

## Original Article

# The lower expression of gonadotropin-releasing hormone receptor associated with poor prognosis in gastric cancer

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**Abstract:** Aims: Expression of gonadotropin-releasing hormone receptor (GnRHR) has been demonstrated in a number of malignancies. The aim is to investigate the expression of GnRHR and prognosis in gastric cancer. Methods and materials: GnRHR mRNA was examined in tumor and non-tumor tissues from 48 gastric cancer patients by Real-time PCR. The GnRHR protein expression was performed by immunohistochemical analysis. Results: The expression of GnRHR mRNA was higher (mean  $\pm$  SD,  $-10.06 \pm 1.28$ ) in gastric tumor tissues than matched non-tumor tissues (mean  $\pm$  SD,  $-12.43 \pm 1.33$ ). GnRHR mRNA expression was associated with lymph node metastasis, distant metastasis, and TNM stage. We found the decreased expression of GnRHR mRNA were significantly correlated with poor overall survival ( $P = 0.003$ ). Immunocytochemical staining of GnRHR in tumor tissues showed mainly weak staining (43.48%, 10/23) and moderate staining (21.74%, 5/23) in high GnRHR mRNA patients, and mainly negative staining in low GnRHR mRNA patients. And the staining of GnRHR was not detection in tumor tissues for more than half of gastric patients (52.08%, 25/48). These results implied that the loss of GnRHR protein could be a main event in gastric cancer. Conclusion: The GnRHR expression is very low in gastric cancer, and the loss of GnRHR expression could be a poor prognostic factor, which implied that GnRHR could play an important role in the development of gastric cancer.

**Keywords:** GnRHR, gastric cancer, prognosis

## Introduction

Gastric cancer is one of the most common malignant diseases in China, which is correlated to many factors such as eating habits, environmental factors, chronic gastritis, and *Helicobacter pylori* infection [1]. Therefore, the underlying molecular mechanisms of gastric cancer and how to overcome them is still a critical issue.

GnRH has been known as a hypothalamic hormone, which is involved in the control of gonadotroph cells. The binding of GnRH to its specific cell surface receptor, the gonadotropin releasing hormone receptor (GnRHR) stimulates gonadotropin secretion [2, 3]. GnRH produces two isoforms, GnRHR-I and GnRHR-II, in humans, which are members of the G-protein

coupled receptor family of proteins and function in the inositol phosphate signaling pathway [4, 5]. But the mRNA for GnRHR-II is not expressed into a functional protein in human [6]. And some reports indicated that the GnRH and its receptor were expressed in other peripheral tissues including the breast, ovary, endometrium, placenta, and prostate. Meanwhile literatures implied that GnRH and GnRHR was expressed in some type of tumor cells, such as breast, prostate, endometrial cells in endometriosis and endometrial cancer, ovarian, pancreatic and hepatoma [7]. GnRH and GnRHR genes are expressed in some cell lines, which act as an autocrine regulator on the growth of cancer cells [4]. And they could be a novel role in tumor progression such as metastasis and angiogenesis, which is closed with proliferation and migration in cancer cells [8].

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Although the expression of GnRH and GnRHR in cancer cells is very low, but it could have a high-affinity/low-affinity binding sites by GnRH in cancer cells [9]. GnRH analogs have an anti-proliferative effect by bound to GnRHR, activating a phosphotyrosine phosphatase which dephosphorylates EGFRs in cancer cells [7]. GnRH and GnRHR were expressed more frequently in breast tumors than in the adjacent mammary tissue, which is correlated significantly with poor prognosis pathological parameters [10].

Several reports implied that GnRH and GnRHR could be classified as a kind of brain-gut hormone, because they were expressed in rat digestive tract, submaxillary gland, thyroid gland and intestine [4, 11, 12]. Lei Chen et al. have demonstrated that GnRH receptor was expressed in cultured gastric smooth muscle cells of rats and GnRH analogue had a direct growth inhibitory effect through GnRHR, which strongly suggested that GnRH play an important role in digestive tract [13]. GnRH analogue could inhibit the gastric acid secretion both by direct actions on parietal cells and by inhibiting vagus function, which modulate physiological function of gastric parietal cells through autocrinal and paracrine way [14].

By now, there still have been few reports concerning the existence and expression of GnRHR in human gastric cancer. We detected the distribution of GnRHR in gastric cancer tissues, and to explore the correlations between GnRHR expression and clinicopathologic variables in gastric cancer patients.

