additional multiple levels of immunohistochemistry leads to significant upgrading. For example, Freneaux et al reported upgrading in 47% of examined cases using four H&E sections and six additional levels of cytokeratin immunohistochemistry at intervals of 0.15 mm.[38]

Most metastatic foci detected only by immunohistochemistry will be either micrometastases or isolated tumour cells. There is a small possibility that cells other than metastatic carcinomas may be positive (false-positive staining), such as some macrophages. Benign transport of breast epithelium and pseudometastasis from noninvasive carcinomas have been reported.[39,40] To avoid pseudometastasis and to detect only clinically significant metastases, it is recommended that the number of immunohistochemistry-positive cells be quantified,[10] e.g. less than 10 cells, 10–100 cells, and more than 100 cells, as represented in two dimensions on a slide.

*Molecular analysis*

RT-PCR has been used for molecular analysis of SLNs. It is more sensitive than immunohistochemistry, but specific markers are lacking. The results of upgrading are still variable, and the procedure is not feasible in all pathology laboratories. At least currently, it is only used for research.[4]

## Assessment of metastases detected in SLNs

The clinical significance of carcinoma metastases in SLNs is important because almost half of SLN-positive cases may have further metastases in non-sentinel nodes.[41] Extranodal invasion from the SLN, the size of the metastatic focus in the SLN, the number of positive SLN nodes, and the size and lymphovascular invasion of the primary tumour are correlated with non-SLN metastases.[41,42] Conversely, small primary tumours (i.e. T1a ) and micrometastases are unlikely to have further metastases in non-SLNs.[20,42]

The negative predictive value of SLNs is considered good,[43] but the probabilities are significantly changed according to pathological procedures. Turner and colleagues analysed 1,087 non-sentinel nodes from 60 patients who were SLN-negative by H&E and immunohistochemistry. Only one node (one case) was positive for carcinoma, and the lesion was only detected by additional immunohistochemistry.[44] Thus, the probability of non-SLN metastasis will be less than 0.1% if SLN negativity is confirmed by both H&E and immunohistochemistry. In other words, isolated tumour cells in SLNs are unlikely to be associated with non-SLN involvement.

Finally, the clinical significance of micrometastases and/or isolated carcinoma cells has not been well elucidated. There are some studies,[4,27,45,46] but the real prognostic significance of micrometastases (i.e. detected by immunohistochemistry only) will only be clarified by future additional studies.

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J Hum Genet (2004) 49:391–395
DOI 10.1007/s10038-004-0160-5





SHORT COMMUNICATION

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# Identification and evaluation of 55 genetic variations in the *BRCA1* and the *BRCA2* genes of patients from 50 Japanese breast cancer families

Received: 29 January 2004 / Accepted: 7 April 2004 / Published online: 27 May 2004


**Abstract** We sequenced approximately 23 kb genomic regions containing all the coding exons and their franking introns of two breast cancer susceptibility genes, *BRCA1* and *BRCA2*, of 55 individuals from 50 unrelated Japanese breast cancer families. We identified 55 single-nucleotide polymorphisms (SNPs) (21 in *BRCA1* and 34 in *BRCA2*) containing nine pathogenic protein-truncating mutations (four in *BRCA1*and five in *BRCA2* from ten patients). Among the remaining 46 SNPs, allele frequencies of 40 were examined in both the breast cancer patients and 28 healthy volunteers with no breast cancer family history by PCR-RFLP or by direct DNA sequencing. Twenty-eight SNPs were common and were also found in the healthy volunteers and/or a SNP database. The remaining 18 were rare (allele frequency $<0.05$) and were not found in the healthy volunteers and/or the database. The pathogenic significance of these coding SNPs (cSNPs) remains to be clarified. The SNP information from this study will be useful in the future genetic testing of both *BRCA1* and *BRCA2* genes in the Japanese population.

**Keywords** Single-nucleotide polymorphism (SNP) · Breast cancer susceptibility gene · *BRCA1* · *BRCA2* · Japanese population · Direct DNA sequencing

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## Introduction

Mutations in the *BRCA1* and the *BRCA2* genes have been linked with the susceptibility to breast and ovarian cancer (Miki et al. 1994; Wooster et al. 1995; Tavtigian et al. 1996). Mutation carriers of these genes are at high risk of breast and ovarian cancer (Narod et al. 1995; Ford et al. 1998; Thorlacius et al. 1998; Neuhausen 1999; Rebbeck 1999; Struewing et al. 1997; Anglian Breast Cancer Study Group 2000). The two genes have large coding sequences consisting of 48 exons in total, and a large number of mutations and SNPs are reported in the Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/) and the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html). The majority of mutations described are protein-truncating mutations containing frame-shift mutations and nonsense mutations. In the BIC database, 55.9% of genetic variants are reported as pathogenic mutations containing mainly protein-truncating mutations and a small fraction of pathogenic missense mutations, and 39.3% of variants are categorized as "unclassified variants." These variants contain coding SNPs (cSNPs) that result in amino-acid substitutions or SNPs located at exon–intron boundaries. We have also reported five protein-truncating mutations and 12 "unclassified variants" that have been found only once in 24 breast cancer families (Sakayori et al. 2003). To elucidate whether detected SNPs are pathogenic mutations or not, it is important to accumulate SNP information (both the type and allele frequency) in patients and the general population.

Table 1 Sequence variations detected in the *BRCA1* and the *BRCA2* genes

| SNP ID[a] | Location | Variation[b] | Flanking sequence (5′ to 3′) | dbSNP ID[c] | BIC[d] | Volunteers[e] |
|---|---|---|---|---|---|---|
| *BRCA1*gene | | | | | | |
| BRCA1-1 | Intron 1 | IVS1-115C > T | tggtttgtat C/T attctaaaac | rs3765640 | | + |
| BRCA1-2 | Exon 3 | K38 (silent) (114G > A) | tctccacaaa G/A tgtgaccaca | rs8176099 | + | + |
| BRCA1-3 | Intron 8 | IVS8-58delT | tacatttttt T/– aacccttta | | + | + |
| BRCA1-4# | Exon 11 | G275D (824G > A) | gagccatgtg G/A cacaaatact | | | |
| BRCA1-5 | Exon 11 | S694 (silent) (2082C > T) | gacatgacag C/T gatactttcc | rs1799949 | + | + |
| BRCA1-6 | Exon 11 | L771 (silent) (2311T > C) | cagtatttca T/C tggtacctgg | rs16940 | + | + |
| BRCA1-7# | Exon 11 | 2389–2390delGA* | ggcaaaaaca GA/– accaaataaa | | | |
| BRCA1-8 | Exon 11 | P871L (2612C > T) | tcatttgctc C/T gttttcaaat | rs799917 | + | + |
| BRCA1-9 | Exon 11 | E1038G (3113A > G) | gtttttaaag A/G agccagctca | rs16941 | + | + |
| BRCA1-10 | Exon 11 | K1183R (3548A > G) | agcgtccaga A/G aggagagctt | rs16942 | + | + |
| BRCA1-11# | Exon 12 | C1372X (4116T > A)* | catctgggtg T/A gagagtgaaa | | | |
| BRCA1-12 | Exon 13 | S1436 (silent) (4308T > C) | taagtgactc T/C tctgcccttg | rs1060915 | + | + |
| BRCA1-13# | Intron 14 | IVS14 + 14A > G | agaaacatca A/G tgtaaagatg | | + | |
| BRCA1-14 | Exon 16 | S1613G (4837A > G) | atctgcccag A/G gtccagctgc | rs1799966 | + | + |
| BRCA1-15# | Exon 16 | M1628T (4883T > C) | tataatgcaa T/C ggaagaaagt | rs4986854 | + | |
| BRCA1-16 | Intron 18 | IVS18 + 66G > A | tacacctaac G/A tttaacacct | rs3092994 | + | + |
| BRCA1-17# | Intron 22 | IVS22 + 33A > T | gagagggagg A/T cacaatattc | | | |
| BRCA1-18# | Intron 23 | IVS23 + 8G > T | atggtaaggt G/T cctgcatgta | | + | |
| BRCA1-19 | Exon 5 | L63X (188T > A)* | cagtgtcctt T/A atgtaagaat | | + | |
| BRCA1-20 | Exon 3 | H41R (122A > G) | aagtgtgacc A/G catattttgcaaa | | | |
| BRCA1-21 | Exon 8 | 470–471delCT* | tccaactct CT/– aaccttggaa | | + | |
| *BRCA2*gene | | | | | | |
| BRCA2-1 | Exon 2 | 5′UTR-26G > A | tatttaccaa G/A cattggagga | rs1799943 | + | + |
| BRCA2-2# | Intron 2 | IVS2-16T > A | taaggtggga T/A ttttttttta | | | |
| BRCA2-3# | Intron 2 | IVS2-9T > G | ggatttttt T/G ttaaatagat | | | |
| BRCA2-4 | Intron 4 | IVS4 + 67A > C | tgttctataa A/C gatgaatctg | | | + |
| BRCA2-5 | Intron 4 | IVS4-89T > C | acaatttata T/C gaatgagaat | | + | + |
| BRCA2-6 | Intron 7 | IVS7 + 183T > A | caaatacatt T/A agtggtagtc | | | + |
| BRCA2-7 | Intron 8 | IVS8 + 56C > T | tttggaatgc C/T ttgttaaatt | | + | + |
| BRCA2-8# | Exon 10 | F266 (silent) (798T > C) | gtcatggatt T/C ggaaaaacat | | | |
| BRCA2-9 | Exon 10 | N289H (865A > C) | gtcaatgcca A/C atgtcctaga | rs766173 | + | + |
| BRCA2-10 | Exon 10 | H372 N (1114C > A) | aaatgtagca C/A atcagaagcc | rs144848 | + | + |
| BRCA2-11 | Exon 10 | S455 (silent) (1365A > G) | taccaaaatc A/G gagaagccat | rs1801439 | + | + |
| BRCA2-12# | Exon 10 | T582P (1744A > C) | tttaatatcc A/C ctttgaaaaa | | + | |
| BRCA2-13 | Exon 11 | H743 (silent) (2229T > C) | cagtacaaca T/C tcaaaagtgg | | + | + |
| BRCA2-14 | Exon 11 | M784V (2350A > G) | aaacctagtc A/G tgatttctag | | + | + |
| BRCA2-15 | Exon 11 | N991D (2971A > G) | tgattacatg A/G acaaatgggc | rs1799944 | + | + |
| BRCA2-16 | Exon 11 | K1132 (silent) (3396A > G) | agtttagaaa A/G ccaagctaca | rs1801406 | + | + |
| BRCA2-17# | Exon 11 | S1140 (silent) (3420T > C) | tgcagaagag T/C acatttgaag | | | |
| BRCA2-18# | Exon 11 | 3830delA* | aagatagaaa A/– tcataatgat | | | |
| BRCA2-19# | Exon 11 | L1522F (4566G > T) | ctactctgtt G/T ggttttcata | | | |
| BRCA2-20# | Exon 11 | G2044V (6131G > T) | tcccaaaaag G/T cttttcatat | | + | + |
| BRCA2-21 | Exon 14 | S2414 (silent) (7242A > G) | aaactaaatc A/G cattttcaca | Rs1799955 | + | + |
| BRCA2-22# | Exon 11 | 6491–6495delAGTTG* | gacaaacaac AGTTG/– gtattaggaa | | | |
| BRCA2-23 | Intron 16 | IVS16 + 47A > G | gtattcccte A/G tccctcttte | | | |
| BRCA2-24 | Intron 16 | IVS16-14T > C | aatattctac T/C tttatttgtt | | + | + |
| BRCA2-25 | Exon 11 | V2109I (6325G > A) | acttcctcgt G/A ttgataagag | | + | |
| BRCA2-26# | Exon 20 | S2835X (8504C > A)* | gagaagacat C/A atctggatta | | | |
| BRCA2-27 | Intron22 | IVS22-147A > G | cagataaagt A/G taaagttagt | | | |
| BRCA2-28 | Exon 25 | R3128X (9382C > T)* | AacctccagtggC/Tgaccagaatcc | | + | |
| BRCA2-29 | Exon 10 | 1278delA* | ttcagaaaa A/– gacctattag | | | |
| BRCA2-30 | Exon 10 | K322Q (964A > C) | aaatctacaa A/C aagtaagaactagc | | + | |
| BRCA2-31 | Exon 10 | E425 (silent) (1275A > G) | aaatatttcaga A/G aaagacct | | | |
| BRCA2-32 | Exon 11 | V1269 (silent) (3807T > C) | catgattctgt T/C gtttcaatgt | | + | |
| BRCA2-33 | Exon 11 | E1455 (silent) (4365A > G) | cagaaaccaga A/G gaattgcata | | | |
| BRCA2-34 | Exon 18 | K2729N (8187G > T) | tatgctgttaa G/T gcccagttagatcct | | + | |

