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PRODUCTION AND APPLICATION OF MOUSE ANTISERUM TO HUMAN ESTROGEN RECEPTORS

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A polyclonal antibody to peptide containing 15 amino acids and corresponding to region - D of human estrogen receptors (hER_D) was obtained in mice by immunization with the coupler of peptide and KLH. Using this antiserum, the ER status of paraffin - embeded sections of 95 human breast carcinomas were studied. The corresponding rate for determination of ER status between immunohistochemical staining (IHC) and dextran coated charcoal (DCC) assay was 89.5%. The concordance for semiquantitative grades was 69.3%. In addition, in *situ* hybridization (ISH) of 15 frozen sections of same sample using digoxigenin labeled dUTP to identify the expression of ER mRNA for confirming the 1HC also be used. This technique revealed more specific, sensitive and convenient than DCC. The results of ISH were fully consistent with IHC (100%). Above results show that the mouse antiserum to hER_D obtained in this study is specific and sensitive for IHC assay of ER and IHC is a valuable adjunct and/or alternative to the biochemical method for determination of the ER status of breast cancer.

Key words: Breast cancer, Estrogen receptor, Immunohistochemistry, In *situ* hybridization.

Studying the method of ER determination in order that estrogen receptor analysis is relatively inexpensive, sensitive, requires no specialized equipment and can be performed at the community hospital level ought to be done. The main interest in estrogen receptor content is because of its ability to predict the response to endocrine treatment and its possible value as an overall prognostic indicator in breast cnacer.[1,2]

We report here the production and characterization of specific polyclonal mouse antibody to definite construction and synthetic peptides containing 15 amino acids representing region D of human estrogen receptor. Antipeptide antibody was used for the IHC of ER in human breast cancer.

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MATERIALS AND METHODS

Materials

Keyhole Limpet Hemocyanin (KLH), M - maleimidobenzoic acid N - Hydroxysuccinimide ester (MBS), 3 - Aminopropyltriethoxysilane (APES) were products of Sigma. DIG labeling and detection kit was purchased from Boehringer Mannheim (Germany). Sephadex G - 25, mouse PAP kit were purchased from Beijing institute for biological product research. Recombinant plasmid

PSG_5 - ER cDNA was obtained from Dr. Chambon (lab. of genetic molecular of eucaryotes, National Scientific Research Center, France). The synthetic peptide corresponding to amino acid Leu^{256} - Glu^{280} of human estrogen receptor was synthesized by College of Life Sciences, Beijing University. 95 specimens of breast carcinoma tissues obtained at surgery of BICR were in the fresh state and were made into routine pathological paraffin - embeded sections for IHC assay. A part of carcinoma tissues remained or 31 in 95 were immediately frozen after operation and stored in liquid nitrogen in order to provide frozen sections for ISH and IHC. All samples above have been diagnosed and assayed by dextran coated charcoal method.

Methods

IHC

The peptide was coupled to KLH with MBS,[3] BALB/C mice were used to raised polycolonal antibody against the peptide conjugate, see reference 3. The reactivity of antiserum towards the immunogen was tested by ELISA,[4] 40 - well plates were coated with the coupler of BSA and hER_D. For immunohistochemical assay, the staining method included following steps: Sections were deparaffin, passed through graded alcohol and entered water. They were then treated with 0.3% H_2O_2 15 min at room temperature, rinsed in PBS, incubated with blocking reagent for 45 min and incubated overnight at 4℃ with primary antibody which was collected from mice. After a rinse in PBS sections were incubated for 60 min in bridging antibody and the peroxidase antiperoxidase complex respectively, then incubated in the diaminobenzidine for 10 min. Sections were then washed in distilled water, lightly counterstained with Harris's hematoxylin, dehy - drated, cleared and mounted. Staining was rated semiquantitatively, incorporating both the intensity and distribution of specific staining.[5,6] The estimated percentage of neoplastic cells with positive nuclear and /or nuclear and cytoplasm staining in each of four categories was recorded as follows: + = weak staining, positive cells cover 10—25% of the total neoplastic cells; + + = moderate staining, positive cells cover 25—50% of the total ones; + + + = strong staining, positive cells cover over 50% of the total ones; - = no staining or positive cells are less than 10% of the total cancer cells. ISH is similar to IHC. These grades were compared with DCC results (Table 1).