### Materials and methods

#### *Subjects and tissues*

The tissues (tumor tissues and matched non-tumor tissues) resected with gastric cancer were retrieved from the surgical pathology files of Changzhou Cancer Hospital (China). The specimens were fixed in 10% formalin and embedded in paraffin wax for immunohistochemistry. Specimens were kept in  $-196^{\circ}\text{C}$  until processing for RNA extraction. These patients included 30 males and 18 females with a median age of 50 years (range: 39-70). All tumors were histologically diagnosed independently by three experienced pathologists without any knowledge of clinical data. Written informed

consent was obtained from each patient, and research protocols for this study were approved by the ethics committee at Soochow University School of Medicine (China).

#### *RNA extraction and reverse transcription reaction*

Total RNA from tissues was extracted using TRIzol reagent. The first-strand cDNA was synthesized from 2  $\mu\text{g}$  of total RNA according to the manufacturer's protocol. Primer sequences of GnRHR for RT-PCR reaction were forward (5'-GACCTTGCTGGAAAGATCC-3') and reverse (5'-CAGGCTGATCACCACCATCA-3') [15]. Quantitative real-time PCR (qPCR) were carried out by using the Mx3000P QPCR System (Stratagene, California, USA). The cDNA was then used for qPCR in a 20  $\mu\text{l}$  SYBR Premix Ex Taq. qPCR was performed under the following conditions: 5 min at  $95^{\circ}\text{C}$ , 40 cycles of 40 seconds at  $95^{\circ}\text{C}$ , 32 seconds at  $60^{\circ}\text{C}$ , and 30 seconds at  $72^{\circ}\text{C}$ . As an internal control,  $\beta$ -actin mRNA expression was amplified from the same cDNA samples.  $C_T$  values for triplicate reactions were averaged and relative GnRHR expression was determined with the comparative  $C_T$  method, using average  $C_T$  values for GnRHR and  $\beta$ -actin.

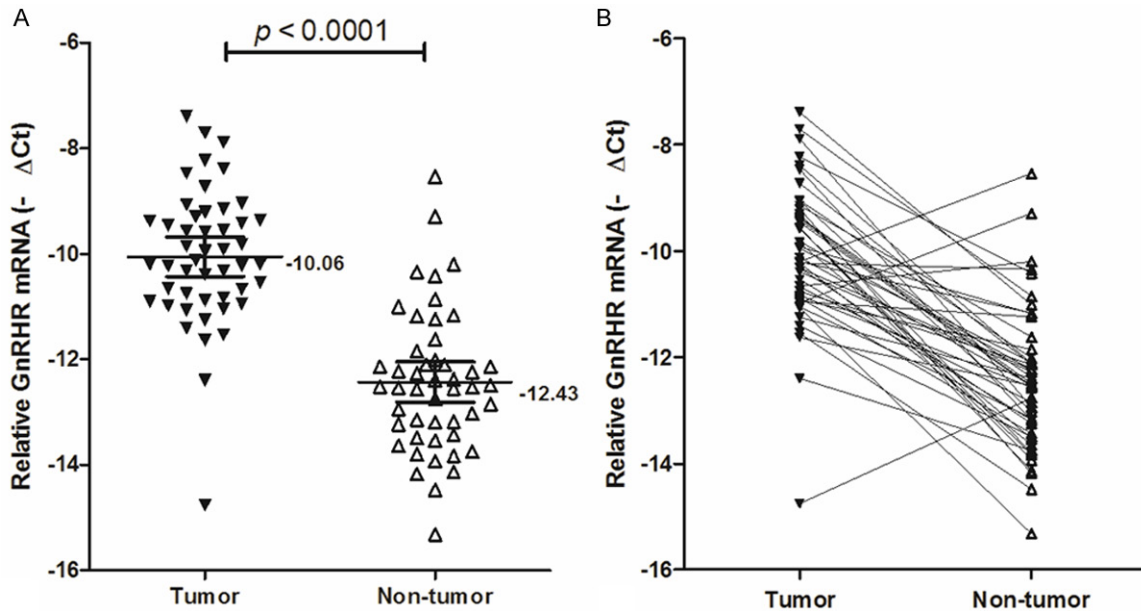
#### *Immunohistochemistry*

Immunohistochemical analysis was performed by goat polyclonal antibody for GnRHR (Santa Cruz Biotechnology, Inc.). The slides were heated at  $120^{\circ}\text{C}$  for 5 min in citric acid buffer. The dilution of GnRHR antibody was 1:400. Normal goat IgG was also used in place of the primary antibodies as a negative control. Slides were incubated with hydrogen peroxide for 30 min and stained with tertiary ABC reagent (Pierce, USA) for 1 h. DAB (Sigma, USA) was added for 2 to 5 min and slides counterstained with Nuclear Fast Red (Sigma, USA). Slides were scanned using the Leica DM RXA2 (Leica Microsystems, Germany) at  $\times 200$  magnifications. The specimens were evaluated by two independent observers.

