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Gonadotropin-releasing hormone neuropeptides and receptor in human breast cancer: Correlation to poor prognosis parameters

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ABSTRACT

Expression of the two gonadotropin-releasing hormone homologue peptides GnRHI and GnRHII and their receptor GnRHR has been demonstrated in a number of malignancies. In hormone-dependent breast cancer, GnRH analogs are used for therapy in premenopausal women. Gene expression of GnRHI, II and R was studied in breast biopsies from primary breast adenocarcinoma obtained from the tumor and the adjacent benign tissue. Levels were evaluated by a multiplex real-time RT-PCR. GnRHI transcripts were detected in 14.7% of the benign and 29.4% malignant biopsies and GnRHII in 21.2% benign and 44.1% malignant biopsies. GnRHR was also more frequent in the malignant (54.2%) than in the benign (24.0%) biopsies, at similar expression levels. No transcripts were detected in biopsies from healthy individuals. There was a strong correlation between the presence of GnRHI and GnRHII transcripts and their receptor in the benign and the malignant biopsies. GnRHI, II and R expression correlated significantly with poor prognosis pathological parameters. Immunohistochemistry for GnRHR revealed expression in malignant cells and in epithelial cells of mammary ducts of the adjacent area with pre-cancerous features. In contrast, GnRH I and II peptides were rarely expressed at low levels in breast cancer cells. In conclusion GnRH peptides and receptor are expressed more frequently in breast tumors than in the adjacent mammary tissue, representing a malignant feature. Their expression correlated to tumor characteristics of poor prognosis and was therefore related to more aggressive malignancies. Concomitant expression of peptides and receptor supports an autocrine/paracrine regulating role.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide produced in the hypothalamus [10]. It interacts with a G-protein coupled receptor (GnRH R) in the anterior pituitary [23], controlling the gonadal function in both sexes. Two human isoforms have been identified, namely GnRH-I and GnRH-II. The first is the hypothalamic isoform responsible for the secretion of LH and FSH. The second differs by three amino acids [6,27] and is widely distributed in the central and peripheral nervous system. It is also expressed at significantly higher than GnRH I levels outside the brain and it has been shown to act as a neuromodulator in the behavioral components of reproduction [16,27,28].

GnRH peptides and GnRH R have been found in extrapituitary tissues and tumors of the reproductive and other systems [4,20,29,34,2]. Extrapituitary GnRH binding sites are often associated with many novel cellular responses [7]. Furthermore, expression of GnRH R seems to be related with advanced cancer stage in ovarian carcinomas [8].

The GnRH system has been reported to play an autocrine/paracrine role in the inhibition of cellular growth and metastatic potential in breast cancer cell lines [24,35], and breast tumor regression in nude mouse [14,26]. However, its expression

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was associated with a protective effect on the chemotherapeutic drug-produced apoptosis [30]. As GnRH agonists (or antagonists) show clinical benefit when used as adjuvant pharmacotherapy in premenopausal breast cancer patients [13], the study of the GnRH system of neuropeptides and receptor in breast tumors remains emerging.

In the present study, the expression of the two GnRH neuropeptide genes (GnRH I, II) and their receptor was evaluated in a series of biopsies from primary breast cancers in a quantitative manner by multiplex real-time RT-PCR. Transcript levels from the malignant tissues were compared to these from the adjacent non-neoplastic tissue and tissues without malignancy, and were correlated to multiple clinicopathological and demographic parameters and clinical output in order to reveal potential prognostic or diagnostic value. Finally, histological mapping of peptide and receptor expression in breast cancer biopsies was done by immunohistochemistry, to reveal specific target cellular types.

2. Materials and methods

2.1. Tissues

Patients newly diagnosed with primary breast adenocarcinoma in the "Theagenio" Cancer Hospital, Thessaloniki, Greece were enrolled in the study. Biopsies were obtained from the tumor and the adjacent non-neoplastic tissue. Diagnosis was confirmed by the histological examination in all patients. Full medical history, follow-up and histopathological data were available. Patients have not been receiving any hormonal treatment chemotherapy or radiation. Patients with previous or present neoplastic disease at any other site were excluded from the study. Biopsies without signs of malignancy or other pathology obtained for diagnostic use were also used. Human term placenta was obtained by the Obstetrics and Gynecology Department of the General University Hospital in Alexandroupolis. The project was approved by the local Ethical Committee. Consent has been obtained from each patient after full explanation of the purpose and nature of all the procedures used, in accordance to the Helsinki Declaration. Tissue samples were stored in RNAlater (Invitrogen, Carlsbad, CA) at −80 °C until used for RT-PCR. Breast cancer tissue sections were also taken from paraffin-embedded archival files and used for immunohistochemistry.

