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Heterogeneity of the estrogen receptor in breast cancer tissues

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= Abstract =

유방암에서 에스트로젠 수용체의 다변성

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Background: Popular immunohistochemical techniques for assay of estrogen receptor(ER) allow the localization of positive cells in specific cell populations. Some of breast carcinomas composed of discrete populations of cells were negative for ER, while neighboring populations of cells were positive for ER. Such heterogeneity might be due to biological or artifactual causes. Methods: We studied 67 tissue blocks for geographic heterogeneity within the level of ER and cytokeratin(CK) by staining ER and CK. Positive distribution of ER and CK was manually assessed. **Results:** The immunohistochemical expression revealed 50 cases for ER-positive and 17 cases for ER-negative. In 50 ER-positive cancers, homogeneity was 38 cases, heterogeneity was 11 cases, and artifactural change was developed in and one case. excluded in the analysis. The rate of heterogeneity of the ER-positive cancers was 22.4%(11/49). Comparisons of homogeneity and heterogeneity according to clinicopathologic risk factors in ER-positive breast cancer demonstrated that the heterogeneity of ER was significantly higher in each subgroups; relatively younger ages($\leq$50yr), premenopausal status, early menarche($\leq$15yr), early stage($\leq$Ib), DCIS in pathology, and lower positive expression rate of ER(< 50%). **Conclusion:** Clinicopathologic risk factoes would be required to discover the heterogeneity of ER-positive breast cancer. Also a long-term follow-up study on risk factors, including disease free survival, response to anti-estrogen therapy, and survival according to heterogeneity of ER would be needed. **(Journal of Korean Breast Cancer Society 2000;2:95~103)**

Key Words: Estrogen receptor, Immunohistochemistry, Heterogeneity, Breast cancer

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Introuction

Immunohistochemical staining has become a common method to determine estrogen receptor status in breast cancer tissues. In some breast cancers, the presence of small population of estrogen receptor negative cells within predominant estrogen receptor positive cancer cells has been found.

Heterogeneity of ER would be one of the risk factors in prognosis of breast cancers. On the assumption that heterogeneity of ER positive breast cancer would be in some way related to clinicopathologic factors, we assessed heterogeneity for ER in ER-positive breast cancers to determine the possible relationships between tumor heterogeneity for ER and clinico-pathologic risk factors.

Matetials and Methods

1. sample preparation

Sixty-seven malignant tissue blocks were selected from consecutive breast cancer patients who were admitted to Korea University Hospital for surgery in 1997.

2. Primary antibodies

The primary antibody for the ER assay used in this study was a mouse monoclonal antibody directed against sites present on human ER antigen. The antibody is known to bind to the receptor in formalin-fixed, paraffin-embedded tissue. The primary antibody for the CK assay was a monoclonal mouse anti-keratin.

3. Immunohistochemical Procedures

The tumors were sectioned and fixed in 10% neutral buffered formalin for routine surgical pathologic evaluation. After fixation, the specimens were routinely processed and paraffin embedded. Study tissues were cut into 5-μm slices and placed on slides. The slides were deparaffinized in three cycles of xylene and then rehydrated in three changes of ethanol. The slides were brought to water and immersed in autoclaving at 120℃ for 15 min with 1,000 ml of 10 mM citrate buffer(pH 6.0) as a routine process. IHC staining procedure was based on the labeled stretavidin-biotin(LSAB) method with the aid of Large volume DAKO LSAB® Kit(DACO, Carpinteria, U.S.A.). Endogenous peroxidase activity was quenched by first incubating the specimen for 5 min in 3% hydrogen peroxidase. Non-specific staining was blocked by a 5-min incubation with blocking reagent(normal goat serum). The specimens were incubated with primary antibody of ER or CK, followed by sequential 10-min incubations with biotinylated anti-rabbit and anti-mouse immunoglobulins. Staining was completed after 10-min incubation with substrate-chromogen solution (DAKO® DAB Chromogen tablets, 3,3'-diaminobezidine). Counterstaining was completed after 10-min incubation with Mayer's hematoxylin. The specimen was dehydrated in three changes of ethanol and was mounted with DAKO Glycergel® Mounting Medium. The primary antibodies of ER and CK were used a monoclonal mouse antibody directed against a mixture of ER (NCL-ER-6F11, clone 6F11, 1:50, Novocastra Co., U.S.A.) for ER and a monoclonal mouse anti-keratin antibody directed against a mixture of cytokeratin (AE1/AE3, 1:50, Zymed, South San Francisco, U.S.A.) for CK.