#### *Statistical analysis*

All data were generated without knowledge of the clinical status of the samples analyzed by SPSS 18.0 software (SPSS, Inc., Chicago, USA). Comparison was done with t test (unpaired or paired). The survival was compared by the

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**Figure 1.** The expression of GnRHR mRNA in patients with gastric cancer. A. GnRHR mRNA was indicated in tumor and Non-tumor samples. Data are shown by the Mean<sub>-ΔCt</sub>. B. The GnRHR mRNA in Non-tumor was lower than that in the matched tumor tissues ( $P < 0.0001$ ). There was a decreased tendency for GnRHR expression from tumor tissues to Non-tumor. Statistical analyses were done using the paired t test.

**Table 1.** Correlation of clinicopathologic variables with GnRHR mRNA in gastric cancer tissues

| Characteristics       | N = 48 | GnRHR mRNA (-ΔCt) $\geq -10.06$ , n = 23 | GnRHR mRNA (-ΔCt) $< -10.06$ , n = 25 | P Value* |
|-----------------------|--------|--|---------------------------------------|----------|
| Sex                   |        |  |                                       | 0.117    |
| Male                  | 30     | 17                                       | 13                                    |          |
| Female                | 18     | 6  | 12                                    |          |
| Mean age (years)      |        |  |                                       | 0.897    |
| $\geq 50$             | 40     | 19                                       | 21                                    |          |
| $< 50$                | 8      | 4  | 4                                     |          |
| Differentiation       |        |  |                                       | 0.102    |
| Well                  | 32     | 18                                       | 14                                    |          |
| Poor                  | 16     | 5  | 11                                    |          |
| Depth of invasion     |        |  |                                       | 0.195    |
| T1 and T2             | 17     | 6  | 11                                    |          |
| T3 and T4             | 31     | 17                                       | 14                                    |          |
| Lymph node metastasis |        |  |                                       | 0.036    |
| Absent                | 35     | 20                                       | 15                                    |          |
| Present               | 13     | 3  | 10                                    |          |
| Distant metastasis    |        |  |                                       | 0.007    |
| Absent                | 28     | 18                                       | 10                                    |          |
| Present               | 20     | 5  | 15                                    |          |
| TNM stage             |        |  |                                       | 0.020    |
| I and II              | 25     | 16                                       | 9                                     |          |
| III and IV            | 23     | 7  | 16                                    |          |

\*The chi-square test was used to compare all variables.

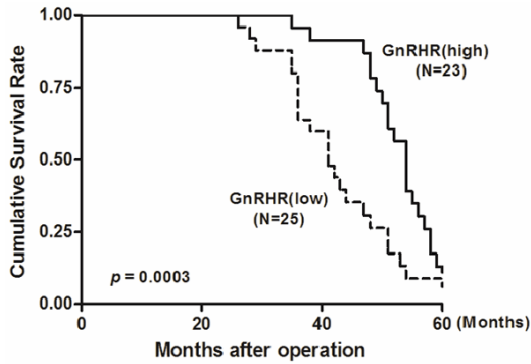
Kaplan-Meier method and the log rank test. All  $P$  values presented were two-sided, and a  $P$  value of less than 0.05 was considered statistically significant.

### Results

#### *GnRHR mRNA expression in gastric cancer tissues*

To determine GnRHR mRNA expression in tumor and non-tumor tissues, we sought to analyze GnRHR mRNA in 48 gastric patients by real-time PCR (**Figure 1A**). The expression of GnRHR mRNA was higher (mean  $\pm$  SD,  $-10.06 \pm 1.28$ ) in gastric tumor tissues than matched non-tumor tissues (mean  $\pm$  SD,  $-12.43 \pm 1.33$ ). Meanwhile, the expression of

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**Figure 2.** Kaplan-Meier survival curves for patients with gastric cancer. GnRHR mRNA was significantly correlated with patient survival ( $P = 0.0003$ ). GnRHR (high): GnRHR mRNA ( $-\Delta Ct \geq -10.06$ ); GnRHR (low): GnRHR mRNA ( $-\Delta Ct < -10.06$ ).

