

Calcium-sensitive receptor expression differs between primary and secondary hyperparathyroidism

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Abstract

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

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

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

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

Background

Secondary hyperparathyroidism (SHPT) 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 to help maintain the mineral balance. SHPT is characterized by phosphorus retention, hyperphosphatemia, elevated parathyroid hormone (PTH) levels, increased fibroblast growth factor 23 (FGF23), 1,25-dihydroxyvitamin D deficiency, hypocalcemia, decreased intestinal calcium absorption, and poor expression of calcium-sensitive receptor (CASR) and vitamin D receptor (VDR)[1]. SHPT is the leading cause of death and cardiovascular events in patients with CKD. 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, expression of PTH mRNA, and proliferation of parathyroid glands. After a long disease course and end-stage renal disease (ESRD), the parathyroid glands show changes from diffuse hyperplasia to nodular hyperplasia, along with a reduction in CASR and VDR; however, these mechanisms remain poorly understood.

Primary hyperparathyroidism (PHPT) is a disease caused by increased secretion of PTH due to 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 likely related, including CASR[2]. Koh et al.[3] found that CASR is one of the genes that play an important role in parathyroid adenoma.

Published literature has shown that both SHPT and PHPT are associated with decreased CASR gene and protein expression, 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 a theoretical basis and ideas for studying the mechanism of CASR down-regulation in SHPT.

Methods

Patients

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

Immunohistochemistry

Immunohistochemical staining of CASR was performed in 31, 20, and 20 tissue specimens of SHPT, PHPT, and normal parathyroid tissue, respectively. Staining assessed the 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 features was selected and sliced into 4- μ m-thick slices with a slicer, and the sections were stained with the EnVision/HRP method using an automatic immunostainer. Microscopic evaluations were performed under 20 \times and 40 \times microscopes. A total of 500 cells were counted (five different regions, 100 cells each) to evaluate the percentage of CASR expression. CASR expression was quantified as follows: cell membrane and cytoplasm staining, CASR staining was weak at +, moderate at ++, strong at +++, and very strong at +++++. Observers were not involved while immunohistochemistry was in progress.

RNA isolation and real-time reverse transcription quantitative PCR

In all, 31 SHPT and 16 PHPT tissue specimens 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). ABI 7500 FAST real-time PCR detection system (ABI, CASRisbad, CA, USA) and SYBR Premix Ex Taq™ (TaKaRa Biochemicals) 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 $2^{-\Delta\text{ct}}$: $\Delta\text{ct} = \text{CASR ct value} - \beta\text{-actin ct value}$.

Statistical methods

The results were analyzed statistically using the SPSS software. Quantitative data between multiple groups were analyzed by ANOVA. Quantitative data between two groups were analyzed by independent

sample *t*-test. Values were expressed in the form of mean±standard deviation. $P \leq 0.05$ was considered to indicate statistical significance

Result

We analyzed CASR protein expression in 71 tissue specimens (31 SHPT, 20 PHPT, and 20 normal parathyroid tissue). The CASR expression in SHPT was lower than that in normal parathyroid tissue (2.42 ± 0.5 vs. 3.2 ± 0.62 , $P < 0.05$) and higher than that in PHPT (2.42 ± 0.5 vs. 1.8 ± 0.83 , $P < 0.05$). The CASR expression of normal parathyroid tissue was higher than both SHPT and PHPT tissues ($P < 0.05$) (Table 2, Figures 1–4). The expression in thyroid tissue was negative (Figure 5). We analyzed CASR mRNA expression in 47 cases (31 SHPT and 16 PHPT). The expression of CASR mRNA in SHPT was higher than that in PHPT (0.29 ± 0.23 vs. 0.01 ± 0.12 , $P < 0.05$) (Table 3 and Figure 6).

Table 1. Primers used for realtimePCR

Gene	Forward	Reverse
CASR[4]	CGGGGTACCTTAAGCACCTACGGCATCTAA	GCTCTAGAGTTAACGCGATCCCAAAGGGCTC
β -actin[3]	ACTCTTCCAGCCTTCCTTCC	CAGGAGGAGCAATGATCTTG

Table 2. CASR protein expression in different parathyroid tissues

Tissue	Quantity	Expression value mean \pm 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 by realtimePCR $2^{-\Delta ct}$

Gene	Quantity	CASR/ β -actin $2^{-\Delta ct}$ ($\bar{x} \pm sd$)	P
SHPT	31	0.29 ± 0.23	≤ 0.05
PHPT	16	0.01 ± 0.12	≤ 0.05