4. Interpretation

Various stainings of geographic heterogeneity for expression of nuclear ER and cytoplasmic CK were assessed by staining with antibodies directed against ER and CK. The first was stained with antiserum directed against ER and the second with antiserum directed against CK.

Following immunohistochemical localization of the antigens and interpretation under light microscopic examination(40x magnification), the slides were examined for geographic distribution of carcinoma as well as cells reacting with antibodies directed against CK and the ER. We examined four zones in one slide. The area of stain and geographic distributions of the carcinoma cells and the immunoreactivity for CK and ER were manually compared. Positive expression for ER was defined as over 10% staining of ER as a brownish stained nucleus on slides. The pattern of staining, area and shape of the zones for CK was assessed for control; the pattern of staining, area and shape of the zones for ER was assessed by similar methods and compared with the staining for CK, whether homogeneous or heterogeneous.

When the zones and geographic patterns of staining between CK and ER were similar, the staining reaction was considered homogenous; when the zones and geographic patterns of staining between the CK and the ER were variable, it was considered heterogeneous(Fig. 1). Variations in staining reaction between alternative slides stained for CK or ER was considered artifactural changes.

5. Data analysis

Comparisons between the homogeneity and heterogeneity of ER in ER-positive breast cancers were performed according to clinicopathologic factors, excluding ER-negative cases. The factors were composed of age BMI (body mass index, $kg/m^{2)}$, menopausal status, age at menarche, menstrual cycle, age at 1st delivery, breast feeding, number of baby, tumor size, lymph node involvement, distant metastasis, histologic grade, staging, pathologic type, and expression rate of the ER.

6. Statistical analysis

The data were expressed as the number of cases. Statistical differences between groups were tested with chi-square test or fisher exact test. A P value of less than 0.05 was regarded as significant.

Results

Immunohistochemical expression of ER was realed as 50 ER-positive and 17 ER-negative cases. In the 50 ER-positive cancers, homogeneity was 38 cases, heterogeneity was 11 cases, and artifactural change was one case. Artifactural change was excluded analysis(Table 1). The rate of heterogeneity of ER-positive cancers was 22.4%(11/49). In contrast, imunohistochemical expressions of CK for control showed up as positive and homogenous in all 67 cases. There was no case of heterogeneity in exession of CK. Comparisons of homogeneity and heterogeneity for 49 ER-positive breast cancers acrding to clinical characteristics were shown in Table 2.

According to clinicopathologic risk factors in ER-positive breast cancer between homogeneity and heterogeneity, it was demonstrated that the heter-

Table 1. Immunohistochemical expression for ER and CK.

Immunohistochemical expression		ER*	CK**
ER - positive	Homogeneity	38	49
	Heterogeneity	11	0
	Artifact	1	1
ER - negative		17	17