GnRHR mRNA was decreased only in 5 gastric patients in tumor tissues than in non-tumor tissues (**Figure 1B**). It implied that GnRHR mRNA was low expression in gastric tumor tissues, but more lower in matched non-tumor tissues. Although there is a statistically significant association between the tissues and GnRHR mRNA expression ( $P < 0.0001$ ), the GnRHR mRNA expression could be very low, whatever in cancer or non-tumor samples.

### *GnRHR mRNA expression with Clinicopathologic parameters in gastric cancer*

The correlation of GnRHR mRNA expression with clinicopathologic parameters in gastric cancer patients was analyzed in **Table 1**. We found that GnRHR mRNA expression was associated with tumor metastasis. And, high GnRHR mRNA was lower frequency with lymph node metastasis (13.04%, 3/23) compared with low GnRHR mRNA patients (40.0%, 10/25;  $P = 0.036$ ). Meanwhile, high GnRHR mRNA was also lower frequency with distant metastasis (21.74%, 5/23) compared with low GnRHR mRNA patients (60.0%, 15/25;  $P = 0.007$ ). There is a significant difference between GnRHR mRNA and TNM stage in gastric cancer ( $P = 0.02$ ). GnRHR mRNA was not associated with the others clinicopathologic parameters evaluated, including sex, tumor differentiation, and other features.

The survival of gastric cancer patients was compared by the Kaplan-Meier method and the log rank test (**Figure 2**). We found the decreased expression of GnRHR mRNA ( $-\Delta Ct < -10.06$ )

were significantly correlated with poor overall survival ( $P = 0.003$ ; HR = 0.42, 95% CI: 0.22-0.87). These results suggested that the loss of GnRHR mRNA could be poor prognostic factors in gastric cancer patients.

### *Immunocytochemical analysis of GnRHR in gastric cancer*

In order to understand the expression of GnRHR protein in gastric cancer, we carried out immunocytochemical analysis of GnRHR in tumor tissues and non-tumor tissues. We found that the staining was mainly localized in the cytoplasm (**Figure 3**). Immunocytochemical staining of GnRHR showed that gastric tumor tissues was mainly immunopositive with weak staining (43.48%, 10/23) and moderate staining (21.74%, 5/23) in high GnRHR mRNA patients. On the other, the staining of GnRHR was mainly negative staining in low GnRHR mRNA patients, while a very small number (3 cases) were found with weak staining. The entirely loss staining of GnRHR was only 5 cases (21.74%) in high GnRHR mRNA patients, but 20 cases (80.0%) in low GnRHR mRNA patients.

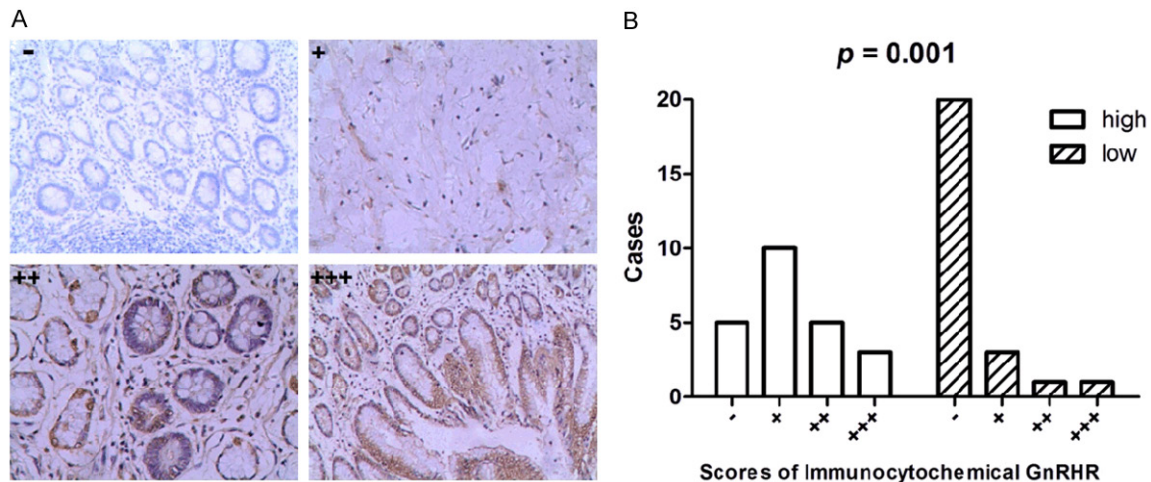
And the staining of GnRHR was not detection in tumor tissues for more than half of gastric patients (52.08%, 25/48). These results implied that the loss of GnRHR protein could be a main event in gastric cancer.