[a]The SNP ID that have been found in this study. #Published previously (Sakayori et al. 2003)

[b]The nucleotide number in the coding region indicates the position downstream of the first nucleotide of ATG (initiation codon) in the *BRCA1* gene or the *BRCA2* gene. *: Nonsense or frame-shift mutation

[c]National Center for Biotechnology Information (NCBI) dbSNP database

[d]Breast Cancer Information Core (BIC) database, +: listed in BIC

[e]28 Japanese healthy volunteers, +: found at least one individual

Table 2 Allele frequencies of SNPs in the *BRCA1* and the *BRCA2* genes

| SNP ID[a] | Allele frequency | | |
|---|---|---|---|
| | Breast cancer patients[b] | Volunteers[c] | dbSNP[d] |
| *BRCA1*gene | | | |
| BRCA1-1 | *C*=0.43, *T*=0.57 | *C*=0.52, *T*=0.48 | *C*=0.343, *T*=0.657 |
| BRCA1-2 | *G*=0.93, *A*=0.07 | *G*=0.98, *A*=0.02 | *G*=0.994, *A*=0.006 |
| BRCA1-3 | *T7*=0.65, *T6*=0.35* | *T7*=0.50, *T6*=0.50 | |
| BRCA1-4 | *G*=0.98, *A*=0.02 | *G*=1.00, *A*=0.00 | |
| BRCA1-5 | *C*=0.62, *T*=0.38 | *C*=0.56, *T*=0.44 | *C*=0.657, *T*=0.343 |
| BRCA1-6 | *T*=0.62, *C*=0.38 | *T*=0.54, *C*=0.46 | *T*=0.678, *C*=0.322 |
| BRCA1-8 | *C*=0.62, *T*=0.38 | *C*=0.54, *T*=0.46 | *C*=0.619, *T*=0.381 |
| BRCA1-9 | *A*=0.62, *G*=0.38 | *A*=0.54, *G*=0.46 | *A*=0.725, *G*=0.275 |
| BRCA1-10 | *A*=0.62, *G*=0.38 | *A*=0.54, *G*=0.46 | *A*=0.703, *G*=0.297 |
| BRCA1-12 | *T*=0.62, *C*=0.38 | *T*=0.54, *C*=0.46 | *T*=0.747, *C*=0.253 |
| BRCA1-13 | *A*=0.98, *G*=0.02* | *A*=1.00, *G*=0.00 | |
| BRCA1-14 | *A*=0.62, *G*=0.38 | *A*=0.54, *G*=0.46 | *A*=0.696, *G*=0.304 |
| BRCA1-15 | *T*=0.98, *C*=0.02 | *T*=1.00, *C*=0.00 | *T*=0.995, *C*=0.005 |
| BRCA1-16 | *G*=0.63, *A*=0.37 | *G*=0.54, *A*=0.46 | *G*=0.693, *A*=0.307 |
| BRCA1-17 | *A*=0.98, *G*=0.02* | *A*=1.00, *G*=0.00 | |
| BRCA1-18 | *G*=0.99, *T*=0.01 | *G*=1.00, *T*=0.00 | |
| BRCA1-20 | *A*=0.99, *G*=0.01 | ND | |
| *BRCA2*gene | | | |
| BRCA2-1 | *G*=0.55, *A*=0.45 | *G*=0.49, *A*=0.51 | *G*=0.762, *A*=0.238 |
| BRCA2-2 | *T*=0.97, *C*=0.03* | *T*=1.00, *C*=0.00 | |
| BRCA2-3 | *T*=0.98, *G*=0.02* | *T*=1.00, *C*=0.00 | |
| BRCA2-4 | *A*=0.84, *C*=0.16* | *A*=0.84, *C*=0.16 | |
| BRCA2-5 | *T*=0.84, *C*=0.16* | *T*=0.84, *C*=0.16 | |
| BRCA2-6 | *T*=0.32, *A*=0.68* | *T*=0.58, *A*=0.42 | |
| BRCA2-7 | *C*=0.96, *T*=0.04* | *C*=0.96, *T*=0.04 | |
| BRCA2-8 | *T*=0.98, *C*=0.02 | *T*=1.00, *C*=0.00 | |
| BRCA2-9 | *A*=0.86, *C*=0.14 | *A*=0.84, *C*=0.16 | *A*=0.838, *C*=0.024, *G*=0.009, *T*=0.129 |
| BRCA2-10 | *A*=0.80, *C*=0.20 | *A*=0.82, *C*=0.18 | *A*=0.607, *C*=0.281, *G*=0.026, *T*=0.085 |
| BRCA2-11 | *A*=0.86, *G*=0.14 | *A*=0.84, *G*=0.16 | *A*=0.875, *G*=0.125 |
| BRCA2-12 | *A*=0.99, *C*=0.01 | *A*=1.00, *C*=0.00 | |
| BRCA2-13 | *T*=0.86, *C*=0.14 | *T*=0.84, *C*=0.16 | |
| BRCA2-14 | *A*=0.93, *G*=0.07 | *A*=0.95, *G*=0.05 | |
| BRCA2-15 | *A*=0.85, *G*=0.15 | *A*=0.84, *G*=0.16 | *A*=0.970, *G*=0.030 |
| BRCA2-16 | *A*=0.47, *G*=0.53 | *A*=0.50, *G*=0.50 | *A*=0.705, *G*=0.295 |
| BRCA2-17 | *T*=0.99, *C*=0.01 | *T*=1.00, *C*=0.00 | |
| BRCA2-19 | *G*=0.98, *T*=0.02 | *G*=1.00, *T*=0.00 | |
| BRCA2-20 | *G*=0.97, *T*=0.03 | *G*=0.98, *T*=0.02 | |
| BRCA2-21 | *A*=0.58, *G*=0.42 | *A*=0.50, *G*=0.50 | *A*=0.758, *G*=0.242 |
| BRCA2-23 | *A*=0.96, *G*=0.04* | *A*=1.00, *G*=0.00 | |
| BRCA2-24 | *T*=0.36, *C*=0.64 | *T*=0.27, *C*=0.73 | |
| BRCA2-25# | *G*=0.99, *A*=0.01 | *G*=1.00, *A*=0.00 | |
| BRCA2-27# | *A*=0.98, *G*=0.02* | *A*=1.00, *G*=0.00 | |
| BRCA2-30 | *A*=0.97, *C*=0.03 | ND | |
| BRCA2-31 | *A*=0.97, *C*=0.03 | ND | |
| BRCA2-32 | *T*=0.86, *C*=0.14 | ND | |
| BRCA2-33 | *A*=0.98, *G*=0.02* | ND | |
| BRCA2-34 | *G*=0.98, *T*=0.02 | ND | |

[a]Identical to the SNP ID listed in Table 1, #: found in a relative but not in the proband
[b]110 alleles from 55 patients, *: data derived from 68 alleles (34 patients)
[c]56 alleles from 28 healthy volunteers, ND: not determined
[d]National Center for Biotechnology Information (NCBI) dbSNP database

In this study, we extended our study on the *BRCA1* and *BRCA2* sequencing project to 55 breast cancer patients from 50 Japanese breast cancer families. We evaluated the detected SNPs by comparing the allele frequency of the SNPs in healthy volunteers.