Table 1. ER status

IHC	ISH	DCC(fmol/mg)
Negative (-)	-	< 10
Low Positive (+)	+	10 - 50
Positive (+ +)	+ +	50 - 100
High Positive (+ + +)	+ + +	> 100

ISH

Preparation of Slides: Slides were treated with 3 - Aminopropyltriethoxysilane (APES).[7] Tumor samples stored in liquid nitrogen were embedded in OCT and cut into 5 μm slides. After drying, the sections were immediately fixed for 20 min in 4% paraformaldehyde in PBS, rinsed 5 min twice in PBS, dehydrated in 70%, 90%, 100% ethanol, stored in -20℃.

ER cDNA Probe Synthesis[3]: In which, probe was labeled by random primed method as the kit recommended.

Prehybridization: All sections were rehydrated with 100%, 90% and 70% ethanol to water, rinsed twice in PBS - 5 mM $Mgcl_2$ for 10 min, followed by a wash in 0.2 N HCl for 10 min, 2 × SSC - 5mM EDTA at 50℃ for 30 min, incubated for 15 min in 25 μg/ml of proteinase K, washed in 0.2% glycine 5 min twice, then post - fixed with 4% paraformaldehyde for 30 min, washed again with PBS - 5mM $Mgcl_2$.

In Situ Hybridization: Each breast cancer section was covered with 50 μl of hybridization buffer containing 45% de - ionized formamide, 10% dextran sulfate, 5 × Denhardt's solution, 6 × SSC, 100 μg/ml sheared salmon sperm DNA. The sections were placed in closed humid boxes and incubated for 1 hr at 42℃. After adding denatured ERcDNA probe, the sections were incubated

overnight at 42℃, then washed the slides 2 times in 45% formamide/2 × SSC at 42℃ for 15 min, followed by 10 min in 2 × SSC, 1 × SSC, 0.1 × SSC at room temperature. Then the slides were was hed twice (10 min each) in 100 mM Tris - Hcl (pH 7.5), 150 mM Nacl buffer (buffer 1), blocked in 0.5% Blocking Reagent in buffer 1 (buffer 2) for 30 min at room temperature, incubated with antibody conjugate (diluted 1:5000 in buffer 1) for 30 min at room temperature. After incubated the slides in buffer 1 for 10 min followed by a 10 min wash in a 100 mM Tris - Hcl (pH 9.5) buffer containing 100 mM Nacl and 50 mM $Mgcl_2$(buffer 3). For detection of the signal slides were incubated overnight in a chromogen solution containing 337.5 μg/ml nitroblue tetrazolium salt (NBT), 175 μg/ml 5 - bromo - 4 - chloro - 3 - indolyl - phosphate (X - phosphate) in buffer 3 overnight at room temperature in the dark. The chromogen reaction was halted by rinsing the slides in 10 mM Tris - Hcl (pH 8.1) and 10 mMEDTA, counterstained with A. O (0.03%) for 10—30 seconds and viewed under a light microscope.

Controls. 1. No antibody conjugate. 2. No ER cDNA probe. 3. For a third control, sections were treated with 25 μg/ml of RNase.

RESULTS

Titer of polyclonal antibody to hER_D was tested by ELISA and fixed 1:800—1:1600. The results showed no difference for both 31 frozen sections and 31 paraffin - embed ones for IHC assay. In positive tumors the staining was located in the nuclei or nuclei and cytoplasms of cancer cells (Figure 1). We determined that 56 of the 95 studied cases were positive and 29 were negative by both ER - DCC and ER - IHC methods. That is to say, there were the same results to 85 cases for both assays, only 10 cases showed opposite. This represents a 89.5% concordance between ER - DCC and ER - IHC (Table 2). In addition, there was 69.3% concordance for semiquantitative grads and there was no cases which are positive in DCC but negative in IHC. Using the anti - ER_D antibody, we also tested 4 lung cancer, 4 colon cancer, 4 esophagus cancer, 4 ovary cancer by IHC. The results were all negative and tallyed with that of DCC.