## Discussion

GnRH is the pivotal hormone in the hypothalamic-pituitary gonadal axis, and GnRHR are trans-membrane G-protein-coupled receptors [16, 17]. Anti-GnRHR monoclonal antibodies in humanized forms could function as GnRH analogs and serve as an ideal candidate of anti-cancer drugs for therapeutic treatments of various cancers [18]. For example, GnRHR represent the most effective molecular target for the treatment of steroid-dependent prostate cancer, which could suppress the pituitary-testicular axis [19].

But the clinical relevance of GnRHR in gastric cancer is not yet known. Moreover, there is little literature dealing with GnRHR in gastric cancer. In this study, we investigated the GnRHR mRNA and protein expression in gastric cancer patients. We detected the distribution of GnRHR in gastric cancer tissues by RT-PCR





**Figure 3.** Immunohistochemical analysis for GnRHR expression in gastric cancer. A. Representative immunostaining for GnRHR expression. (-): negative expression for normal gastric tissue. (+): low GnRHR expression in gastric cancer tissue. (++): middle GnRHR expression in gastric cancer tissue. (+++): high GnRHR expression in gastric cancer tissue. Magnification,  $\times 200$ . B. Semi-quantitative immunocytochemical GnRHR scores in tumor tissues. (-): negative expression; (+): low expression; (++): middle expression; (+++): high expression. High: GnRHR mRNA ( $-\Delta Ct \geq -10.06$ ); low: GnRHR mRNA ( $-\Delta Ct < -10.06$ ). Statistical analyses were done using the Pearson chi-square test.

methods. And immunocytochemical analysis was carried out to confirm the local expression of GnRHR. We found that very low transcripts levels were detected in matched non-tumor tissues in gastric cancer patients. There was some high expression of GnRHR mRNA in tumor tissues. In summary, we have demonstrated that GnRHR is partly increased in gastric cancers. On the basis of these results, it is not easy to predict what exactly might happen with low level of GnRHR mRNA in gastric tissues.

Meanwhile, our results indicated that GnRHR mRNA was associated with lymph node metastasis, distant metastasis, and TNM stage. We have also shown that patients with lower express of GnRHR have an overall poorer survival. Nevertheless, our findings seem to support that the increased expression of GnRHR may provide some benefits related to gastric cancer patients. Similarly, GnRHR transcripts were also found at higher frequency the malignant (54.2%) than the benign biopsies examined (24.0%) in human breast cancer [10]. GnRHR expression was also studied at the protein level by immunohistochemistry in our study. We found that GnRHR was localized in the membranes or in the cytoplasm of malignant cells. But few strong positive cells were detected in both non-tumor tissues and tumor tissues. And more than half of gastric patients (52.08%, 25/48) had not staining of GnRHR in

tumor tissues. Differential staining of GnRHR in the tumor tissues could explain the great variability at the translational level.

GnRH was demonstrated that it also exists in a number of organs beyond the hypothalamus and acts on extrapituitary organs [20]. Huang W, et al. have found that the epithelium of gastric pit and the cells above in digestive tract showed GnRHR immunoreactivity, which implied that the digestive tract can also produce GnRHR [11]. And our analysis showed that GnRHR expression in the benign and malignant tissues correlated positively with the presence of GnRH neuropeptide transcripts. Our study also confirms that the loss of GnRHR protein is an important event in gastric cancer. Although gastric tissue is a hormonally non-responsive tissue, GnRH and GnRHR system could play a key role in the development of this type of cancer.

Our results could increase the pathophysiological significance of the GnRH system in gastric cancer, which could contribute to the clinicopathological profiling of gastric cancer with potential clinical exploitation in prognosis and treatment.

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#### Disclosure of conflict of interest

None.