## Subjects and methods

Fifty-five enrolled patients (including 50 probands and five relatives) with a history of breast cancer from 50 unrelated high-risk breast cancer families were selected according to the criteria defined by the Tohoku Familial Cancer Society (Sakayori et al. 2003). An additional 28 Japanese volunteers with no breast cancer family history were also enrolled to analyze the specific *BRCA1* and *BRCA2* variations detected in the breast cancer patients. We obtained informed consent from all patients and volunteers, and the independent studies were approved by the Familial Cancer Society and the Ethical Committee of Tohoku University Graduate School of Medicine. To identify the sequence variations in the *BRCA1* and *BRCA2* genes of the familial breast cancer patients, we sequenced approximately 23 kb genomic regions of the *BRCA1* (8.4 kb) and the *BRCA2* (14.6 kb) containing all coding exons and their franking introns using a method described previously (Sakayori et al. 2003). The identified SNPs found in the familial breast cancer

patients were also examined in the genomic DNA from the healthy volunteers by PCR-RFLP analysis using PCR primers, cycle conditions and restriction enzymes for PCR-RFLP analysis (http://www.idac.tohoku.ac.jp/dep/co/data/saka/brca02.htm) or by DNA sequence analysis. We used Genbank (U14680 and U61268 for *BRCA1*, U43746 and X95152-77 for *BRCA2*) and the BIC database as the reference sequences of *BRCA1* and *BRCA2* genes.

## Results and discussion

By DNA sequencing analysis of the *BRCA1* and *BRCA2*genes for 55 patients from 50 unrelated breast cancer families; we detected 55 SNPs (21 SNPs in *BRCA1* and 34 SNPs in *BRCA2*). Among these SNPs, we found nine protein-truncating mutations (four in *BRCA1*and five in *BRCA2*) in ten patients containing six novel mutations that were not found in the BIC database (http://research.nhgri.nih.gov/bic/) (Table 1). The existence of mutations were also confirmed by the stop codon assay in yeast (Ishioka et al. 1997; Sakayori et al. 2003). The percentage of protein-truncating mutations in the examined families was 20% (ten of 50), comparable with several reports describing the frequency of protein-truncating mutations of the two genes in Japanese breast cancer families (Inoue et al. 1995; Takano et al. 1997; Inoue et al. 1997; Ikeda et al. 2001). Although the frequency was also similar to results from Western populations, the number of patients studied in our and other Japanese populations was smaller than that in Western countries. Therefore, it is necessary to study a larger number of patients to clarify the mutation frequency in Japanese familial breast cancer.

Among the remaining 46 SNPs (17 SNPs in *BRCA1* and 29 SNPs in *BRCA2*), 18 and 31 have been reported in the dbSNP database and in the BIC database, respectively, (Table 1). To evaluate whether the SNPs are also found in Japanese healthy volunteers, all but six SNPs were further examined by PCR-RFLP analysis or by DNA sequence analysis. Twenty-six SNPs were found in the volunteers at least once with different allele frequencies (Table 2). In these 40 SNPs, we predicted that 27 SNPs were common polymorphisms and probably played no direct role in the tumorigenesis of breast cancer. The remaining 13 SNPs were quite low in allele frequency ($<0.05$) and were not found in the healthy volunteers or the dbSNP database. In addition, there were six SNPs that failed to examine in the volunteer group. In these six SNPs, one was predicted to be common and five to be rare from the allele frequencies in the patients. Overall, we predicted the 18 rare SNPs are candidates of pathogenic mutations and that the remaining 28 were common polymorphisms and probably have no direct roll in tumorigenesis of breast cancer. In the 18 rare SNPs, five (BRCA1-13, BRCA1-17, BRCA1-18, BRCA2-2, BRCA2-3) were located at the exon–intron boundaries and two (BRCA2-23, BRCA2-27) were located in introns far from exon–intron boundaries. Although SNPs at the exon–intron boundaries may affect normal RNA splicing, our RT-PCR analysis showed negative data for splicing abnormalities. In the remaining 11 rare cSNPs, seven (BRCA1-4, BRCA1-20, BRCA2-12, BRCA2-19, BRCA2-25, BRCA2-30 and BRCA2-34) were nonsynonymous substitution resulting in the amino-acid substitutions, and four (BRCA2-8, BRCA2-17, BRCA2-31 and BRCA2-33) were synonymous (silent) changes. These SNPs may directly affect the functions of BRCA1 and BRCA2 proteins or affect normal splicing by acting as possible cryptic splicing sites. Unfortunately, we have failed to clarify this issue mainly because there are no reliable functional assays of either BRCA1 or BRCA2 protein for many nonsynonymous changes.

To confirm the pathogenic effect of rare SNPs, both the development of functional assays for these gene products and more intensive SNP analysis including an investigation into whether these SNPs cosegregate with breast cancer onset in families are necessary.

**Acknowledgements** We thank patients and their families for participating in this study. We also thank Yuka Fujimaki for technical assistance. This study was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan.

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Colloids and Surfaces A: Physicochem. Eng. Aspects 242 (2004) 47–52

COLLOIDS AND SURFACES A

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# Silica-coating of fluorescent polystyrene microspheres by a seeded polymerization technique and their photo-bleaching property

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Received 30 October 2003; accepted 20 April 2004

Available online 15 June 2004

**Abstract**

This paper describes silica-coating of polystyrene microspheres incorporated with fluorescence dyes (fluorescent microspheres) by means of a seeded polymerization technique based on Stöber method. The silica-coating of the fluorescent microspheres was performed in the presence of 0–10 g/l polyvinylpyrrolidone (PVP), 1.13–17 M water, 0–1.2 M aqueous ammonia and 0.00038–0.2 M tetraethoxyorthosilicate (TEOS). The addition of PVP was found to suppress the generation of free silica particles and improve the uniformity of shell thickness. The silica shell thickness increased from 13 to 138 nm with an increase in TEOS concentration at 10 g/l PVP, 0.4 M aqueous ammonia and 10.9 M water. The thickness also increased with the ammonia concentration and the water concentration. However, excess ammonia and water caused aggregation of free silica particles and the polystyrene microspheres. The silica-coated fluorescence microspheres showed more stable fluorescence to laser-irradiation than uncoated microspheres.


*Keywords:* Fluorescent microsphere; Core-shell; Silica-coating; Sol–gel; Seeded polymerization

## 1. Introduction

Microspheres incorporated with fluorescence dyes (fluorescent microspheres) have been used widely as cell-surface antigen detection, neutral retrograde tracers, phagocytosis tracers, sensitive diagnostic reagents and blood flow measurements [1–4]. It is desirable that fluorescences of dyes in the microspheres are strong and persistent for long periods. The photostability of the dyes is environmentally sensitive, and singlet state oxygen molecules play the main role of photo-bleaching of the fluorescence dye molecules in the excited state [5–7]. Core-shell type particles are good candidates for preventing decomposition because the shell materials can keep dyes from contact with oxygen molecules.

The core-shell types particles show various unique properties owing to their composite structures. They are applicable to a wide variety of materials such as magnetism [8–12], electronics [13–16] and optics [17–19]. Liz-Marzán and co-workers demonstrated silica-coating on CdS nanoparticles inhibited light-induced surface reactions, so that photostability of CdS was improved [20]. Our group also reported a protection effect of silica shell using silica-coated Co nanoparticles, in which the silica-coating prevented Co nanoparticles from oxidization and provided crystallization to cubic metal Co phase that showed magnetic properties [21]. In addition, we employed direct silica-coating on gold nanoparticles by a seeded polymerization techniques [22].

In this article, the silica-coating technique is extended to the fluorescence microspheres. The fluorescence microspheres have been coated with silica shell at different concentrations of polyvinylpyrrolidone (PVP), water, ammonia and tetraethoxysilane (TEOS). The photo-bleaching of the

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doi:10.1016/j.colsurfa.2004.04.052

fluorescence dyes within the microspheres has been monitored under irradiation of laser in the presence of air.

## 2. Experimental

### 2.1. Chemicals

FluoSpheres® beads (F-8803) commercially available from Molecular Probes Inc. were used as fluorescent microspheres. The fluorescent microspheres were composed of a host matrix of polystyrene and a dopant of fluorescent dyes. Fig. 1 shows their fluorescence spectrum and photograph taken by a transmission electron microscope (TEM). The fluorescent microspheres have a fluorescence peak at 512 nm and an average size of 193 nm. The chemicals of ethanol (99.5%), $NH_4OH$ (25% aqueous solution) and tetraethylorthosilicate (TEOS, 95%) obtained from Wako Pure Chemicals Ltd., and polyvinylpyrrolidone (PVP, average molecular weight: 36000) from Nacalai Tesque Ltd. were used as received. Ultrapure deionized water (resistivity higher than 18 MΩ cm) was used in the preparations.

### 2.2. Preparation of materials

Silica-coating of the fluorescent microspheres was carried out with ammonia-catalyzed reaction of TEOS in ethanol–water solution in a hermetically sealed reactor equipped with a magnetic stirrer at room temperature. Ethanol solution of TEOS was added to aqueous PVP solution under vigorous stirring after addition of the suspension of the fluorescent microspheres. Hydrolysis reaction of TEOS was initiated by the addition of the aqueous ammonia solution to form silica shell on the microspheres, which

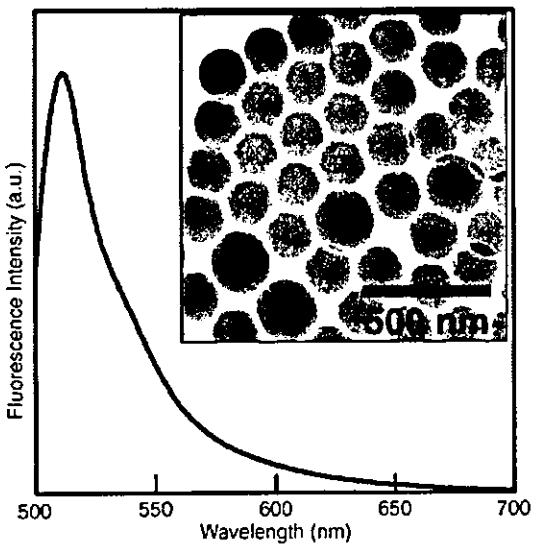


Fig. 1. Fluorescence spectrum of FluoSpheres® (F-8811) and their TEM image shown in the inset. The excitation wavelength was 488 nm.

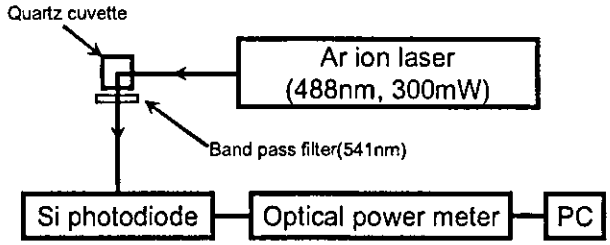


Fig. 2. Experimental set-up for measurements of photo-bleaching.

was reacted for 24 h under stirring. Concentrations of PVP, water, ammonia and TEOS and were in ranges of 0–10 g/l, 1.13–17 M, 0–1.2 M and 0.00038–0.2 M, respectively.