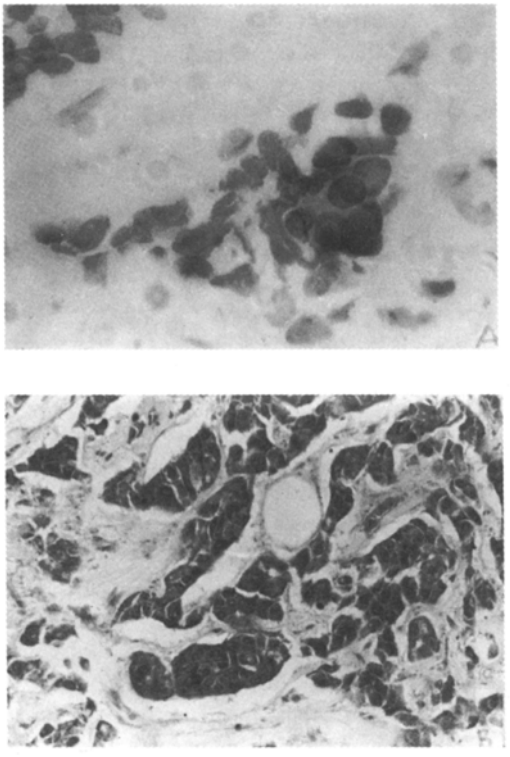

Fig. 1. Immunohistochemical staining using the peroxidase - antiperoxidase to demonstrate localization of estrogen receptor with anti - human estrogen receptor antibody. A. frozen section of breast carcinoma (DCC 51.9 fmol/mg), × 1000. showing strong labeling of malignant cells. B. paraffin - embeded section of breast carcinoma (DCC 292.7 fmol/) with a positive staining of virtually all nuclei of malignant cells, × 400. Counterstain with Harris' hematoxylin.

Table 2. Comparison of ER between IHC and DCC

Status	IHC(%)	DCC(%)
ER -	29/95(31)	39/95(42)
ER +	66/95(69)	56/95(58)

The cellular localization of ERmRNA is easily detected using digoxigenin labeled cDNA probe in conjunction with ISH (Table 3, Figure 2). Table 3 described a good correlation between DCC and ISH. This represents a 93.3% rate of agreement between DCC and ISH. The more the DCC level, the more the ER mRNA expressed. Only one discrepancy was observed. There were also 3 discordance in four grades, see Table 4. Overall concordance of 100% between ISH and IHC was found. In addition, tumors having a low level of ER shown by IHC invariably had a low level of ER showen by ISH, there was 80% concordance for semiquantitative grades.

Each control sections have no hybridized signal.

Table 3. Comparison of ER results in 15 breast cancer tissue samples obtained by ISH, DCC and IHC

ER status	ISH(%)	DCC(%)	IHC(%)
ER+	11/15(73.3)	10/15(66.7)	11/15(73.3)
ER-	4/15(26.7)	5/15(33.3)	4/15(26.7)

Table 4. Difference between DCC and ISH

No.	DCC	ISH
2293	-	+
2275	16.7	+ +
2299	38	+ + +
2268	50.8	+ + +

DISCUSSION

The DCC assay has been considered the gold standard for hormone status determination, but it requires specialized equipment and is expensive. In addition, abundant fresh frozen tissue is needed. It has been shown that much as 20% of tumors sampled at two different sites can give divergent results by as much as 35%.[8] Finally, and most importantly, the assay has relatively poor specificity and sensitivity in predicting hormone response.[2] The low rate of specificity is due to the false - positive rate, possibly from interference by benign components or an inability to detect tumor heterogenicity and a dual populations of cells. The low rate of sensitivity is because of its inability to detect ER - positive tumors with very low tumor cellularity.

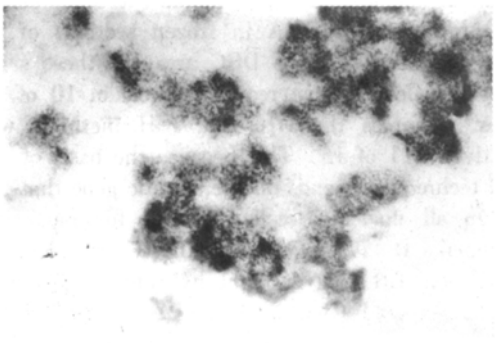

Fig.2. In *situ* Hybridization using digoxigenin labeled estrogen receptor cDNA probe to demonstrate localization of estrogen receptor mRNA on frozen section of a case of breast carcinoma (DCC 228 fmol/mh), ×1000.

