行政院國家科學委員會補助專題研究計畫成果報告

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※ 婦科癌症促性腺素釋放激素 GnRH 受體的偵測 ※

※ Detection of Gonadotropin-Releasing Hormone ※

※ (GnRH) Receptor in Gynecologic Cancers ※

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計畫主持人：周松男教授

執行單位：臺大醫學院婦產科

中　華　民　國　　91　年　10　月　25　日

婦科癌症促性腺素釋放激素 GnRH 受體的偵測

Detection of Gonadotropin-Releasing Hormone (GnRH) Receptor in Gynecologic Cancers

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一、ABSTRACT

In order to demonstrate whether GnRH receptor is present in human normal ovary and/or human ovarian epithelial cancer, we use the monoclonal antibodies of GRX-6 from Dr. Lee of University of British Columbia, to perform this study. Tissue specimens of normal ovaries and ovarian cancers were obtained from appropriate patients. Immunohistochemical procedures are employed to localize the presence of GnRH receptor (RGnRH) on human tissue sections. The molecular size of the RGnRH from each of the different tissue extracts is determined by Western blot assay. The amount of GnRH receptor mRNA in ovarian tissues (normal and malignant) is detected by RT-PCR. Our present experiments revealed the presence of RGnRH and its mRNA in human epithelial ovarian cancer. Mechanism of autocrine regulation of tumorigenesis in human epithelial ovarian cancer can be explained by the coexistence of RGnRH, GnRH and their mRNA.

Keywords: GnRH, GnRH receptor, Immunohistochemical analysis, Western blot assay, RT-PCR, Ovarian cancer

二、INTRODUCTION

The hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) severs a key role in regulating mammalian reproduction function. An extrapituitary role for GnRH in the normal and malignant reproductive tissues has been postulated, following the description of GnRH binding sites in placenta and breast, ovarian, and endometrial cancers [1-4]. These extrapituitary binding sites are often reported to exhibit properties different from those of the pituitary and may, therefore, represent a new GnRH receptor entity. In addition, transcription of the GnRH precursor gene and synthesis of the peptide have been shown in human placenta [5], and breast and endometrial cancers [6-7]. Studies demonstrating in vitro GnRH and/or GnRHa growth-regulatory effects on tumor cell proliferation support an autocrine role for GnRH in sites distant from the pituitary gland [3,8-9]. Direct growth factor activity of GnRH in endometrial and ovarian cancers might explain prolonged remissions after GnRHa treatment in women with recurrent gynecologic cancers [10-20].

The gonadotropin-releasing hormone receptor (RGnRH) from several species has been molecularly cloned and nucleotide-sequenced [21-23]. It is composed of 327-328 amino acids and appears to belong to the superfamily of G-protein coupled receptors which are of the seven transmembrane types [24]. However, whether GnRH and its binding sites are present in the human ovary is somewhat controversial.

Ohno et al (1993) detected GnRH mRNA in human ovarian epithelial carcinoma [25], but not in normal ovary, whereas Dong et al (1993) reported that GnRH mRNA is expressed in this tissue [26].

Polyclonal and monoclonal antibodies against GnRH receptor have been raised and characterized [27-28]. Unfortunately the later didn't work well. Dr. Lee recently success-

fully raised and characterized monoclonal antibody to GnRH receptor using the improved hybridoma technique [29]. In order to demonstrate whether GnRH receptor is present in human normal ovary and/or human ovarian epithelial cancer, we will use the monoclonal antibodies of GRX-6 from Dr. Lee, to perform this study. Tissue specimens of normal ovaries and ovarian cancers, as well as the malignant tissues, will be obtained from appropriate patients. Immunohistochemical procedures are employed to localize the presence of GnRH receptor on human tissue sections. The molecular size of the GnRH receptor from each of the different tissue extracts is determined by Western blot assay. The amount of GnRH receptor mRNA in ovarian and endometrial tissues (normal or malignant) is detected by RT-PCR.

三、MATERIALS AND METHODS

1. Monoclonal antibodies and Chemicals

The monoclonal antibodies of GRX-6, which is specific to human GnRH receptor, were generously supplied by Dr. Gregory Lee from University of British Columbia, Vancouver, Canada.

Biotinylated second anti-mouse antibodies, streptavidin-horseradish peroxidase conjugate, and DAB (diaminobenzidine) substrate were from BioGenex, San Ramon, CA.

The MESSAGEMAKER mRNA Isolation System was purchased from GIBCO・BRL.

Chemical regents for electrophoresis were from BIORAD, Richmond, CA.

The other chemical reagents, including Triton X-100, ethidium bromide, RNase H were from Sigma Chemical Co. St. Louis, MO.

2. Collection of tissues specimens

Specimens of normal ovarian tissue and ovarian cancer tissue were obtained from gynecologic patients immediately after operation at National Taiwan University Hospital. Fresh specimens were put into liquid nitrogen tank, stored at -70 ℃ refrigerator until use for Western blot assay and RT-PCR.