### 2.3. Characterization

The silica-coated fluorescent microspheres were observed with a transmission electron microscope (TEM) (Zeiss LEO 912 OMEGA) operated at 100 kV accelerating voltage. Samples for TEM were prepared by dropping the suspension of the fluorescent microspheres onto the top of a collodion-coated copper grid and drying. Fluorescence spectra were measured with a Hitachi F-4500 fluorophotometer. Fig. 2 shows a set-up for analysis of photo-bleaching. The silica-coated microspheres in a quartz cuvette were irradiated by an argon ion laser (Coherent, INOVA90) with an emission wavelength of 488 nm and a power of 300 mW. Fluorescence at 541 nm was selected using a band pass filter with a bandwidth of 10 nm and detected with an Si photodiode (Anritsu, MA9411A) connected with an Anritsu ML9001 optical power meter.

## 3. Results and discussion

### 3.1. Effect of PVP concentration

Fig. 3 shows TEM micrographs of silica-coated fluorescent microspheres prepared at various PVP concentrations. In whole images, many core-free silica particles with sizes of 50–80 nm were observed. According to Kawahashi and Shiho [23–25], PVP is required for preventing aggregation of particles. However, no aggregations of the fluorescent microspheres were observed even without the addition of PVP (Fig. 3(a)). The fluorescent microspheres used have carboxyl groups on their surfaces according to a commercial catalog of FluoSpheres® beads. These carboxyl groups probably prevent such aggregation. In Fig. 3(a) and (b), silica particles with sizes of 45–90 nm deposited on the surfaces of fluorescent microspheres, which indicated that silica did not have a strong affinity for the fluorescent microsphere surfaces during growth from silica nuclei to silica nanoparticle. Such deposition decreased with the increase in PVP concentration. In Fig. 3(c)–(e), the silica shell with a size of 40–45 nm was formed on the surfaces, though the deposited

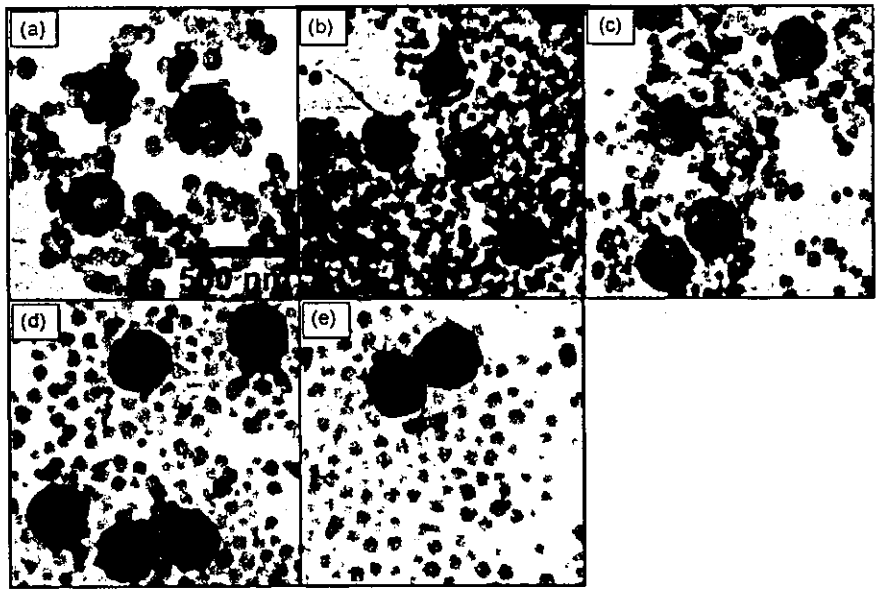


Fig. 3. TEM images of fluorescence microspheres coated with silica for 10.9 M water, 0.4 M ammonia and 0.02 M TEOS at PVP concentrations of (a) 0 g/l, (b) 0.01 g/l, (c) 0.1 g/l, (d) 1.0 g/l and (e) 10 g/l.

silica particles were still observed in Fig. 3(c). The surfaces of silica shell were smoother with the increase in PVP concentration. In our research, it can be considered that PVP improved an affinity between the silica nuclei and the fluorescent microsphere surfaces.

### 3.2. Effect of water concentration

Fig. 4 shows TEM micrographs of silica-coated fluorescent microspheres prepared at various water concentrations. In Fig. 4(a)–(c), homogeneous silica shells were observed on the surfaces of the fluorescent microspheres and their thickness increased from 13 to 60 nm with the water concentration. As Bogush and Zukoski reported [26], an increase in water concentration in TEOS/$NH_3$/water/ethanol solution dissociates ammonium hydroxide and brings about an increase in electric conductivity that corresponds to ionic strength. Since the increase in ionic strength reduces electrostatic repulsion between particles, the growth of silica shells was probably promoted.

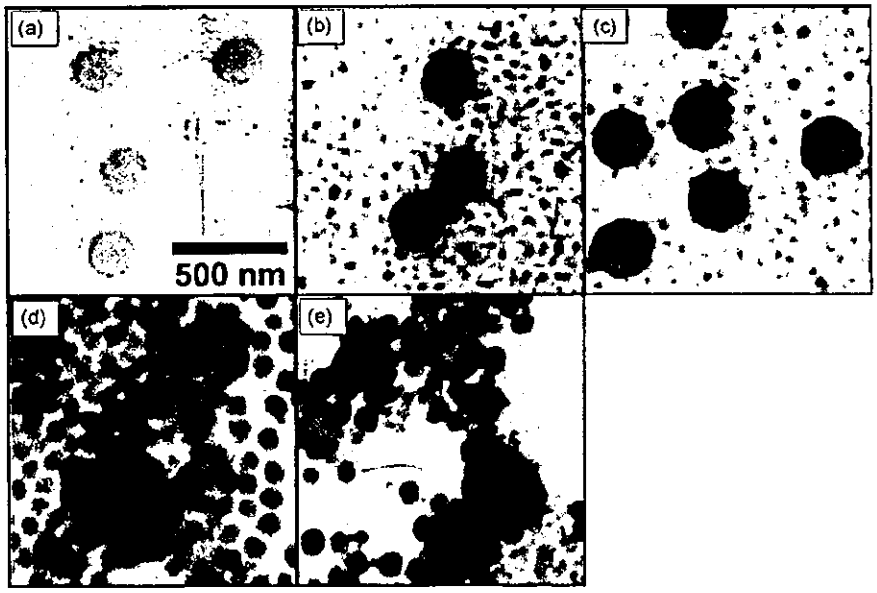


Fig. 4. TEM images of fluorescence microspheres coated with silica for 10 g/l PVP, 0.4 M ammonia and 0.02 M TEOS at initial water concentrations of (a) 1.13 M, (b) 5.0 M, (c) 10.9 M, (d) 13.0 M and (e) 17 M.

Many core-free silica particles were also observed and their average sizes increased from 41 to 92 nm with the water concentration. In Fig. 4(d) and (e), such silica particles adhered to the fluorescent microsphere surfaces and no more homogeneous shell was observed, which can not be explained by the change in ionic strength that was the factor causing the silica shell growth as shown in Fig. 4(a)–(c). Because the dielectric constant of water/ethanol mixture increases with water concentration, silanol groups on the silica particle surface probably tend to ionize with increasing water concentration. This might increase affinity of the silica particles for the dispersant. Therefore, silica nuclei generated during the early stages of the sol–gel reaction probably grew as stable core-free silica particles.

### 3.3. Effect of ammonia concentration

Fig. 5 shows TEM micrographs of silica-coated fluorescent microspheres prepared at different ammonia concentrations. At an ammonia concentration of 0 M (Fig. 5(a)), no silica shell and no silica particle was observed because of a shortage of catalyst. At ammonia concentrations of 0.2–0.8 M (Fig. 5(b)–(d)), the thickness of silica shell increased from 38 to 43 nm with the increase in ammonia concentration. Addition of ammonia increases the ionic strength of the solution and catalyzes the hydrolysis and condensation of the alkoxysilanes [27]. Thus, the high ammonia concentration should reduce the double layer repulsion between the fluorescent microspheres and the silica nuclei. As a result, the silica shells grew on the microsphere surfaces. At an ammonia concentration as high as 1.2 M, the fluorescent microspheres aggregated with the secondary generated silica particles (Fig. 5(e)). The high ammonia concentration extensively accelerated the sol–gel reaction of TEOS and then the core-free silica particles were generated from the silica nuclei and grew much before the silica nuclei was used for the silica shell formation.

### 3.4. Effect of TEOS concentration

Fig. 6 shows TEM micrographs of silica-coated fluorescent microspheres formed at various TEOS concentrations. Some silica particles were observed and their size tended to increase with the TEOS concentration. The ionic strength decreases as a sol–gel reaction of TEOS proceeds and then secondary silica particles are generated [28,29]. Since the high TEOS concentration should increase a source of silica, the silica shell grew. The silica shell thickness was varied from 13 to 138 nm as initial TEOS concentration increased from 0.00038 to 0.2 M. This means the shell thickness can be controlled within a certain threshold. The thickness of silica was smaller than those estimated from initial TEOS concentrations, because of the generation of the core-free silica particles.