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#### References

- [1] Shi J, Qu YP and Hou P. Pathogenetic mechanisms in gastric cancer. *World J Gastroenterol* 2014; 20: 13804-13819.
- [2] Millar RP. GnRHs and GnRH receptors. *Anim Reprod Sci* 2005; 88: 5-28.
- [3] La Rosa S, Celato N, Uccella S and Capella C. Detection of gonadotropin-releasing hormone receptor in normal human pituitary cells and pituitary adenomas using immunohistochemistry. *Virchows Arch* 2000; 437: 264-269.
- [4] Naor Z. Signaling by G-protein-coupled receptor (GPCR): studies on the GnRH receptor. *Front Neuroendocrinol* 2009; 30: 10-29.
- [5] Grosse R, Schmid A, Schoneberg T, Herrlich A, Muhn P, Schultz G and Gudermann T. Gonadotropin-releasing hormone receptor initiates multiple signaling pathways by exclusively coupling to G(q/11) proteins. *J Biol Chem* 2000; 275: 9193-9200.
- [6] Pawson AJ, Morgan K, Maudsley SR and Millar RP. Type II gonadotrophin-releasing hormone (GnRH-II) in reproductive biology. *Reproduction* 2003; 126: 271-278.
- [7] Limonta P, Moretti RM, Montagnani Marelli M and Motta M. The biology of gonadotropin hormone-releasing hormone: role in the control of tumor growth and progression in humans. *Front Neuroendocrinol* 2003; 24: 279-295.
- [8] Cheung LW and Wong AS. Gonadotropin-releasing hormone: GnRH receptor signaling in extrapituitary tissues. *FEBS J* 2008; 275: 5479-5495.
- [9] Aguilar-Rojas A and Huerta-Reyes M. Human gonadotropin-releasing hormone receptor-activated cellular functions and signaling pathways in extra-pituitary tissues and cancer cells (Review). *Oncol Rep* 2009; 22: 981-990.
- [10] Pazaitou-Panayiotou K, Chemonidou C, Poupri A, Koureta M, Kaprara A, Lambropoulou M, Constantinidis TC, Galaktidou G, Koffa M, Kiziridou A, Kakolyris S, Kolios G, Kortsaris A and Chatzaki E. Gonadotropin-releasing hormone neuropeptides and receptor in human breast cancer: correlation to poor prognosis parameters. *Peptides* 2013; 42: 15-24.
- [11] Huang W, Yao B, Sun L, Pu R, Wang L and Zhang R. Immunohistochemical and in situ hybridization studies of gonadotropin releasing hormone (GnRH) and its receptor in rat digestive tract. *Life Sci* 2001; 68: 1727-1734.
- [12] Yao B, Huang W, Huang Y, Chui Y, Wang Y, Li H, Pu R, Wan L and Zhang R. A study on the localization and distribution of GnRH and its receptor in rat submaxillary glands by immunohistochemical, in situ hybridization and RT-PCR. *Life Sci* 2003; 72: 2895-2904.
- [13] Chen L, He HX, Sun XD, Zhao J, Liu LH, Huang WQ and Zhang RQ. Expression of gonadotropin-releasing hormone receptor and effect of gonadotropin-releasing hormone analogue on proliferation of cultured gastric smooth muscle cells of rats. *World J Gastroenterol* 2004; 10: 1780-1784.
- [14] Chen L, Sun XD, Zhao J, Yang AG and Huang WQ. Distribution, cloning and sequencing of GnRH, its receptor, and effects of gastric acid secretion of GnRH analogue in gastric parietal cells of rats. *Life Sci* 2005; 76: 1351-1365.
- [15] Angelucci C, Lama G, Iacopino F, Ferracuti S, Bono AV, Millar RP and Sica G. GnRH receptor expression in human prostate cancer cells is affected by hormones and growth factors. *Endocrine* 2009; 36: 87-97.
- [16] Naor Z and Huhtaniemi I. Interactions of the GnRH receptor with heterotrimeric G proteins. *Front Neuroendocrinol* 2013; 34: 88-94.
- [17] Pincas H, Choi SG, Wang Q, Jia J, Turgeon JL and Sealson SC. Outside the box signaling: secreted factors modulate GnRH receptor-mediated gonadotropin regulation. *Mol Cell Endocrinol* 2014; 385: 56-61.
- [18] Lee G and Ge B. Growth inhibition of tumor cells in vitro by using monoclonal antibodies against gonadotropin-releasing hormone receptor. *Cancer Immunol Immunother* 2010; 59: 1011-1019.
- [19] Limonta P and Manea M. Gonadotropin-releasing hormone receptors as molecular therapeutic targets in prostate cancer: Current options and emerging strategies. *Cancer Treat Rev* 2013; 39: 647-663.
- [20] Hapgood JP, Sadie H, van Biljon W and Ronacher K. Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. *J Neuroendocrinol* 2005; 17: 619-638.