3. Immunohistochemical analysis

Specimens collected from normal and malignant ovarian tissues were stained immunohistochemically. The general procedures for immunohistochemical staining were performed as previous reported [30–31]. Paraffin-embedded tissue specimens were sectioned at 6-μm, transferred to subbed microscope slides deparaffinized in xylene for 15min. They were rehydrated first in 100% alcohol for 10 min, then in 90% alcohol, 70% alcohol, and PBS (PH7.4, containing 0.3% Triton X-100) for 2 min, respectively. Tissue sections were incubated in 10% nonimmune serum for 10 min, drained by blotting, and incubated with mouse anti-GnRH receptor monoclonal antibody for 60 min at noon temperature. Sections were washed with PBS and treated with secondary anti-mouse biotinylated antibodies for 20 min at noon temperature, followed by incubation in stredtavidin-horseradish peroxidase complex for 20 min at noon temperature. Peroxidase labeling was visualized by incubation of the sections with liquid DAB (3,3'-diaminobenzidine) containing H_2O_2 in Tris HCL buffer (PH7.6). The sections were washed with PBS, counterstained with hematoxylin (IM 1486), dehydrated and mounted. Slides were examined under Olympus microscope.

4. Western blot assay

Membrane proteins are solubilized in Laemmli's SDS sample buffer and heated at 95 ℃ for 5 min. Samples are electrophoresed on 7.5 % polyacrylamide gel. Proteins are transferred onto the nitrocellulose membrane and probed with an anti-GnRHR antibody. Membrane is incubated with anti-mouse HRP-conjugated IgG. Immunoactive bands are visualized with Supersignal ECL-HRP chemiluminescent substrate. After draining, the membrane is covered in clear plastic wrapping and expose to X-ray film for 3min. Resulting bands are imaged using an image analyzer.

5. RT-PCR detection

Total RNA are isolated from cells and frozen tissues after homogenization using the RNAgents Total Isolation System. First-stand cDNA is transcribed by SuperScript RNase H-Reverse Transcriptase from 5 μg total RNA using random hexamer primers for the GnRHR mRNA. cDNA is amplified in a 50-μl PCR reaction buffer containing specific oligonucleotide primers designed according to the sequence published for GnRHR.

Primer 1:

5'-CTGACCTTCATCCTCTGCTGGA CACC-3'

Primer 2:

3'-GGAGAGCAGGAGTAGAAGTGA G-5'

Thirty-five cycles of amplification are carried out in a thermal controller : denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 1 min. The DNA products are run on a 1.5% agarose gel, and the bands are visualized by incubation for 10 min in a solution containing 10 ng/ml of ethidium bromide. Resulting gel bands are imaged using an image analyzer.

四、RESULTS

1. Immunohistochemical staining

Thirteen normal ovarian and 39 ovarian cancer tissue specimens were subjected to immunohistochemical staining. Representative immunohistochemical staining of GnRH receptor is shown in *Figure 1*. The immunohistochemical staining of GnRH receptor in different tissue categories and different stages of ovarian cancers are shown in *Table 1*. There was no any positive staining of GnRH receptor from 13 normal ovaries. In contrast, the rate of positive staining in tissues of ovarian cancers was 53.8% (21/39). As shown in *Table 1*, the positive rate of staining of GnRH receptor in late stage of ovarian cancer (stage 3 and 4) was 73.9% (17/23), which was significantly higher as compared with 25.0% (4/16) in early stage (stage 1 and 2) of ovarian cancer.

2. Western blot assay

The representative electrophoretic gel of Western blot analysis is presented in *Figure 2*. On the contrary, no protein band was detected from tissue specimens of 13 normal ovaries. The results of Western immunoblots from 39 ovarian cancers are demonstrated in *Table 2*.

3. Detection of mRNA for GnRH receptor by RT-PCR

Total RNA was successfully isolated from tissue specimens of normal ovaries and ovarian cancers. RT-PCR was performed to detect the presence of mRNA expression for GnRH receptor in normal ovaries and ovarian cancers by using the designated primer pairs. Typical results of RT-PCR products on electrophoretic gels are demonstrated in *Figure 3*. The RT-PCR products were of the size 486 bp (base pairs). Through these experiments, it was clearly shown that mRNA for GnRH receptor is not expressed in any of normal ovarian tissues tested (n = 13), but is expressed at different levels in different stages of ovarian cancers. Details of detection of mRNA expression for GnRH receptor by RT-PCR are summarized in *Table 3*.

五、DISCUSSION

The fundamental function of gonadotropin-releasing hormone (GnRH) is to stimulate the release of many animal specific receptor (RGnRH) localized on the external membrane of certain types of cells. GnRH binding sites have been identified on extrapituitary system, such as human placenta, ovarian granulosa cells, human breast cancer, normal heart cells, hepatoma cells epithelial ovarian cancer and endometrial cancer [2,32-35].