### 3.5. Photo-bleaching

Fig. 7 shows the time-dependence of the fluorescence intensity. The fluorescence intensity of the silica-coated fluorescent microspheres was lower than that of the uncoated ones up to 15 min. However, the laser-irradiation over 15 min reversed the order of the fluorescence intensites. For making clear a difference between the silica-coated microspheres and the uncoated ones, the fluorescence intensities were normalized by the value of fluorescence intensity measured before the laser-irradiation, as shown in

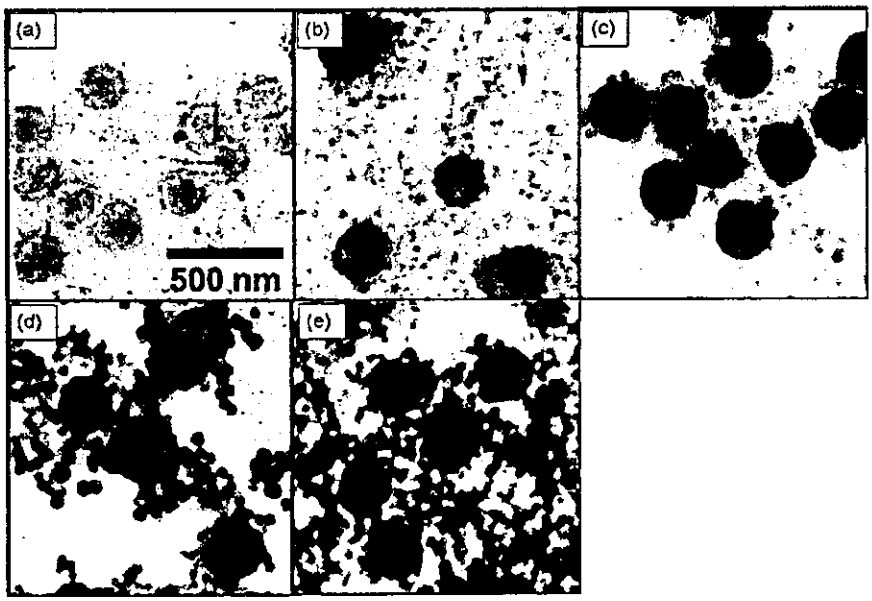


Fig. 5. TEM images of fluorescence microspheres coated with silica for 10 g/l PVP, 10.9 M water and 0.02 M TEOS at initial ammonia concentrations of (a) 0 M, (b) 0.2 M, (c) 0.4 M, (d) 0.8 M and (e) 1.2 M.

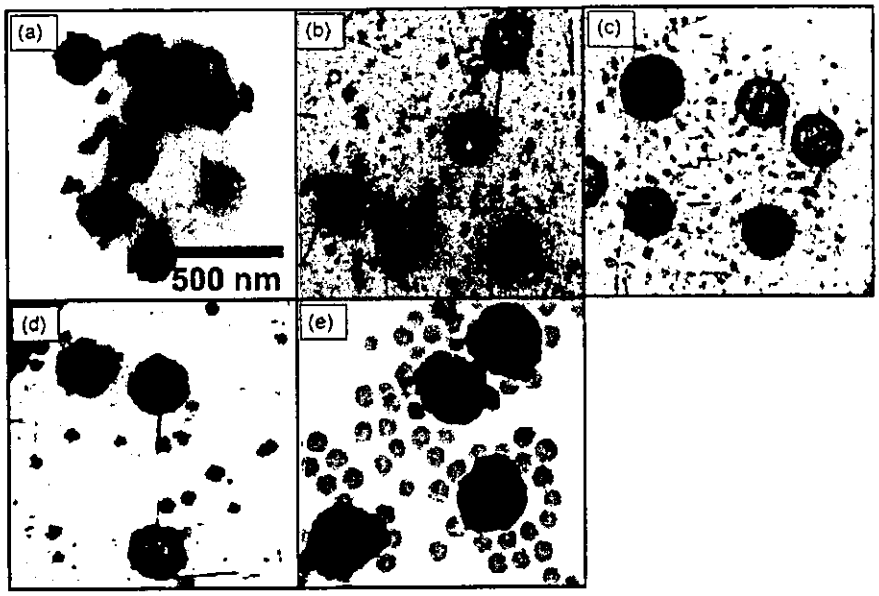


Fig. 6. TEM images of fluorescence microspheres coated with silica for 10 g/l PVP, 10.9 M water and 0.4 M ammonia at initial TEOS concentrations of (a) 0.00038 M, (b) 0.0015 M, (c) 0.009 M, (d) 0.02 M and (e) 0.2 M.

the inset. Time-dependence of the normalized fluorescence intensity for the silica-coated fluorescent microspheres was weak compared to that of the uncoated fluorescent microspheres, which is evidence that the silica-coated fluorescent microspheres were more stable in respect to their luminescence property than the uncoated ones. Singlet state oxygen molecules decompose dye molecules in their exited stage. [5–7]. This stable fluorescence property is probably related to the diffusional limitations of oxygen molecules inside of the fluorescent microspheres through the silica shell. Such stabilization by the silica-coating will be of importance in the preparation of stable materials for practical applications.

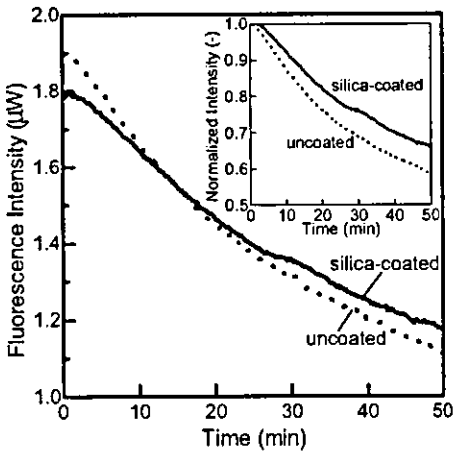


Fig. 7. Fluorescence intensities of silica-coated fluorescent microspheres and uncoated ones as a function of laser-irradiation time. The silica-coating was employed at 10 g/l PVP, 10.9 M water, 0.4 M ammonia and 0.02 M TEOS. The inset shows fluorescence intensity normalized by the value of fluorescence intensity measured before the laser-irradiation.

## 4. Conclusion

A synthetic method was developed for the stabilization of fluorescent microspheres. The method was based on the deposition of a silica shell on the fluorescent microsphere cores. The silica-coating was performed with a sol–gel reaction of TEOS in the presence of PVP and the fluorescent microspheres. Homogeneous silica shells were formed on the fluorescent microspheres in the presence of PVP. At high water and ammonia concentrations, no formation of homogeneous silica shells could be performed. With increasing TEOS concentration, the silica shell thickness increased. Concentration effects can probably be explained by differences in ionic strength of the solution. It was observed that the silica-coated fluorescent microspheres provided high luminescence stability, compared with uncoated ones. This property is significant for biomedical application.

## Acknowledgements

This research was partially supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for the COE Project, Giant Molecules and Complex Systems), and by the Ministry of Health, Labor and Welfare of Japan.

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[CANCER RESEARCH 64, 4670–4676, July 1, 2004]

# Estrogen-Related Receptor $\alpha$ in Human Breast Carcinoma as a Potent Prognostic Factor

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## ABSTRACT

**Estrogen-related receptor $\alpha$ (ERR$\alpha$) was identified as a gene related to estrogen receptor $\alpha$ (ER$\alpha$) and belongs to a class of nuclear orphan receptors. ERR$\alpha$ binds to estrogen responsive element(s) (ERE) and is considered to be involved in modulation of estrogenic actions. However, biological significance of ERR$\alpha$ remains largely unknown. Therefore, we examined the expression of ERR$\alpha$ in human breast carcinoma tissues using immunohistochemistry ($n$ = 102) and real-time reverse transcription-PCR ($n$ = 30). ERR$\alpha$ immunoreactivity was detected in the nuclei of carcinoma cells in 55% of breast cancers examined, and relative immunoreactivity of ERR$\alpha$ was significantly ($P$ = 0.0041) associated with the mRNA level. Significant associations were detected between ER$\alpha$ and ERE-containing estrogen-responsive genes, such as pS2 ($P$ < 0.0001) and EBAG9/RCAS1 ($P$ = 0.0214), in breast carcinoma tissues. However, no significant association was detected between ER$\alpha$ and pS2 ($P$ = 0.1415) in the ERR$\alpha$-positive cases ($n$ = 56) or between ER$\alpha$ and EBAG9/RCAS1 ($P$ = 0.8271) in the ERR$\alpha$-negative group ($n$ = 46). ERR$\alpha$ immunoreactivity was significantly associated with an increased risk of recurrence and adverse clinical outcome by both uni- ($P$ = 0.0097 and $P$ = 0.0053, respectively) and multi- ($P$ = 0.0215 and $P$ = 0.0118, respectively) variate analyses. A similar tendency was also detected in the group of breast cancer patients who received tamoxifen therapy after surgery. Results from our study suggest that ERR$\alpha$ possibly modulates the expression of ERE-containing estrogen-responsive genes, and ERR$\alpha$ immunoreactivity is a potent prognostic factor in human breast carcinoma.**

## INTRODUCTION

Estrogens are well known to contribute immensely to the development of hormone-dependent breast carcinomas (1, 2). Biological effects of estrogens are mediated through an interaction with estrogen receptor (ER) $\alpha$ and/or $\beta$ (3). ERs activate transcription of various target genes (*i.e.*, estrogen responsive genes) in a ligand-dependent manner by direct DNA interaction through the estrogen-responsive element(s) (ERE) or by tethering to other transcription factors (4, 5). Therefore, antiestrogens such as tamoxifen, which blocks ER, have been mainly used as an endocrine therapy in breast carcinoma for many years.

Estrogen-related receptor (ERR) family belongs to nuclear hormone receptors, and consists of three closely related members ($\alpha$, $\beta$, and $\gamma$; Refs. 6 and 7). ERRs share significant homology to ER$\alpha$ at the DNA-binding domain and recognize the ERE (8–10), which indicates that ERRs modulate the actions of ERs (11–13). However, ERRs are not activated by known natural estrogens and are therefore classified as orphan receptors (14). ERRs can also bind to steroidgenic factor 1 (SF1)-binding element within the promoter regions of various steroidogenic P450 genes including aromatase (15, 16).

Previous *in vitro* studies have demonstrated the mRNA expression of ERR$\alpha$ in breast cancer cell lines (17) and breast carcinoma tissues (18). ERR$\alpha$ activated the expression of pS2, one of the estrogen responsive genes (17), in breast cancer cells, and it has also been reported that ERR$\alpha$ regulated aromatase expression in breast fibroblasts (11). However, a detailed examination of ERR$\alpha$ expression, including at the protein level, has not been examined in human breast carcinoma tissues, and the biological significance of ERR$\alpha$ remains largely unclear. Therefore, in this study, we examined the immunolocalization of ERR$\alpha$ in 102 cases of human breast carcinoma tissues and correlated these findings with various clinicopathological factors including the clinical outcome. In addition, we also examined mRNA expression of ERR$\alpha$ in 30 cases of breast carcinoma tissues using real-time reverse transcription-PCR and analyzed the correlation with the ERR$\alpha$ immunoreactivity or aromatase mRNA expression.