Discussion

The human *CASR* gene is located on chromosome 3q13.3-21 and is abundantly expressed in the 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 maintaining calcium balance. Under normal circumstances, calcium-activated CASR triggers the mitogen-activated protein kinase C (MAPK) cascade, promotes the synthesis of phospholipase A2, and production of arachidonic acid, which 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 CKD and has a poor prognosis, especially for those undergoing hemodialysis. In the United States, the prevalence of CKD patients with SHPT ranges from 2 to 5 million, with 30–50% patients with ESRD having SHPT[6]. According to the results of Dialysis Outcomes and Practice Patterns Study (DOPPS), 27% patients with 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 including high-transport bone disease, which can reduce bone mass and is often accompanied by bone pain and fractures; an estimated 40–87% of dialysis patients are affected by this[8]. Extra-skeletal manifestations include calcification of soft tissues and blood vessels and increased risk of cardiovascular disease, and may lead to very high cardiovascular mortality in dialysis patients. In fact, >50% patients with CKD die of cardiovascular disease, and patients with CKD receiving dialysis are 10 times more likely to develop cardiovascular disease and die than the general population[9].

According to current treatments, a significant proportion of patients have insufficient control of PTH, phosphorus, and/or calcium levels, and their range often exceeds the recommended values[10]. Data from DOPPS indicate that in patients receiving hemodialysis over 180 days, the risk of cardiovascular and all-cause death is greater when the levels of calcium, phosphorus, and PTH exceed 10 mg/dL, 7 mg/dL, and 600 pg/mL, respectively. The 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; controlling 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 new hope for treatment of SHPT. The target of these drugs is to increase CASR, but there are also bottlenecks such as drug side effects, high cost, resistance, and poor response. 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]. Given the limitations of SHPT treatment standards, the PTH index of many patients does not reach the ideal range [17].

CASR protein was down-regulated in both SHPT and PHPT compared with normal parathyroid tissues (2.42 ± 0.5 vs. 3.2 ± 0.62 , $P < 0.05$; 1.8 ± 0.83 vs. 3.2 ± 0.62 , $P < 0.05$). Further, SHPT tissue showed higher expression of both CASR protein (2.42 ± 0.5 vs. 1.8 ± 0.83 , $P < 0.05$) and CASR mRNA (0.29 ± 0.23 vs. 0.01 ± 0.12 , $P < 0.05$) than PHPT tissue, respectively. 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 it due to 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 *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 rats with SHPT; however, it was unclear whether this difference was due to the higher cell proliferation rate. 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 the present study, we found that in the same tissue with SHPT, the expression of CASR in areas with significant nodular hyperplasia was relatively weak at the 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, rather 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 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 rather by gene-related 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, and 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 in this process.

At present, the mechanism of down-regulation of CASR expression in SHPT is not very clear. One reason is the lack of an effective control group. It is difficult to obtain parathyroid tissue of the normal population as a negative control, and the parathyroid tissue from animal models cannot fully reflect the true condition of the human body. Primary parathyroid tissue provides a good positive control. The study of the differences between SHPT and PHPT provides further effective research ideas. Under the influence of multiple factors, SHPT development undergoes a gradual process, from diffuse to nodular to tertiary hyperplasia. CASR downregulation occurs at this stage. The mechanism of CASR downregulation is the key to exploring the pathogenesis and progression of SHPT. Despite the differences in pathogenesis of SHPT and PHPT, there are some similarities in clinical manifestations. Both conditions are associated with decreased CASR expression. The present understanding is that 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 that found in PHPT.

Our study compared the expression of CASR in SHPT and PHPT and found that although CASR was down-regulated in both conditions, the degree of down-regulation in CASR expression in SHPT 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 further ideas for identifying new targets to increase CASR expression.

Conclusion

SHPT is a common complication in patients undergoing dialysis for CKD, which can lead to decreased quality of life, increased cardiovascular events, and increased mortality. There is currently no particularly effective treatment for SHPT. CASR expression is down-regulated at the onset of SHPT and plays an important role in its progression. 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 and gene levels. By further analyzing this difference, we can find the cause and mechanism of CASR down-regulation, which presents new perspectives for the treatment of SHPT.

Declarations

Ethics approval and consent to participate

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

Consent for publication

Not applicable.

Availability of data and materials

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.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

YM collected the clinical information and drafted the manuscript. LJ, SXLand 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.