*estrogen receptor, ** cytokeratin

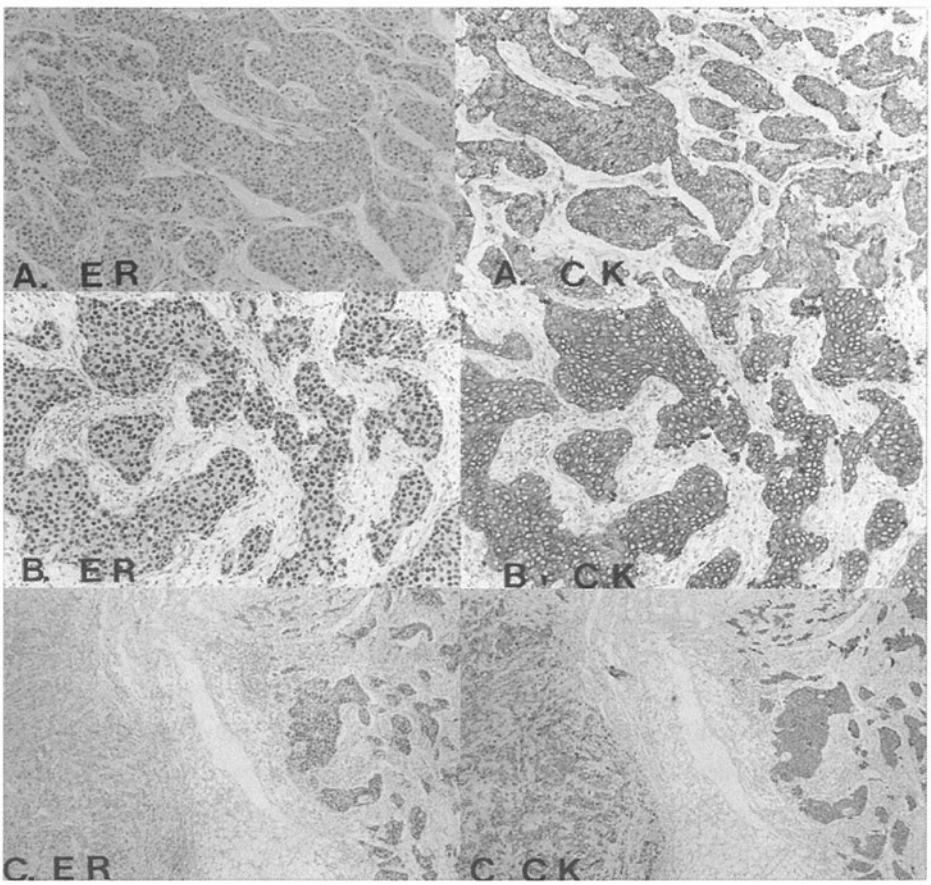

Fig. 1. Patterns of staining for the estrogen receptor(ER) and cytokeratin(CK). Immunohistochemical stain ing for ER shows nuclear staining, but that for CK shows cytoplasmic staining along the cell border. A. Homogeneously negative for ER and homogeneously positive for CK. B. Homogeneously positive for ER and CK. C. Heterogeneously positive for ER and homogeneously positive for CK.

ogeneity of ER was significantly higher in each subgroup; relatively younger ages($\leq$50yr), premenopausal status, early menarche($\leq$15yr), early stage($\leq$ Ib), DCIS in pathology, and lower positive expression rate of ER(< 50%)(Table 3, 4).

Discussion

Evaluation of ER status by immunohistochemical staining has evolved significantly since McCarty et al[1] stated that "several areas will require clarificaon before histochemical techniques can begin to be considered as a method for estrogen "receptor" analyses in the clinical evaluation of breast neoplasm." It had been demonstrated that patients with ER positive neoplasms had longer survival than with negative neoplasms and immunocytochemical analysis of estrogen receptors was the strong single prognostic indicator[2].

ER level by immunohistochemistry has become the standard method at many institutions. However, the implications of heterogeneity in the expression of hormone receptor within tissue section remains questionable[3].

Table 2. Comparisons of homogeneity and heterogeneity for ER-positive breast cancers according to clinical characteristics.