## MATERIALS AND METHODS

**Patients and Tissues.** One hundred and two specimens of invasive ductal carcinoma of the breast were obtained from female patients who underwent mastectomy from 1985 to 1990 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. Breast tissue specimens were obtained from patients with a mean age of 53.6 years (range 27–82). None of the patients examined used oral contraceptives. The patients did not receive chemotherapy or irradiation before surgery. Eighty-eight patients received adjuvant chemotherapy, and ten patients received tamoxifen therapy after the surgery. The mean follow-up time was 106 months (range 4–157 months). The histological grade of each specimen was evaluated based on the method of Elston and Ellis (19). All specimens were fixed with 10% formalin and embedded in paraffin wax.

Thirty specimens of invasive ductal carcinoma were obtained from patients who underwent mastectomy in 2000 in the Departments of Surgery at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan. Specimens of adipose tissue adjacent to the carcinoma and non-neoplastic breast tissues were available for examination in 7 and 5 of these 30 cases, respectively. Specimens for RNA isolation were snap-frozen and stored at −80°C, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin-wax. Informed consent was obtained from all patients before their surgery and examination of specimens used in this study.

Research protocols for this study were approved by the Ethics Committee at both Tohoku University School of Medicine and Tohoku Kosai Hospital.

**Antibodies.** Mouse monoclonal antibody for ERR$\alpha$ (2ZH5844H) was purchased from Perseus Proteomics Inc. (Tokyo, Japan). This antibody was produced by immunizing mice with a systemic peptide corresponding to amino acids 98–171 of ERR$\alpha$ (GenBank accession number; X51416), and the characterization was confirmed by immunoblotting analyses.[4] Rabbit polyclonal antibody for estrogen sulfotransferase (EST; *SULT 1E1* gene; PV-P2237; Ref. 20) was purchased from Medical Biological Laboratory (Nagoya, Japan). EBAG9/RCAS1 antibody was a rabbit polyclonal antibody (21, 22) and was kindly provided from Dr. S. Inoue (Department of Biochemistry, Saitama Medical School, Saitama, Japan). Monoclonal antibodies for ER$\alpha$ (ER1D5), progesterone receptor (PR; MAB429), Ki-67 (MIB1), pS2 (M7184), cyclin D1 (P2D11F11), and c-myc (1-6E10) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA), DAKO (Carpinteria, CA), DAKO, Novocastra Laboratories (Newcastle, United Kingdom), and Cambridge Research Biochemical (Cambridge, United Kingdom), respectively. Rabbit polyclonal antibodies for ER$\beta$ (06–629) and human epidermal growth factor

Received 1/26/04; revised 4/1/04; accepted 5/4/04.

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receptor 2 (HER2; A0485) were obtained from Upstate Biotechnology (Lake Placid, NY) and DAKO, respectively.

**Immunohistochemistry.** A Histofine kit (Nichirei, Tokyo, Japan), which uses the streptavidin-biotin amplification method, was used for the identification of ERRα, ERα, PR, EST, HER2, Ki-67, pS2, EBAG9/RCAS1, cyclin D1, and c-myc immunoreactivity, whereas EnVision$^+$ (DAKO) was used for ERβ immunohistochemical analysis. Antigen retrieval for ERRα, ERα, ERβ, PR, HER2, Ki-67, EBAG9/RCAS1, and cyclin D1 immunostaining was performed by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer [2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)], and similarly, antigen retrieval for EST and pS2 immunostaining was done by heating the slides in a microwave oven for 15 min in a citric acid buffer. Dilutions of primary antibodies used in this study were as follows: ERRα, 1:1000; ERα, 1:50; ERβ, 1:50; PR, 1:30; EST, 1:9000; HER2, 1:200, Ki-67, 1:50; pS2, 1:30; EBAG9/RCAS1, 1:20; cyclin D1, 1:40; and c-myc 1:600. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution (1 mM 3,3′-diaminobenzidine, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% $H_2O_2$) and counterstained with hematoxylin.

Human tissues of heart were used as positive controls for ERRα immunohistochemistry (23). As a negative control for ERRα immunohistochemistry, normal mouse IgG was used instead of the primary antibody for ERRα, and no specific immunoreactivity was detected in these sections.

**Real-Time Reverse Transcription-PCR.** Total RNA was carefully extracted with guanidinium thiocyanate followed by ultracentrifugation in cesium chloride. A reverse transcription kit (SUPERSCRIPT II Preamplification system; Life Technologies, Inc., Grand Island, NY) was used in the synthesis of cDNA.

The Light Cycler System (Roche Diagnositics GmbH, Mannheim, Germany) was used to semi-quantify the mRNA level of ERRα, aromatase, and ribosomal protein L 13a (RPL13A) by real-time reverse transcription-PCR (24). Settings for the PCR thermal profile were as follos: initial denaturation at 95°C for 1 min followed by 40 amplification cycles of 95°C for 1 s, annealing at 62°C (ERRα), 60°C (aromatase), or 68°C (RPL13A) for 15 s, and elongation at 72°C for 15 s. The primer sequences used in this study are as follows: ERRα [X51416; forward 5′-TGCTCAAGGAGGGAGTGC-3′ (cDNA position; 785–802) and reverse 5′-GGCGACAATTTCTGGTTCGGGTCAGGCATGGCATAG-3′ (cDNA position; 981–998)], aromatase [(X13589; Ref. 20; forward 5′-GTGAAAAAGGGGACAAACAT-3′ (cDNA position; 1286–1305) and reverse 5′-TGGAATCGTCTCAGAAGTGT-3′ (cDNA position; 1481–1500)], and RPL13A [(NM012423; 25; forward 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′ (cDNA position; 487–509) and reverse 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′ (cDNA position; 588–612)]. Oligonucleotide primers for ERRα were designed in different exons to avoid the amplification of genomic DNA or human ERRα pseudo-gene (U85258). To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Human heart tissue was used as a positive control for ERRα, whereas human placental tissue was used as a positive control for aromatase. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA, and no amplified products were detected under these conditions. mRNA level for ERRα and aromatase in each case has been summarized as a ratio of RPL13A and subsequently evaluated as a ratio (%) compared with that of the positive controls.

**Scoring of Immunoreactivity and Statistical Analysis.** ERRα, ERα, ERβ, PR, and Ki-67 immunoreactivity was scored in >1000 carcinoma cells for each case, and the percentage of immunoreactivity, *i.e.*, labeling index (LI), was determined. In this study, cases that were found to have ERRα LI of >10% were considered ERRα-positive breast carcinomas, according to a report for ERα and PR by Allred *et al.* (26). Immunoreactivity of EST was classified into the following three categories: ++, >50% positive cells; +, 1–50% positive cells; and −, no immunoreactivity, according to a previous report (20).

Values for LIs for ERRα, ERα, ERβ, PR, Ki-67, ERRα mRNA level, patient age, and tumor size were summarized as a mean ± 95% confidence interval. The association between immunoreactivity for ERRα status and these parameters were evaluated using a one-way ANOVA and Bonferroni test. The association between ERRα and PR LIs, and the association between ERRα mRNA and ERRα LI or aromatase mRNA were performed using a correlation coefficient ($r$) and regression equation. Statistical difference between ERRα status and menopausal status, stage, lymph node status, histological grade, ERα status, EST, or HER2 status was evaluated in a cross-table using the $\chi^2$ test. Overall and disease-free survival curves were generated according to the Kaplan-Meier method, and the statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated by Cox proportional hazards model using PROC PHREG in our SAS software. Differences with $Ps < 0.05$ were considered significant.

## RESULTS

**Immunohistochemistry for ERRα in Breast Carcinoma Tissues.** Immunoreactivity for ERRα was detected in the nuclei of invasive ductal carcinoma cells (Fig. 1*A*). A mean value of ERRα LI in the 102 breast carcinoma tissues examined was 23.0% (range 0–75%), and a number of ERRα-positive breast carcinomas (*i.e.*, ERRα LI ≥ 10%) was 56 of102 cases (54.9%). ERRα immunoreactivity was focally detected in epithelial cells of morphologically normal glands (Fig. 1*B*), whereas the stroma or adipose tissue was immunohistochemically negative for ERRα. A mean value of ERRα LI in non-neoplastic mammary epithelia was 14.6% (range 0–33%), and the number of cases showing higher ERRα LI in carcinoma cells than that in non-neoplastic mammary epithelia was 49 of 102 (48.0%). In positive control sections for ERRα immunohistochemistry, ERRα immunoreactivity was markedly detected in the nuclei of myocardial cells of the heart (Fig. 1*C*).

Associations between ERRα immunoreactivity and clinicopathological parameters in 102 breast carcinomas are summarized in Table 1. ERRα immunoreactivity tended to be positively associated with ERα status and ERα LI and negatively associated with EST; however the correlation did not reach a statistical significance ($P = 0.0848$, $P = 0.1485$, and $P = 0.1224$, respectively). No significant association was detected between ERRα immunoreactivity and the other clinicopathological parameters examined, including patient age, menopausal status, stage, tumor size, lymph node status, histological grade, ERβ LI, PR LI, HER2 status, and Ki-67 LI, in this study.

**Influence of ERRα Status on the Association between ERα and Estrogen Responsive Genes.** pS2, EBAG9/RCAS1, PR, cyclin D1, and c-myc are all well recognized as estrogen-responsive genes in human breast cancers. As shown in Table 2, a significant positive association was detected between ERα LI and the status of these immunoreactivity genes except for c-myc in the 102 breast cancer tissues examined ($P < 0.0001$ for pS2, $P = 0.0214$ for EBAG9/RCAS1, $P < 0.0001$ for PR LI, $P = 0.0002$ for cyclin D1, and $P = 0.9372$ for c-myc), which agrees well with previous immunohistochemical studies (22, 27–30). However, when the breast cancers were classified into two groups according to ERRα status, no significant association was detected between ERα LI and pS2 in the group of ERRα-positive breast carcinomas ($P = 0.1415$; $n = 56$) or between ERα LI and EBAG9/RCAS1 in ERRα-negative breast cancers ($P = 0.8271$; $n = 46$). On the other hand, significant association was detected between ERα LI and PR LI ($P < 0.0001$ in ERRα-positive cases; $P < 0.0001$ in ERRα-negative cases) or cyclin D1 ($P = 0.0126$ in ERRα-positive cases; $P = 0.0082$ in ERRα-negative cases), regardless of the ERRα status in the breast cancer cases examined.

