helps maintain mineral balance. SHPT will show phosphorus retention, hyperphosphatemia, elevated PTH, increased fibroblast growth factor 23 (FGF23), lack of 1,25-dihydroxyvitamin D, hypocalcemia, intestinal calcium absorption and poor expression of CASR and vitamin D receptor (VDR)[1]. SHPT is the leading cause of death and cardiovascular events in CKD patients. The main determinants of parathyroid dysfunction in CKD are CASR and VDR. CASR directly regulates the secretion of PTH, and CASR and VDR signaling pathways affect the transcription of PTH genes, the expression of PTH mRNA, and the proliferation of parathyroid glands. After a long course of disease and end-stage renal disease, the parathyroid glands change from diffuse hyperplasia to nodular hyperplasia, and the reduction of CASR and VDR occurs in this process, but the mechanism is not clear.

Primary hyperparathyroidism (PHPT) is a disease caused by increased secretion of PTH caused by tumor-like hyperplasia of one or more parathyroid glands. Parathyroid adenoma is the most common cause. Sporadic adenoma accounts for 85% of PHPT, and hereditary adenoma accounts for 10–20%. Genetically related genes include the MEN1 and RET genes. The molecular mechanism of sporadic parathyroid tumors is not clear. Some clinical studies have revealed that Germ cell and somatic gene mutations are related to this, including CASR[2]. KOH[3] found that CASR is one of the genes that play an important role in parathyroid adenoma.

It has been reported in the literature that CASR has decreased protein and gene expression in both secondary and primary hyperparathyroidism, but there are fewer reports on the differences between the
two. The different expression of proteins and genes in secondary hyperthyroidism, compared with primary hyperparathyroidism, provides theoretical basis and ideas for studying the mechanism of CASR down-regulation in SHPT.

Materials And Method

Patient

The specimens of this study were collected from patients who underwent surgery at the China-Japan Friendship Hospital from 2013 to 2016. Both secondary hyperparathyroidism and primary hyperparathyroidism were confirmed by pathological diagnosis. Normal parathyroid tissue was taken from parathyroid tissue that was accidentally removed during thyroidectomy.

Immunohistochemistry

Immunohistochemical staining of CASR was performed on 31 cases of secondary hyperparathyroidism, 20 cases of primary hyperparathyroidism, and 20 cases of normal parathyroid tissue. Staining assesses parathyroid adenomas, nodular hyperplasia glands, and normal glands. The parathyroid tissues of 71 patients with formalin-fixed paraffin were evaluated. Mouse CASR monoclonal antibody (Thermo Fisher, MA1-934) was diluted 1:1500. Formalin-embedded tissue showing the best histological characteristics was selected, and sliced into 4 micron slices with a slicer, and the sections were stained with the EnVision / HRP method using an automatic immunostainer. Microscopic evaluations were performed under 20x and 40x microscopes. Count 500 cells (5 different regions, 100 cells each) to evaluate the percentage of CASR expression. CASR expression was defined as: cell membrane and cytoplasm staining, CASR staining was weak at +, moderate at ++, strong at +++ and very strong at ++++. Observers are not involved while immunohistochemistry is in progress.

RNA isolation and real-time reverse transcription quantitative PCR

31 cases of secondary hyperparathyroidism tissue and 16 cases of primary hyperparathyroidism tissue were stored at 80 °C until RNA isolation. Total RNA was isolated using the TaKaRa MiniBest Universal RNA Extraction Kit (TaKaRa Biochemicals, Osaka, Japan). The first-strand cDNA extraction was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa Biochemicals, Osaka, Japan). ABI 7500 FAST real-time PCR detection system (ABI, CASRisbad, CA, USA) and SYBR Premix Ex Taq™ (TaKaRa Biochemicals, Osaka, Japan) were used for real-time quantitative PCR analysis. The CASR primer sequences and internal reference gene sequences used for real-time reverse transcription quantitative PCR are shown in Table 1. The specificity of the PCR product was verified by melting curve analysis. The mRNA expression of the target gene CASR was measured with $2^{-\Delta ct}$, $\triangle ct = \text{CQR ct value - \beta-actin ct value}$.
Statistical methods

The results were analyzed statistically using SPSS software. Quantitative data between multiple groups were analyzed by analysis of variance test. Quantitative data between two groups were analyzed by independent sample T test. Values were expressed in the form of mean ± standard deviation. P value ≤ 0.05 was considered statistically significant.