Risk factors	Category	Cases (n)	Homo-geneity	Hetero-geneity
Age(yrs)	Mean±S.E.*	49	49.87±1.76	42.9±2.65
	Ranges		25.0 - 73.0	33.0 - 65.0
BMI(kg/m2)	Mean±S.E.	49	23.45±0.57	23.93±0.91
	Ranges		16.2 - 30.5	19.5 - 29.1
Menopausal status	Mean±S.E.	17	49.12±1.37	57.0 -
	Ranges		39.0 - 58.0	57.0
Age at menarche	Mean±S.E.	49	17.00±0.40	15.11±0.39
	Ranges		14.0 - 21.0	13.0 - 17.0
Menstrual cycle	Regular	25	19	6
	Irregular	4	3	1
Age at 1st delivery	Mean±S.E.	37	26.46±0.66	24.75±0.73
	Ranges		20 - 33	21 - 28
Breast feeding	Yes	28	24	4
	No	9	9	0
No. of baby	Mean±S.E.	37	2.68±0.27	2.25±0.41
	Ranges		1 - 6	0 - 4
Tumor size	Tis	4	1	3
	T1	10	8	2
	T2	31	26	5
	T3	4	3	1
LN Involvement	N0	25	18	7
	N1	19	15	4
	N2	5	5	-
Distant Metastasis	M0	46	35	11
	M1	3	3	-
Histologic Grade	G1	7	6	1
	G2	31	25	6
	G3	-	-	-
Staging	0	3	1	2
	Ia	3	1	2
	Ib	2	1	1
	IIa	21	19	2
	IIb	12	9	3
	IIIa	5	4	1
	IIIb	-	-	-
	IV	3	3	-
Pathology	DCIS	5	1	4
	Invasive	41	34	7

* Mean Standard error

Table 3. Comparisons of homogeneity and heterogeneity for ER-positive breast cancers according to clinico-pathologic risk factors.

Risk factors	Category	Cases (n)	Homo-geneity	Hetero-geneity	P value*
Age (yrs)	≤50	31	21	10	0.05
	>50	18	17	1	
BMI (kg/m2)	<22	12	10	2	NS
	>25	11	8	3	
Menopausal status	Pre-	29	19	10	0.05
	Post-	17	16	1	
Age at menarche	≤15	14	6	8	0.01
	≥17	17	16	1	
Menstrual cycle	Regular	25	19	6	NS
	Irregular	4	3	1	
Age at 1st delivery	<30	28	24	4	NS
	≥30	9	9	0	
Breast feeding	Yes	28	24	4	NS
	No	9	9	0	
No. of baby	≤2	23	18	5	NS
	≥3	14	11	3	
Tumor size	<2cm	14	9	5	NS
	≥2cm	35	29	6	
	Tis, 1, 2, 3				NS
LN	Negative	25	18	7	NS
Involvement	Positive	24	20	4	
Distant Metastasis	No	46	35	11	NS
	Yes	3	3	0	
Histologic Grade	G1	7	6	1	NS
	G2	31	25	6	
Staging	≤I b	8	3	5	0.01
	≥II a	41	35	6	
Pathology	DCIS	5	1	4	0.01
	Invasive	41	34	7	

Immunohistochemical staining for ER has become a common method for quantitating ER within tumor tissue. Heterogeneity within cancer tissue has important implications for treatment and prognosis[4]. Heterogeneity in expression of the hormone receptor within histologic sections of breast carcinoma may arise from biological or artifactural causes. True biologic heterogeneity of hormone ex- pression could reflect multiple populations of tumor cells within the carcinoma[4].

Heterogeneity and homogeneity may have a different response to anti-estrogen, chemotherapeutic agents, or both.

Measuring estrogen and progesterone receptor levels has been shown to be of predictive for both responses to endocrine therapy and overall survival in patients with breast cancer[5-8].