No significant association was detected between ERRα LI and these estrogen-responsive genes regardless of ERα status in 102 breast carcinoma tissues (Table 3).

**Correlation between ERRα Immunoreactivity and the Clinical Outcome of the Patients.** ERRα immunoreactivity was significantly associated with an increased risk of recurrence ($P = 0.0071$, log-rank test; Fig. 2*A*). After univariate analysis by Cox proportional hazards model (Table 4), lymph node status ($P < 0.0001$), tumor size ($P < 0.0001$), EST ($P = 0.0035$), and ERRα immunoreactivity

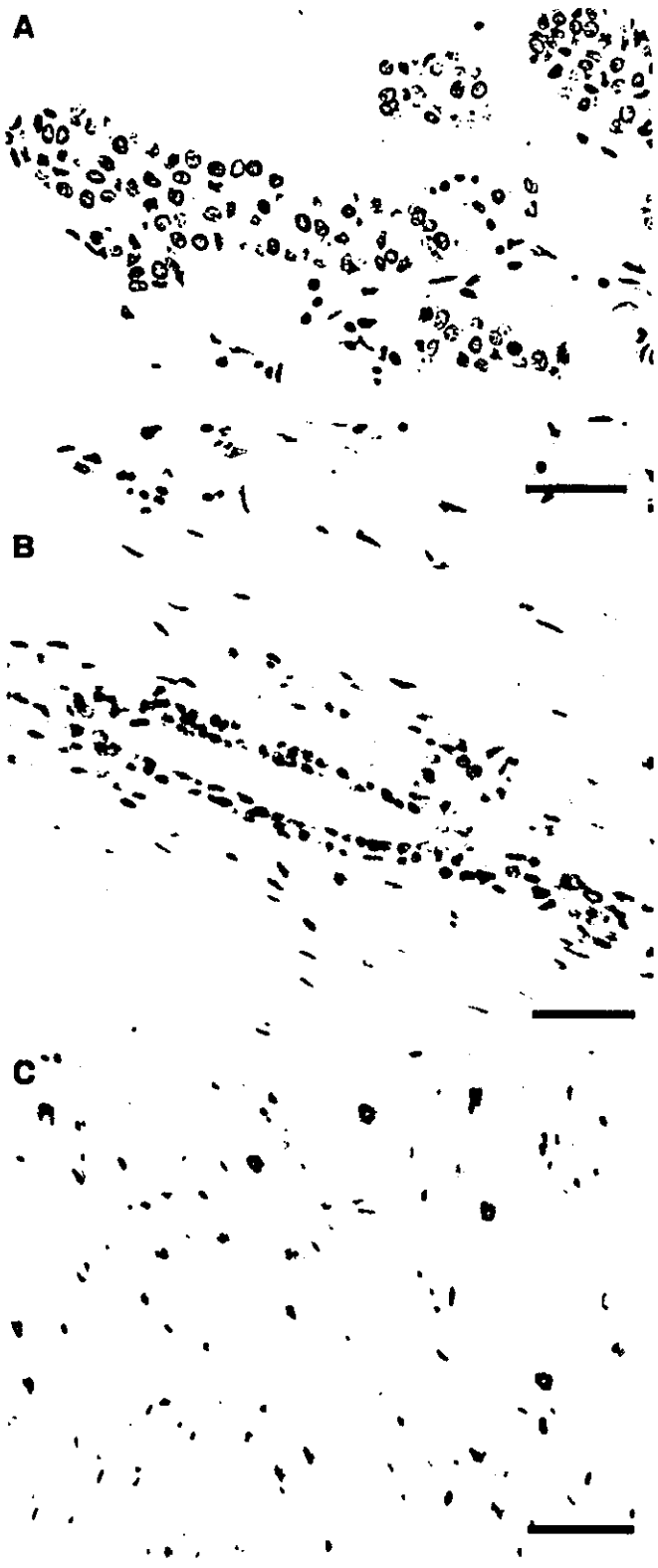


Fig. 1. Immunohistochemistry for ERRα in invasive ductal carcinoma. *A*, ERRα immunoreactivity was detected in the nuclei of invasive ductal carcinoma cells. *ERR*, estrogen-related receptor α. *B*, in morphologically normal mammary glands, immunoreactivity for ERRα was weakly detected in the nuclei of epithelial cells. *C*, in the positive control for ERRα immunohistochemistry, ERRα immunoreactivity was detected in the nucleus of myocardial cells in the heart. *Bar* = 50 μm, respectively.

Table 1 *Association between ERRα immunoreactivity and clinicopathological parameters in 102 breast carcinomas*

| | ERRα immunoreactivity | | |
|---|---|---|---|
| | + ($n$ = 56) | − ($n$ = 46) | $P$ |
| Age (yrs)[b] | 54.3 ± 1.6 | 52.8 ± 1.8 | 0.5271 |
| Menopausal status | | | |
| Premenopausal | 27 (26.5%) | 20 (19.7%) | |
| Postmenopausal | 29 (28.4%) | 26 (25.5%) | 0.6329 |
| Stage | | | |
| I | 14 (13.7%) | 15 (14.7%) | |
| II | 35 (34.3%) | 26 (25.5%) | |
| III | 7 (6.9%) | 5 (4.9%) | 0.6852 |
| Tumor size (mm)[b] | 25.6 ± 1.8 | 24.8 ± 1.8 | 0.7443 |
| Lymph node status | | | |
| Positive | 27 (26.5%) | 19 (18.7%) | |
| Negative | 29 (28.4%) | 27 (26.5%) | 0.4849 |
| Histological grade | | | |
| 1 | 14 (13.7%) | 13 (12.7%) | |
| 2 | 22 (21.6%) | 14 (13.7%) | |
| 3 | 20 (19.7%) | 19 (18.6%) | 0.6462 |
| ERα status | | | |
| Positive | 45 (44.1%) | 30 (29.4%) | |
| Negative | 11 (10.8%) | 16 (15.7%) | 0.0848 |
| ERα LI[b] | 47.5 ± 4.5 | 38.1 ± 5.2 | 0.1485 |
| ERβ LI[b] | 15.3 ± 2.4 | 14.6 ± 2.7 | 0.8493 |
| PR LI[b] | 45.6 ± 4.8 | 40.7 ± 5.1 | 0.4894 |
| EST | | | |
| − | 35 (34.3%) | 24 (23.5%) | |
| + | 10 (9.8%) | 15 (14.7%) | |
| ++ | 11 (10.8%) | 7 (6.9%) | 0.1224 |
| HER2 status | | | |
| Positive | 20 (19.6%) | 15 (14.7%) | |
| Negative | 36 (35.3%) | 31 (30.4%) | 0.7421 |
| Ki-67 LI[b] | 24.7 ± 2.0 | 27.4 ± 2.7 | 0.4045 |

[a] ERRα, estrogen-related receptor α; ERα, estrogen receptor α; LI, labeling index; EST, estrogen sulfotransferase; HER2, human epidermal growth receptor 2.
[b] Data are presented as mean ± 95% confidence interval. All other values represent the number of cases and percentage.

($P$ = 0.0097) were demonstrated as significant prognostic parameters for disease-free survival in 102 breast carcinoma patients. A multivariate analysis (Table 4), however, revealed that only lymph node status ($P$ = 0.0015) and ERRα immunoreactivity ($P$ = 0.0215) were independent-prognostic factors with relative risks over 1.0, whereas tumor size and EST were not significant.

Overall survival curve was demonstrated in Fig. 2*B*, and a significant correlation was detected between ERRα immunoreactivity and adverse clinical outcome of the patients ($P$ = 0.0018, log-rank test). Using a univariate analysis (Table 5), lymph node status ($P$ < 0.0001), tumor size ($P$ = 0.0002), ERRα immunoreactivity ($P$ = 0.0053), EST ($P$ = 0.0065), HER2 status ($P$ = 0.0175), adjuvant chemotherapy ($P$ = 0.0233), and histological grade ($P$ = 0.0310) turned out to be significant prognostic factors for overall survival in this study. Multivariate analysis revealed that lymph node status ($P$ = 0.0085), ERRα immunoreactivity ($P$ = 0.0118), and EST ($P$ = 0.0382) were independent-prognostic factors with a relative risk over 1.0; however other factors were not significant in this study (Table 5).

Ten patients received tamoxifen therapy after surgery, and these cases were ERα-positive breast cancers. The disease-free and overall survival curves in these patients were summarized in Fig. 2, *C* and *D*. ERRα immunoreactivity was also markedly associated with an increased risk of recurrence and worse prognosis in the group of breast cancer patients who received tamoxifen therapy, although *P*s were not available because no patient had a recurrence or died in the group of ERRα-negative breast cancers. Association between ERRα immunoreactivity and clinical outcome of the patients was not significantly changed regardless of the status of adjuvant chemotherapy after surgery in this study (data not shown).

Table 2 *Correlation between ERα[a] and estrogen responsive gene immunoreactivities associated with ERRα status in 102 breast carcinomas*

| | Total ($n$ = 102) | | ERRα positive ($n$ = 56) | | ERRα negative ($n$ = 46) | |
|---|---|---|---|---|---|---|
| | ERα LI | $P$ | ERα LI | $P$ | ERα LI | $P$ |
| pS2 | | | | | | |
| Positive | 54.7 ± 4.0 | | 54.3 ± 5.5 | | 53.8 ± 6.5 | |
| Negative | 28.8 ± 5.1 | **<0.0001** | 38.5 ± 7.2 | 0.1415 | 14.8 ± 6.3 | **<0.0001** |
| EBAG9/RCAS1 | | | | | | |
| Positive | 46.7 ± 4.4 | | 51.6 ± 5.9 | | 40.8 ± 6.7 | |
| Negative | 27.9 ± 7.3 | **0.0214** | 17.4 ± 6.1 | **0.0093** | 45.0 ± 16.4 | 0.8271 |
| PR LI | | **<0.0001** | | **<0.0001** | | **<0.0001** |
| | | ($r$ = 0.590) | | ($r$ = 0.515) | | ($r$ = 0.675) |
| Cyclin D1 | | | | | | |
| Positive | 57.3 ± 4.5 | | 59.6 ± 6.0 | | 54.5 ± 7.0 | |
| Negative | 32.2 ± 4.4 | **0.0002** | 37.2 ± 6.0 | **0.0126** | 26.5 ± 6.5 | **0.0082** |
| c-myc | | | | | | |
| Positive | 43.7 ± 5.3 | | 47.9 ± 6.8 | | 37.4 ± 8.3 | |
| Negative | 44.2 ± 4.5 | 0.9372 | 48.4 ± 6.1 | 0.9583 | 39.8 ± 6.7 | 0.8321 |

*P*s < 0.05 were considered significant, and described as boldface.
[a] ERα, estrogen receptor α; ERRα, estrogen-related receptor α; PR, progesterone receptor; LI, labeling index.