Immuohistochemical staining overcomes many of those drawbacks. Since the primary structure of human ER has been determined.[9] It has been possible to produce site - specific antibodies against different regions of the receptor. Here, we chose this peptide (Leu^{265} - Glu^{280} because this stretch of amino acids lies in the hinge or D region of the receptor between the highly conserved DNA - binding (C) and steroid - binding (E) domans, and no sequences similar to those of the selected peptide are found in other members of the closely related steroid and thyroid hormone receptor family. this region is extremely hydrophilin in character and may contain a β - turn in its secondary structure.[10] It suggests that the peptide might exist on the surface

of the native folded protein.

In 85 of 95 of the cases (89.5%), ER IHC results were correlated with ER DCC assays and a discrepancy was observed in 10 cases (10.5%). Among the 66 ER IHC positive cases, 10 had ER levels < 10 fmol/mg in DCC. In these 10 cases, 2 were progesterone receptor (PR = 13.6, and 87.6 fmol/mg protein), 2 were postmenopause, 2 had no node metastasis. These date suggest that DCC assay values most probably result from unsuitable tissue sampling, In this study, we used IHC with the anti - ER_D antibody made ourselves and ISH analysis with a digoxigenin labeled probe to detect ER protein and mRNA in frozen sections of 15 breast carcinomas. The DCC assay of these same cases revealed positive receptor levels in 10 of 15 cases. Whereas the IHC and ISH methods was positive in 11 of 15. This because the basis of the ISH technique depends on the specific gene that lies within all nucleated cells, coding for particular proteins. It is highly sensitivity and highly specificity. ER mRNA of the hybridized signal was rapidly detected (within 3 days) and provides a degree of cell resolution that is unattainable with conventional methods using radiolabled probes. The method of this study also reveals many other advantages, such as probe stable and more convenient in operation. A further advantage is that the background signal obtained with the present method of detection is much lower than that observed using radiolabeled probes. IHC is used to test the expression fo gene products. It is less sensitive than ISH but more easier, simpler, quicker and cheaper than ISH. Staining of ER protein in frozen sections correlates highly with the ER mRNA detection in this report suggest that the possibility of using the peptide sequence Leu^{265} - Glu^{280} of the ER to produce antipeptide antibody that can be employed for the ER IHC in human tissues.

Of course, under the optimal conditions. ER IHC is a better method than the current biochemical assays. But we should further explore the correlation between semiquantitative grades of ER IHC and effects of antihormonal therapy (such as tamoxifen).

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REFERENCES

1. Jesen EV, Smith S, Desombre E, et al. Hormone dependency in breast cancer. J Steroid Bichem 1976; 7: 911.
2. Osborne K, Yochmowitz G, Knight A, et al. The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer 1980; 46:2884.
3. Sambrook J, Fritsch EF, Maniatis T, et al. Molecular Cloning : a lab manual. New York: Cold Spring Harbor Lab. 1989.
4. Sulian S, Hong DU. Production and application of anti - HRP monoclonal antibody to rat. Acta Anatomica Sinica 1990; 21(4):414.
5. Helin H, Helle M, Kallidniemi O, et al. Immunohistochemical determination of estrogen and progesterone receptors in human breast carcinoma. Cancer 1989; 63: 1761.
6. Wittliff J. Steroid hormon receptors in breast cancer. Cancer 1984; 53:630.
7. Maddox PH. 3 - Aminopropyltriethoxysilane (APES): A new advance in section adhesion. J Clin Pathol 1987; 40: 1256.
8. Esteban J, Battiford H, Warsi Z, et al. Quantification of estrogen receptors on paraffin - embeded tumors by image analysis. Mod Pathol 1991; 4(1):53.
9. Green S, Walter P, Kumar V, et al. Human estrogen receptor cDNA sequence, expression and homology to V - *erb* - A. Nature 1986; 320:134.
10. Furlow D, Ahrens H, Mueller GC, et al. Antisera to a synthetic peptide recognize native and denatured rat estrogen receptors. Endocrinology 1990; 127:1028.