2.2. Cell culture

The human breast cancer cell lines MDA MB231, MCF7 and T47 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (all purchased from Invitrogen, UK), at 37 °C in a 5% CO_2 humidified atmosphere. Cells were plated at a concentration of 2×10^5 cells/ml and were harvested for total RNA extraction when they had reached approximately 80% confluence.

2.3. Multiplex real-time quantitative RT-PCR

Total RNA was extracted from biopsies using Trizol Reagent, according to the manufacturer's instructions. Reverse transcription (RT) was performed using the SuperScript Preamplification System (Invitrogen) and random hexamers in a total volume of 20 μl. Two microliter of the same RT product was used as a template for each gene, amplified by PCR using 2 mM $MgCl_2$, PCR buffer, 0.2 mM of sense and antisense primers, 0.2 mM dNTPs and 2.5 U Taq Polymerase (Invitrogen) in a final reaction volume of 50 μl. Quantitative PCR was performed using the Light Cycler MX3005P (Stratagene, La Jolla, CA) with the following cycling parameters: a pre-amplification cycle (denaturation for 10 min at 95 °C), 40 cycles of amplification (denaturation for 30 s at 95 °C, annealing for 40 sec at 53 °C, 54 °C, 50 °C for GnRH I, GnRH II and GnRH R respectively, and extension for 50 s at 72 °C), and a final dissociation cycle (1 min at 95 °C, 40 s at 57 °C and 30 sec at 95 °C). Primers were designed according to the GenBank published sequences as follows: for human GnRH R sense 5′-CCTTGTCTGGAAAGATCCGA-3′ and antisense 5′-GGAGCGGTCCAGGCTGAT-3′ [33], for human GnRH I sense 5′-CTACTGACTTGGTGCGTGGA-3′ and antisense 5′-CTGCCCAGTTTCCTCTTCAA-3′ and for human GnRH II sense 5′-TCC-TGCTGCTGCTGACTG-3′ and antisense 5′- CTAAGGGCATTCTGGG-GAT-3′ [25]. Product sizes were 319, 240 and 119 bp for GnRH R, GnRH I and GnRH II respectively. Reactions in duplicate were carried out using the SYBER Green MM QPCR Brilliant mix (Stratagene), 0.4 μM of each primer, 2 mM $MgCl_2$ and 0.5 μL of cDNA in a final volume of 20 μL. Results were calculated using MaxPro QPCR Software Version 4.0 (Stratagene) using the comparative threshold cycle method. Analysis of relative gene expression data was performed according to the $2^{-\Delta\Delta C_T}$ method [21] using β-actin as a reference gene and RNA from human placenta as a positive control. Results are expressed as the mean from duplicate values of gene expression in relation to β-actin in the same RNA preparation. Samples with poor β-actin gene amplification were excluded from the study. Negative control samples, where no RT enzyme was added (no RT) or without DNA template (no DNA), were included in every assay in order to exclude the possibility of genomic or other DNA contamination.

2.4. Immunohistochemistry

Immunohistochemistry was conducted as previously described [32]. Briefly, tissue specimens were fixed in formalin and embedded in paraffin. Sections (4 μm) were deparaffinized, rehydrated, and treated with 0.3% H_2O_2 for 5 min in methanol. Slides were incubated for 75 min with primary mouse monoclonal antibodies for human GnRH R (ab22168, Abcam, UK), GnRH I (HU11B, SantaCruz Biotechnology Inc., CA, USA) and GnRH II (D-9, SantaCruz Biotechnology Inc.) diluted 1:250, 1:100 and 1:100 respectively in 10% normal mouse serum in PBS. Negative control slides were incubated for the same period with normal mouse serum IgG. Immunostaining was detected by the Kwik Kit (Thermo Shandon, Pittsbutgh, PA, USA). Finally, bound antibody complexes were stained for 10 min with 0.05% diaminobenzidine, counterstained with Mayer's hematoxylin, mounted and examined under an Olympus BX40 microscope.

2.5. Statistical analysis

All measurements were done in duplicate. Statistical significance was assessed by Mann–Whitney *U*–Wilcoxon Rank Sum W Test, using the SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Group differences were assessed by chi square test. Significance was set at a p value < 0.05. Analysis of the data in pairs of benign and malignant biopsies from the same patient was performed by the McNemar test. Kaplan Meier survival analysis was also performed.

3. Results

3.1. Patient and tumor information

Thirty-five women with primary breast cancer were enrolled in the study, with mean age 61 ± 13 years, mean BMI 28.9 ± 5.4 kg/m^2 and mean age of menarche 13 ± 1.3 years. At the time of diagnosis, 27/35 (71.4%) were menopausal with mean age of menopause 48.5 ± 3.9 years. Two of them did not report any history of pregnancy, whereas for the rest the mean number of full-term pregnancies was 1.9 ± 0.8, with mean age of first pregnancy 25.1 ± 4.3