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Figures

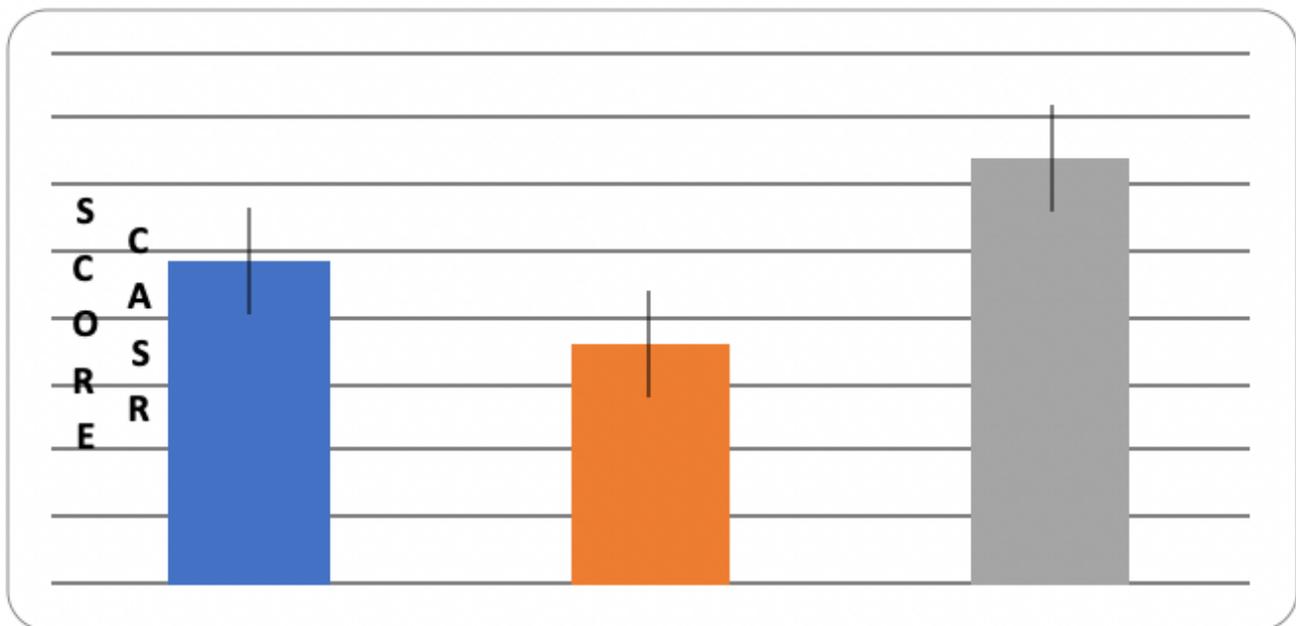


Figure 1

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

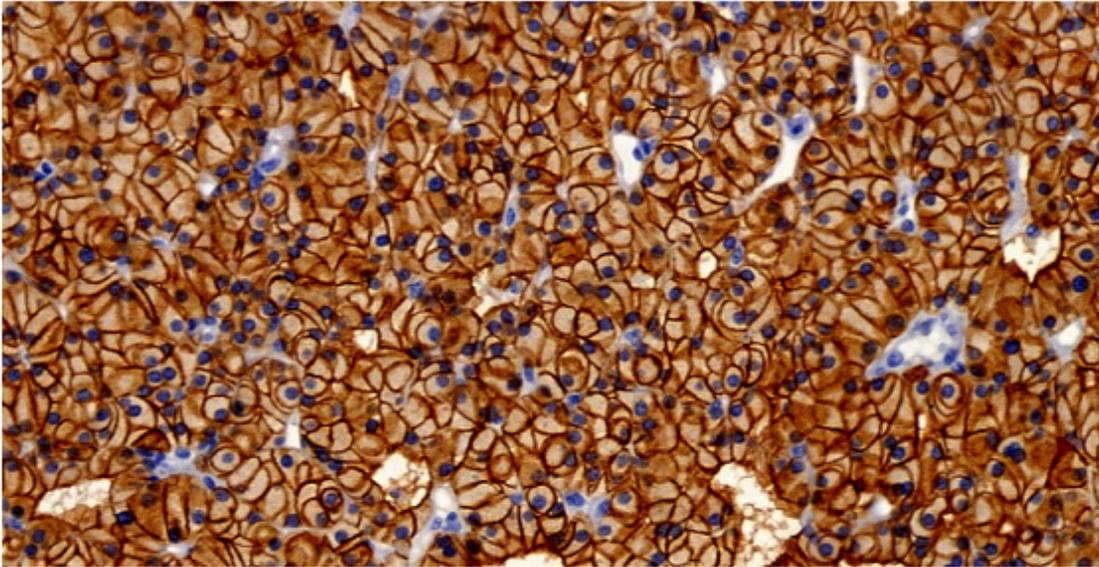


Figure 2