Result

We analyzed CASR protein expression in 71 specimens (31 cases of secondary hyperparathyroidism, 20 cases of primary hyperparathyroidism and 20 cases of normal parathyroid tissue). The CASR expression of secondary hyperparathyroidism was lower than that of normal parathyroid tissue (2.42 ± 0.5 vs 3.2 ± 0.62 P < 0.05), and higher than that of primary hyperparathyroidism tissue (2.42 ± 0.5 vs 1.8 ± 0.83 P < 0.05). The CASR expression of normal parathyroid tissue is higher than both of SHPT and PHPT (P < 0.05) (Table 2, Fig. 1,2,3,4,). The expression in thyroid tissue is negative (Fig. 5). We analyzed CASR mRNA expression in 48 cases (31 cases of secondary hyperparathyroidism, 16 cases of primary hyperparathyroidism). The expression of CASR mRNA in secondary hyperparathyroidism was higher than that in primary hyperparathyroidism (0.29 ± 0.23 vs 0.01 ± 0.12 P < 0.05) (Table 3 and Fig. 6)

| Gene            | 5'-3' primer         | 3'-5' primer         |
|-----------------|----------------------|----------------------|
| CASR[4]         | CGGGGTACCTAAGCCTACCTACGGCATCTAA | GCTCTAGAGTAAACGCGATCCCAAAGGGCTC |
| β-actin[3]      | ACTCTTCCAGGCTTTCTTCC  | CAGGAGGAGCAATGATCTTG |

| Quantity | Expression value mean ± SD | P value |
|----------|---------------------------|---------|
| SHPT     | 31                        | 2.42 ± 0.5 | <0.05  |
| PHPT     | 20                        | 1.8 ± 0.83 | <0.05  |
| Normal parathyroid | 20                      | 3.2 ± 0.62 | <0.05  |
Table 3
T test for SHPT and PHPT CASR mRNA expression (realtimePCR $2^{-\Delta ct}$)

| Quantity | CASR/β-actin | P value |
|----------|--------------|---------|
| SHPT     | 0.29 ± 0.23  | <0.05   |
| PHPT     | 0.01 ± 0.12  | <0.05   |

Discuss

The human CASR gene is located on chromosome 3q13.3-21, and is abundantly expressed in parathyroid gland, kidney, and C cells near the thyroid follicles. CASR is a G protein-coupled receptor that is sensitive to extracellular calcium and plays a key role in calcium balance. Under normal circumstances, calcium-activated CaSR triggers the mitogen-activated protein kinase C (MAPK) cascade, promotes the synthesis of phospholipase A2 and the production of arachidonic acid, and ultimately reduces the synthesis and secretion of PTH[5], CASR activation can also inhibit parathyroid cell proliferation, 1,25-dihydroxyvitamin D3 synthesis, and renal calcium reabsorption.

SHPT is a chronic progressive disease that is common in patients with chronic kidney disease and has a poor prognosis, especially for hemodialysis patients. In the United States, the prevalence of CKD patients with SHPT ranges from 2 to 5 million, with 30–50% of end-stage renal disease (ESRD) patients having SHPT[6]. According to the results of dialysis and practice model studies (DOPPS), 27% of patients with end-stage renal disease (ESRD) have higher parathyroid hormone levels than recommended by the Kidney Disease Outcome Quality Initiative (KDOQI)[7].

