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LACKING EXON5 OF VARIANT ESTROGEN RECEPTOR IN HUMAN BREAST CANCER

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Methods: The target sequence of ER RNA covering exon4~6(1082~1520bp) was amplified in 7 clinical human breast cancer tissues by reverse transcription and polymerase chain reaction (RT-PCR) techniques. Results: PCR products were transferred to nitrocellulose membranes and hybridized using a [r-^{32}P]-ATP labeled ER 29 oligonulceotide probe representing the antisense strand of the ER cDNA sequence 1271~1299. Specific bands were found at 438 and 300 base pairs in two tumors. The 300 base pair of PCR product was recovered from ER+/PR+ and ER+/PR- tumor, respectively. Conclusion: Dideoxy sequence analysis revealed that they contained the variant ER completely missing exon 5.

Key words: Breast cancer; Estrogen receptor; RT-PCR; Variation

ER is an excellent marker of differentiation. It predicts improved disease free survival in breast cancer and, the most important is to predict the likelihood of benefit from endocrine therapy.[1] But 50% of patients fail to respond to hormone despite the presence of ER.[2] Recent evidence of foreign countries showed that several variant ER forms had been found in tissues of breast cancer. The variant ERs would lead to alter function and sometimes to hormone resistance.[3] In this paper, our objective was to determine whether defects in ER occur in tumors taken directly from patients with breast cancer in our country for further studying on the relationships between ER protein structure and function as well as between ER protein structure and phenotype. The results may make us to find new prognostic factors and formulate the scheme for the treatment of breast cancers more accurately. They may also be fit for other endocrine-dependent tumors except breast cancer.

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MATERIALS AND METHODS

Human Tumor Specimens

Frozen human breast tumor specimens, stored at -70℃, were obtained from the surgery of Beijing Institute for Cancer Research (BICR) and consisted of tissue remaining after report assays performed in the pathology laboratory of BICR. All of the tumors containing 1ER-/PR-, 3ER+/PR-, 2ER+/PR+ and 1ER-/PR+ were examined by dextran coated charcoal (DCC) assay.

PSG5 -ERcDNA

Recombinant plasmid PSG5-ERcDNA was obtained from Dr. Pierre Chambon (Lab. of Genetic Molecular Eucaryotes, National Scientific Research Center, France).

Reverse Transcription-polymerase Chain Reaction (RT-PCR)

RNA was isolated with RNATRIZOL™ reagent (GibcoBRL) using 1g of tumor specimens. RNA integrity was assessed through electrophoresis by denaturing agarose gels containing formaldehyde and concentration was determined by UV absorption prior to reverse transcription and PCR amplification. After an initial denaturation at 94℃ for 2 min, 2 units of AMV reverse transcriptase was added, and reverse transcription was allowed to proceed at 42℃ for 2 h. The cDNA product was then amplified addition. The ER up-stream primer (nucleotide 1082 base pair to 1102 base pair of human cDNA sequence) was 5'-GGA GAC ATG AGA GCT GCC AAC-3'; the down-stream primer (nucleotide 1501 base pair to 1520 base pair of human cDNA sequence) was 5'-CCA GCA GCA TGT CGA AGA TC-3'. PCR was performed according to following protocols: Denaturation was carried out at 94℃ for 4 min first. Then each cycle of amplification consisted of a 1 min of denaturation at 94℃, followed by 1 min of annealing (59℃) and 1 min of extension (72℃) steps. After 30 cycles, the final product was extended for 10 min, then on ice at once.

Southern Blotting and Hybridization

One-tenth of the PCR product was applied to a 1.2% agarose gel electrophoresis, and transferred to nitrocellulose by the method of Southern.[4] The nitrocellulose membranes were hybridized with 5'-[r-^{32}P]-ATP labeled 3# oligonucleotide probe (5'-TGA ACC AGC TCC CTG TCT GCC AGG TTG GT-3') representing the antisense strand of the ERcDNA sequence 1272~1300. Selected PCR products which had been doubted the variant ER were purified from acrylamide for dideoxy sequence analysis.

Dideoxy Sequence Analysis

After PCR, 100μl of the PCR product was loaded onto a 5% polyacrylamide gel. After electrophoresis, the gel was stained in ethidium bromide solution (1 μg/ml) for 40 min. The suspect variant DNA bands were excised under UV illumination. Then the gel was soaked in 100 μl of 0.1×TE overnight at 4℃ in a 0.5 ml microcentrifuge tube. Then the supernatant was transfered to a new microcentifuge tube. The 100 μl of 0.1×TE was added to old tube overnight at 4℃ again. The released DNA recovered from twifold supernatant was then analyzed by dideoxy sequencing using fmol PCR cycle sequence kit (Promega). Sequencing primer is the up-stream primer of ER exon4 to exon6.

RESULTS

Denaturing agarose gel containing formal-dehydes showed that there were 28S and 18S bands clearly in every RNA extracted. It suggested that the RNA isolated can be the template for RT-PCR. The amplified RT-PCR products were analyzed by gel electrophoresis and hybridization with an internal ER oligonucleotide probe (Fig. 1). No ER-specific PCR products were detected in the ER-/PR- tumors. There are two specific bands in one ER+/PR+ and one ER+/PR- tumor. That is a substantial amount of 438 base pair wild-type ER and a minor component of 300 base pair variant ER products, respectively. The selected bands (300 base pair) were recovered from the agarose gel for dideoxy sequence analysis. The variant also contained the wild-type sequence for exon 4 and 6. Exon 5, however, was precisely missing (Fig. 2). These results suggest that alternative splicing may occur in the hormone binding domain of the ER.