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

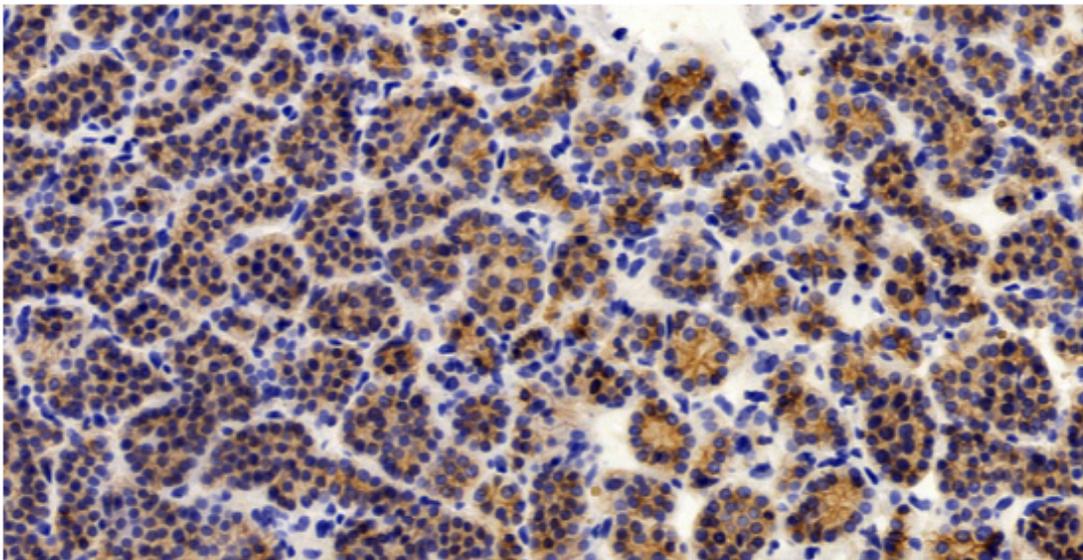


Figure 3

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

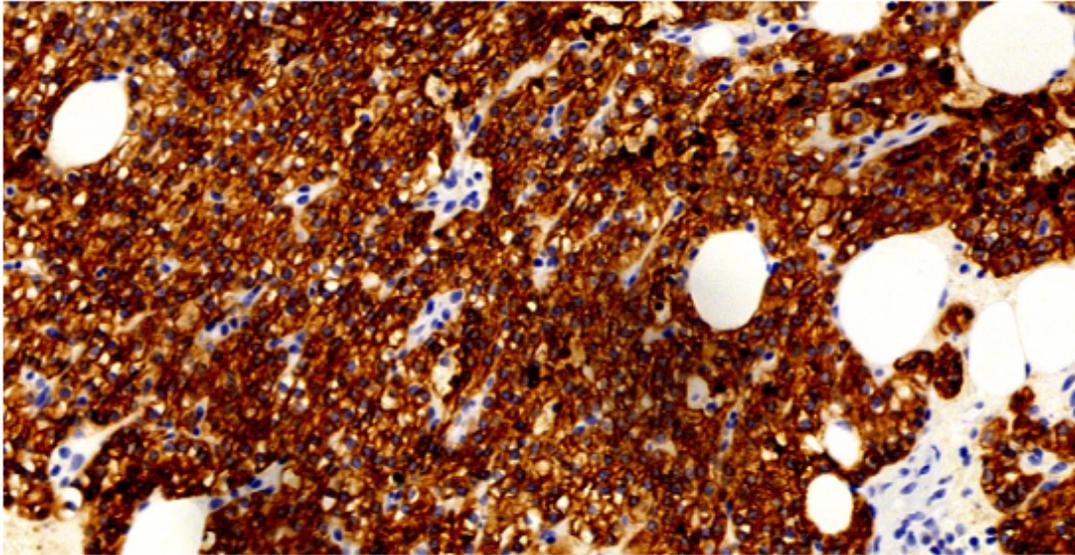


Figure 4