The consequence of SHPT is a disorder of bone metabolism, affecting 40–87% of dialysis patients, including high-transport bone disease, which can reduce bone mass, often accompanied by bone pain and fractures [8]. Extra-skeletal manifestations include calcification of soft tissues and blood vessels, increasing the risk of cardiovascular disease, and may lead to very high cardiovascular mortality in dialysis patients. In fact, more than 50% of patients with chronic kidney disease die of cardiovascular disease, and patients with chronic kidney disease receiving dialysis are 10 times more likely to develop cardiovascular disease and death than the general population[9].

In current treatments, a significant proportion of patients have insufficient control of PTH, phosphorus, and / or calcium levels, and their range often exceeds the recommended range [10]. Data from dialysis results and practice model studies (DOPPS) indicate that in patients receiving hemodialysis over 180 days, the risk of cardiovascular and all-cause death is greater when calcium levels exceed 10 mg / dL, phosphorus levels exceed 7 mg / dL, and PTH levels exceed 600 pg / mL; risks are likewise increased in patients with combinations of these high-risk categories.[11, 12]
At present, the treatment of SHPT should follow three steps: reducing the absorption of phosphorus through dietary restriction or using phosphate binders; the control of vitamin D metabolites on PTH and the use of calcimimetics[13]. Researchers have generally recognized the basic role of CaSR in regulating PTH secretion, and emphasized the potential therapeutic value of drugs that regulate CaSR activity in parathyroid tissue[14, 15]. The emergence of new types of calcimimetics such as cinacalcet and etelcalcetide brings hope for treatment of SHPT patients. The target of these drugs is to increase CASR, but there are also bottlenecks such as drug side effects, high cost, resistance and poor treatment. Parathyroidectomy is usually the last treatment strategy after failed drug treatment, but there are problems such as surgical complications, recurrence of hyperparathyroidism, severe hypocalcemia and hypokinetic bone disease. The goal of treatment is to maintain serum calcium, serum phosphorus, and PTH within acceptable target ranges[16]. Due to the limitations of SHPT treatment standards, the PTH index of many patients does not reach the ideal range [17].

In present study, we found that CASR protein was down-regulated both in SHPT and PHPT compared with normal parathyroid tissue(2.42 ± 0.5 vs 3.2 ± 0.62, P < 0.05; 1.8 ± 0.83 vs 3.2 ± 0.62, P < 0.05), meanwhile SHPT tissue was higher than PHPT in the expression of CASR protein[2.42 ± 0.5 vs 1.8 ± 0.83, P < 0.05]. Similarly, we also found that CASR expression in SHPT tissue was higher than PHPT in mRNA expression[0.29 ± 0.23 vs 0.01 ± 0.12, P < 0.05]. Many scholars have reported that the down-regulation of CASR expression in secondary parathyroidism is an important factor in the pathogenesis of SHPT, but the cause and mechanism of CASR down-regulation are unclear. It is unknown whether CASR itself or external factors. KOH et al. observed that in primary parathyroidism, abnormal elevation of RGS5 has the effect of down-regulating CASR[3]. Mizobuchi et al [18]found that Gcm2 can regulate the expression level of CASR in vitro experiments. Brown et al. [19]found that the expression of CASR mRNA and protein is often more severely suppressed in the nodular region of secondary hyperparathyroidism rats, and they noted that whether this difference is due to the higher cell proliferation rate is unclear. Ritter ‘s research on uremic rat models found that the decrease in CASR in uremic rats was mainly detected in the hyperparathyroidism area[20]. Yano et al. [21]also confirmed this observation in proliferative human parathyroid tissue. In this study, we also found that in the same tissue with secondary hyperparathyroidism, the expression of CASR in areas with significant nodular hyperplasia was relatively weak in protein level. Ritter et al.[22] pointed out that the decrease in CASR content occurred after hyperparathyroidism, so it is not the initial event of SHPT development, but may be the result of proliferation. They found that after parathyroid hyperplasia in uremia rats, limiting phosphate can reverse the down regulation of parathyroid CASR [20], and FGF23 will directly affect parathyroid proliferation and / or differential regulation of CASR and VDR expression, which It may be the direction of future research[23].