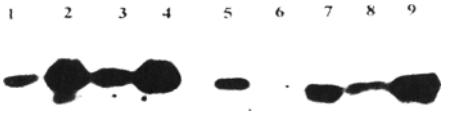

Fig. 1. Expression of ER transcripts in human breast tumors. cDNA was prepared from PSG5-ER cDNA (4,9) and 7 tumors (1-3, 5-8) and analyzed by electrophoresis and hybridization with an ER-specific oligonucleotide probe.

GGA GAC ATG AGA GCT GCC AAC CTT TGG CCA AGC CCG CTC ATC ATC AAA
CGC TCT AAG AAG AAC AGC CTG GCC TTG TCC CTG ACG GCC GAC CAG ATG
GTC AGT GCC TTG TTG GAT GCT GAG CCC CCC ATA CTC TAT TCC GAG TAT
GAT CCT ACC AGA CCC TTC AGT GAA GCT TCG ATG ATG GGC TTA CTG ACC
AAC CTG GCA GAC AGG GAG CTG GTT CAC ATG ATC AAC TGG GCG AAG AGG
GTG CCA GGC TTT GTG GAT TTG ACC CTC CAT GAT CAG GTC CAC CTT CTA
GAA TGT GCC TGG CTA GAG ATC CTG ATG ATT GGT CTC GTC TGG CGC TCC
ATG GAG CAC CCA GTG AAG CTA CTG TTT GCT CCT AAC TTG CTC TTG GAC
AGG AAC CAG GGA AAA TGT GTA GAG GCC ATG GTG GAG ATC TTC GAC ATG
CTG CTG G

Fig. 2. The nucleotide sequence of RT-PCR amplification using primer#1 and #2 (1082-1520bp, 438bp). ▬▬ represent exon 5.

DISCUSSION

ER Variation and Endocrine Therapy of Breast Cancer

Faithful expression of genetic information is lost in tumor cells due to the formation of spontaneous cell variants. In breast cancer, this evolution is marked by progression of tumors from hormone dependent, through hormone-responsive, to hormone-resistant states. It has been well known that ERs play a critical role in the development, progression, and hormone-responsiveness of breast cancers. A product of ER action can be monitored and PR serves this role. In all estrogen/progesterone target tissues, estradiol is required for PR induction. This relationship holds true for breast cancers, and led us to propose that the presence of PR could be used as a tool to predict the hormone dependence of human breast tumors. Thus, a tumor that contains PR would, of necessity, have a functional ER. However, even when both ER and PR are positive it does not give a perfect correlation with endocrine response because about 12% ~ 35% of breast tumors do not show a hormonal response.[5] Some tumors are ER+/PR-,even ER-/PR+. These phenomena which have been long-standing clinical problems make us to think that since the two functions, DNA binding and hormone binding, are carried out by separate parts of the protein, they are to some extent independent.[6] Thus, it is possible to have variant receptors that can bind to DNA with limited affiniting without first binding hormone, and vice versa. Either PR synthesis in these tumors is entirely independent of ER, or a variant or other unmeasured form of ER is stimulating PR synthesis.

The Mutation in Exons of ER

Polymophic forms of the ER gene have been isolated in recent years.[7] They include gene amplification or rearrangement, PVUIIRFLP, gene methylation and so on. The majority of these genetic changes are found in introns, which do not directly encode the mRNA or, in turn, the protein. Recent evidence of foreign countries shows that the mutation in exons of ER has an effect on the structure and function. Lab. of McGuire has isolated three deletions of exon 3,5 and 7.[8] Researchers found that exon5 deletion was cloned from ER-/PR+ tumors, exon 3 and 7 deletion were cloned from ER+/PR- tumors. But in our studies exon 5 deletion variant of ER existed in one ER+/PR- and one ER+/PR+ tumor. These results consist with that of Zhang.[9] Zhang found that exon 5 deletion was in ER+/PR-, ER-/PR+ and ER+/PR+ tumors and the variant ER expressed in minor, excess, or great excess, as compared to wild-type ER in those tumors. The biological consequences of increased variant ER expression in human tumors are unknown at present. If the variant does confer a selective advantage during tumor progression, cells overexpressing the variant may predominant, resulting in a more agressive phenotype. Although the exon 5 deletion variant of ER has lost its hormone binding function, it is capable of dimerization, DNA binding and constitutive transcriptional activity from TAF, in the absence of ligand.[5] Thus to study the location of the variant ER, the ratio of variant ER and wild-type ER in tumors and the method of determination of variant ERs are very important for understanding the development, progression and guiding of endocrine therapy as well as estimating of breast cancer.

The Molecular Heterogeneity of ER

As other authors, we found that Exon 5 deletion variant of ER is always coexpressed with wild-type ER in human breast cancer. This phenomenon illustrates the molecular heterogeneity of ER. Neither variant ER nor wild-type ER found in tumors suggests that ER gene may not be transcribed.

In this experiment, we only did some initial test for ER variation. We would increase the samples of human breast cancer and combine together with the effects of clinical endocrine therapy. Preliminary results showed that there is lacking exon 5 of variant estrogen receptor in human breast cancer of our country.

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