Coexpression of both GnRH and RGnRH mRNA in human granulosa cells suggests an autocrine regulation in ovary tissue [33]. GnRH binding sites was proved to exist in human epithelial ovarian cancer by

the technique of photoaffinity labeling [35]. Further more, GnRH and GnRH mRNA were also detected in human ovarian epithelial carcinoma [25]. Our present experiments revealed the presence of RGnRH and its mRNA in human epithelial ovarian cancer. The result was in consistency with the previous report [35].

Mechanism of autocrine regulation of tumorigenesis in human epithelial ovarian cancer can be easily explained by the coexistence of RGnRH, GnRH and its mRNA, according our own and previous studies [25,35].

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Table 1. Distributions of immunohistochemical staining of GnRH receptor in normal ovaries and different stage of ovarian cancers

Tissue categories	Number of specimens	Number of immunohistochemical staining			
		Negative	+	++	+++
Normal ovaries	13	13	0	0	0
Ovarian cancers	**39**	**18**	**11**	**7**	**3**
Stage I	8	5	1	2	0
Stage II	8	7	0	1	0
Stage III	20	6	8	3	3
Stage IV	3	0	2	1	0

Table 2. Distributions of results of Western immunoblots of GnRH receptor extracted from different stages of ovarian cancers

stage	Number of specimens	Western blot assay	
		Negative	Positive
Ovarian cancers	39		
Early stage (stage I & II)	**16**	**8**	**8**
stage I	9	4	5
stage II	7	4	3
Late stage (stage III & IV)	**23**	**8**	**15**
stage III	21	8	13
stage IV	2	0	2

Table 3. Distributions of results of RT-PCR products on electrophoretic gels for detection of mRNA for GnRH receptor extracted from different stages of ovarian cancers

stage	Number of specimens	RT-PCR	
		Negative	Positive
Ovarian cancers	28		
Early stage (stage I & II)	**11**	**4**	**7**
stage I	7	4	3
stage II	4	0	4
Late stage (stage III & IV)	**17**	**3**	**14**
stage III	15	3	12
stage IV	2	0	2

Figure IA

Figure IB

Figure 1A and 1B. Immunonhistochemical localisation of GnRH receptors on human ovarian cancer. Figure 1A showed negative staining and 1B showed strong positive staining.

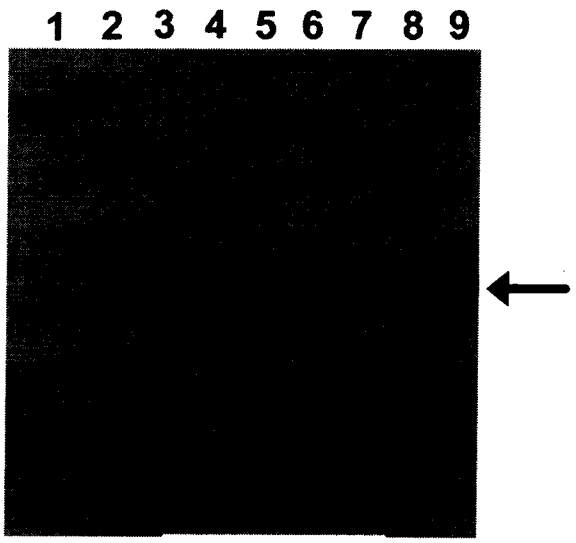

Figure 2. Western blot analysis to determine the molecular weight of GnRH receptor.

Lanes1&2: tissue extracts from normal ovaries.

Lanes3-9: tissue extracts from different stages of ovarian cancers.

Arrow (←) indicates the presence of GnRH receptor protein.

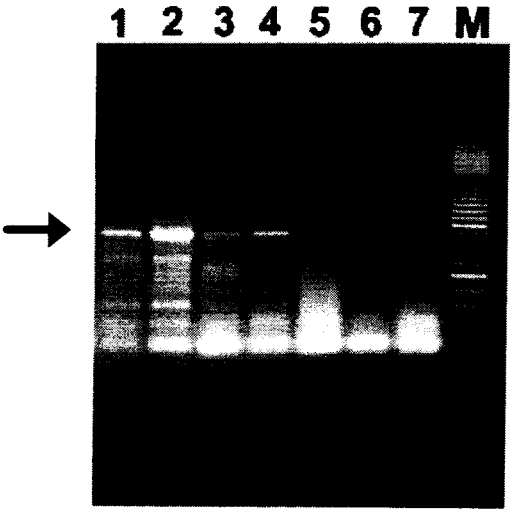

Figure 3. RT-PCR products of GnRH receptor mRNA on electrophoretic gels.

Lanes 1-5: tissue extracts from different stages of ovarian cancers.

Lanes6&7: tissue extracts from normal ovaries.

Lane M is DNA size markers with sizes given in base pairs.

Arrow (→) indicates the location of RT-PCCR products on electrophoretic gels.