Wittliff[6] demonstrated a good correlation between ER level and response to hormone therapy; with 55% of women with ER-positive breast cancer

Table 4. Comparisons of homogeneity and heterogeneity for ER-positive breast cancers according to expression rate of ER

Risk factor	Category*	Case	Homogeneity	Heterogeneity	p value **
ER	1+	19	12	7	NS
	2+	7	5	2	
	3+	20	18	2	
	4+	3	3	0	
ER	1+, 2+	26	17	9	<0.05
	3+, 4+	23	21	2	

* 1+: 10-25% of staining for ER, 2+: 25-50%, 3+:50-75%, 4+:>75%

** Chi square test or fisher exact test

responding to hormone therapys and ER-negative carcinoma appeared to have an increased response rate to cytotoxic chemotherapy. The primary reason for clinicians to order steroid hormone receptor assay was to identify receptor-negative carcinoma that were more likely to relapse and not respond to anti-estrogen therapy such as tamoxifen[9].

Thus, both accuracy of receptor determination of ER level and non-artifactual heterogeneity in the immunohistochemical expression of ER are extremely important issues for validating measuring ER levels[4]. Layfield et al[4] showed that the manual semigantitation of ER might be as accurate as quantitation of ER by image analysis. Homogenous positive staining was seen in 63 of 84 cases for ER and 71 of 84 cases for CK. The heterogeneity of ER-positive cancer in their study was seen in 2 (3.1%) of 65 cases. But also in their study, 7 of 82 cases except heterogeneous cases had shown geographic variation for ER while the CK controls for these slides revealed uniform positive staining. It was demonstrated that approximately 10 % of the cases of breast carcinoma show geographic and potentially biologic heterogeneity of expression for ER. We performed manual semiquantitation for expression of ER. Homogenous positive staining was seen in 38 of 67 cases for ER and 67 of 67 cases for CK. The heterogeneity of ER-positive cancers was revealed in 11(22.4%) of 49 cases in our study.

We experienced that some of breast carcinomas composed of discrete populations of cells were negative for ER, while neighboring populations of cells were positive for ER. Such heterogeneity might be due to biological or artifactual causes.

In cell selection technique to control for artictural heterogeneity of stain, Battifora[10] and Esteban et al[11] proposed that only cells from areas in which some positive staining was seen should be selected for the quantitation process. While this would control for artifactual heterogeneity of staining, it would preclude the detection of true biological heterogeneity. The prognostic and therapeutic implication of such biological heterogeneity are unknown, but it is expected that clones of breast cancer cells negative for ER dominated by a clone positive for ER might have a poorer prognosis and be less responsive to tamoxifen therapy. Hence, the recognition of these negative cases could be of clinical importance. In multiple ER assays with a microsample technique in 26 surgical breast cancer specimens, nine of the 26 breast cancers, although positive overall, were devoid of ER in some regions. For ER-positive tumors the average coefficient of variation(CV) for intra-tumor ER levels was 86%, ranging from 25% to 200%. This is well above the CV obtained with repeat samples of homogeneous

tissue(14%). These results suggest that many ER-positive cancers may be composed of cells with a variety of ER levels. An assessment of individual intra-tumor ER variability may have biologic and clinical significance[12]. The study of van Netten JP et al[13] also showed that heterogeneity in ER level as well as distribution was found in some tumors. In addition, a "checkerboard" type of staining with intermixed ER positive and ER negative cells was observed. A biochemical and immunohistochemical method may provide specific information about intra-tumor ER heterogeneity not available from either method alone.

In our comparison study of homogeneity and heterogeneity according to clinicopathologic risk factors in ER-positive breast cancer, it was demonstrated that the heterogeneity of ER was significantly higher in each subgroups; younger ages($\leq$50 yr.), premenopausal status, early menarche($\leq$15 yr.), early stage ($\leq$Ib), DCIS in pathology, and lower positive exession of ER (< 50%).

In conclusion, the expression of estrogen receptor for ER-positive breast cancer showed heterogeneous pattern in some cases. Status of ER for breast cancer would be important in therapeutic planing and determining responsiveness to hormonal therapy. Clinicopathologic risk factors would be required to find out the heterogeneity of ER-positive breast cancer. Also a long-term follow-up study on risk factors, including disease free survival, response to anti-estrogen therapy, and survival according to heterogeneity of ER would be needed.

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