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

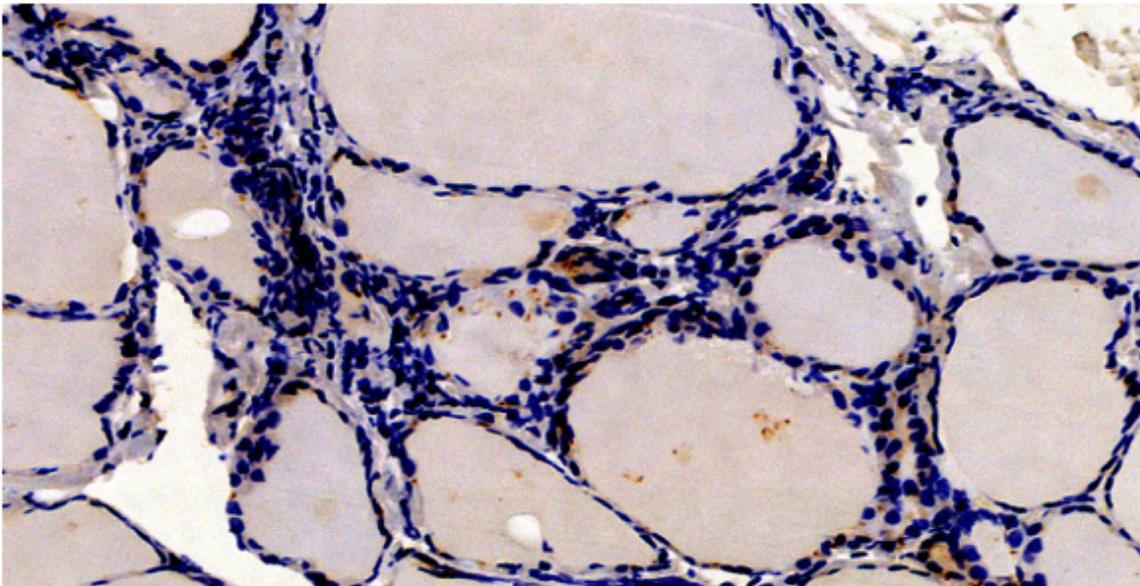


Figure 5

CASR expression in thyroid glands (0, × 20)

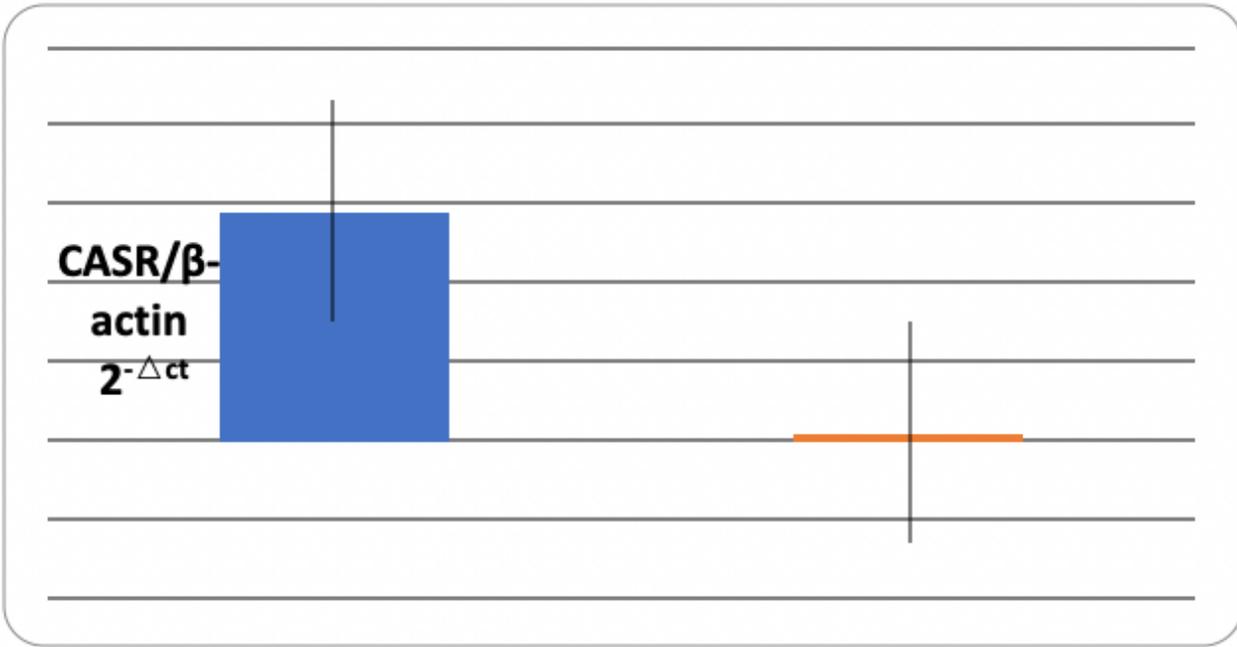


Figure 6

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