Similarly, a decrease in CASR expression also occurred in PHPT, when parathyroid gland proliferation was not triggered by external factors but by genetic abnormal stimulation[24]. Signaling pathways from CASR or controlling CASR activity may be altered in proliferating parathyroid cells. Cell proliferation may trigger a series of events that directly or indirectly lead to the down-regulation of CASR in surrounding cells. During the process, transforming growth factor-alpha (TGF-α) [25], acidic fibroblast growth factor (acidic-
FGF)[26], endothelin-1 (ET-1)[27], cyclin D1[28], parathyroid hormone related peptide (PTHrP)[29] and c-myc[30] may play a role.

At present, the mechanism of the down-regulation of CASR expression in SHPT is not very clear. One reason is the lack of an effective control group. As for the negative control, the parathyroid tissue of the normal population is more difficult to obtain, and the parathyroid tissue of animals cannot fully reflect the true condition of the human body. Apart from them, Primary parathyroidism provides a good positive control. The study of the differences between SHPT and PTHP provides us with effective research ideas. Under the influence of multiple factors, the development of secondary hyperparathyroidism goes through a gradual process, from diffuse hyperplasia to nodular hyperplasia to tertiary hyperplasia. CASR downregulation occurs at this stage. Mechanism of CASR downregulation is the key to exploring the pathogenesis and progression of SHPT. There is a difference in the pathogenesis of secondary hyperparathyroidism and primary hyperparathyroidism, and there are some similarities in clinical manifestations. Both of them have decreased CASR expression. It is currently found that the decrease in CASR in PHPT is more related to its genetic factors. The decrease of CASR in SHPT is related to the proliferation of parathyroid glands, but the mechanism of how CASR decreases during the proliferation process is unknown, and there may be changes in gene levels similar to PHPT. Our study compared the expression of CASR in SHPT with that in PHPT, and found that although both CASRs were down-regulated, the degree of down-regulation was not the same. The decrease in CASR expression in secondary hyperparathyroidism was not as obvious as in primary parathyroidism. This indicates that the down regulation of CASR in primary parathyroidism may occur early and severely, and changes in gene levels or signaling pathways may play a role in the difference between the two. Research around this difference will provide ideas for finding new targets to increase CASR expression.

In conclusion, SHPT is a common complication of dialysis patients with chronic kidney disease, which can lead to decreased quality of life, increased cardiovascular events, and increased mortality. There is currently no particularly effective treatment. CASR expression is down-regulated in the onset of SHPT and plays an important role in progress. Our research found that although CASR was down-regulated in both SHPT and PHPT, the degree of CASR reduction was different at both the protein level and the gene level. Through the study of this difference, we can find the cause and mechanism of CASR down-regulation, which can open up new perspectives for the treatment of SHPT.

**Declarations**

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**Conflicts of interest/Competing interests**
The authors declare that they have no conflict of interest.

Availability of data and material

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Authors' contributions

YM collected the clinical information and drafted the manuscript. LJ, SXL and JHY supported the data collection, interpretation of the data, and writing of the manuscript. SAP and ZHL carried out Immunohistochemical studies and evaluated the results. PB and lx carried out PCR study and evaluated the results. LY, ZL and HLP reviewed the draft and made critical modifications.

Ethical approval

This study has been approved by China Japan Friendship Hospital ethics committee.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Figures

![Figure 1](image)

**Figure 1**

CASR protein expression in different parathyroid tissues. Analysis of variance. P<0.05.
Figure 2

CASR expression in secondary hyperparathyroidism tissue (++, × 20)

Figure 3

CASR expression in tissues of primary hyperparathyroidism (+, × 20)
Figure 4

CASR expression in normal parathyroid glands (+++ × 20)

Figure 5

CASR expression in thyroid glands (0 × 20)
Figure 6

T test for SHPT and PHPT CASR gene expression using real time PCR $2^{-\Delta Ct}$, $P < 0.05$. 