**ERRα mRNA Expression in the Breast Carcinoma Tissues.** mRNA expression for ERRα, aromatase, and RPL13A was detected as a specific single band (214, 215, and 126 bp, respectively) and was semi-quantified by real-time reverse transcription-PCR. Expression of ERRα mRNA was detected markedly in the breast carcinoma tissues (65.7 ± 9.0%) but was low in non-neoplastic breast tissues (25.4 ± 6.0%, $P$ = 0.0448 *versus* carcinoma tissues) or adipose tissues adjacent to the carcinoma (12.6 ± 7.3%, $P$ = 0.0174 *versus* carcinoma tissues; Fig. 3*A*). ERRα mRNA expression was closely correlated with the ERRα immunoreactivity evaluated as ERRα LI ($P$ = 0.0041, $r$ = 0.509) in 30 breast carcinoma tissues examined (Fig. 3*B*). However, mRNA expression of ERRα was not significantly associated with that of aromatase ($P$ = 0.6441, $r$ = -0.088) in this study (Fig. 3*C*).

## DISCUSSION

In this study, ERRα immunoreactivity was detected in the nuclei of carcinoma cells in 55% of breast cancer tissues and was significantly associated with its mRNA level. ERRα mRNA expression was demonstrated previously in various human breast cancer cell lines, breast carcinoma tissues, and normal mammary epithelial cells (17, 18), and our present findings were in good agreement with these previous reports. Results in our present study also demonstrated that ERRα immunoreactivity tended to be positively or inversely associated with ERα or EST, respectively. The possible correlation between ERRα and ERα expression remains controversial. Ariazi *et al.* (18) reported that increased ERRα mRNA levels were associated with ER-negative and PR-negative tumor status in 38 breast cancer tissues and suggested a possible unfavorable marker in the breast cancers. However, Liu *et al.* (31) demonstrated that estrogens stimulate the expression of ERRα in the human breast cell lines, and suggested that ERRα is a downstream target of ERα. On the other hand, EST catalyzes estrogens to biologically inactive estrogen sulfates (32, 33) and is considered to diminish estrogen actions in the breast cancers (20). Therefore, our present results suggest that expression of ERRα is, at least in a part, associated with estrogenic actions.

In our present study, significant associations were detected between ERα and estrogen responsive genes such as pS2, EBAG9/RCAS1, PR, and cyclin D1, as was reported previously (22, 27–29). However, the significant association between ERα and pS2 or EBAG9/RCAS1 disappeared in the group of ERRα-positive or -negative breast cancers, respectively. On the other hand, correlation between ERα and PR, cyclin D1, or c-myc was not influenced by ERRα status in these breast cancer patients examined. Both *pS2* and *EBAG9/RCAS1* genes are induced by ERα through an ERE in the promoter region (34, 35). However, functional ERE has not been identified in *PR* (36) and *cyclin D1* (5), and these are considered to be induced by ER through the interaction between ER and other DNA-binding transcription factors. Considering that ERα and ERRα directly compete for binding EREs (13), our present data suggest that ERRα mainly modulates ERα-mediated ERE-dependent transcription and changes the expression pattern of estrogen-responsive genes in the breast cancer cells.

ERRα immunoreactivity was significantly associated with an increased risk of recurrence or adverse clinical outcome of the patients, and results of multivariate analyses demonstrated that ERRα immunoreactivity is an independent-prognostic factor. Estrogens induce

Table 3 *Correlation between ERRα[a] and estrogen responsive gene immunoreactivities associated with ERα status in 102 breast carcinomas*

| | Total ($n$ = 102) | | ERα positive ($n$ = 75) | | ERα negative ($n$ = 27) | |
|---|---|---|---|---|---|---|
| | ERRα LI | $P$ | ERRα LI | $P$ | ERRα LI | $P$ |
| pS2 | | | | | | |
| Positive | 23.5 ± 2.7 | | 23.0 ± 2.9 | | 27.7 ± 8.6 | |
| Negative | 22.4 ± 3.4 | 0.7981 | 27.7 ± 4.5 | 0.3777 | 16.3 ± 4.8 | 0.2776 |
| EBAG9/RCAS1 | | | | | | |
| Positive | 22.6 ± 2.6 | | 24.2 ± 3.0 | | 18.5 ± 4.9 | |
| Negative | 23.6 ± 5.8 | 0.8834 | 21.8 ± 6.8 | 0.7542 | 29.0 ± 11.3 | 0.5341 |
| PR LI | | 0.5072 | | 0.9069 | | 0.9671 |
| | | ($r$ = 0.066) | | ($r$ = 0.014) | | ($r$ = 0.008) |
| Cyclin D1 | | | | | | |
| Positive | 24.7 ± 3.4 | | 24.8 ± 3.6 | | 23.3 ± 11.0 | |
| Negative | 21.8 ± 2.8 | 0.5134 | 24.2 ± 3.4 | 0.9058 | 18.1 ± 4.7 | 0.6770 |
| c-myc | | | | | | |
| Positive | 25.0 ± 3.3 | | 25.8 ± 3.7 | | 22.5 ± 7.7 | |
| Negative | 22.0 ± 2.8 | 0.4943 | 23.7 ± 3.4 | 0.6648 | 17.8 ± 5.3 | 0.6100 |

[a] ERRα, estrogen-related receptor α; LI, labeling index; ERα, estrogen receptor α; PR, progesterone receptor.

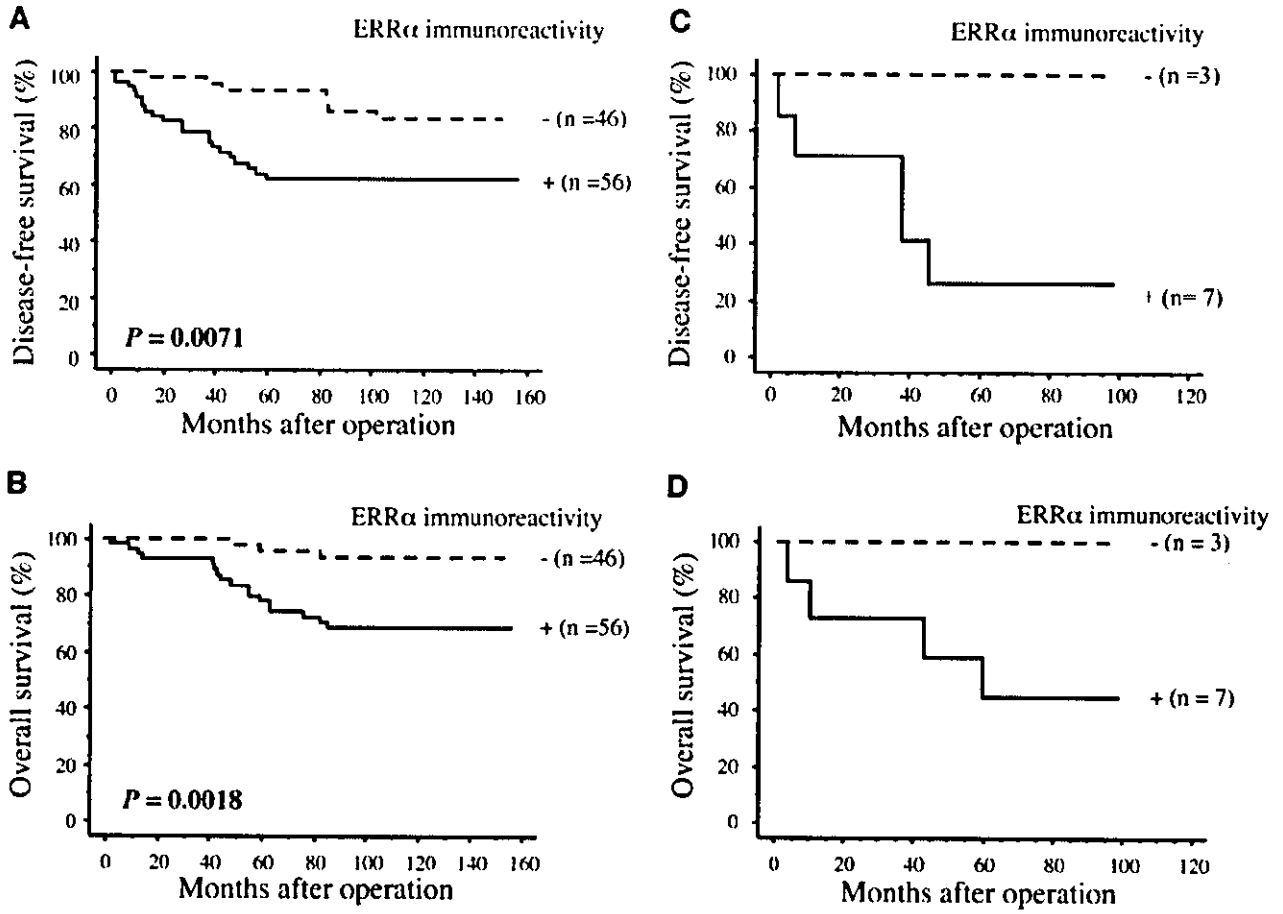


Fig. 2. *A* and *B*, disease-free (*A*) and overall (*B*) survival of 102 patients with breast carcinoma according to ERRα immunoreactivity (Kaplan-Meier method). ERRα immunoreactivity was significantly associated with an increased risk of recurrence ($P = 0.0071$, log-rank test; *A*, and worse prognosis ($P = 0.0018$, log-rank test). *C* and *D*, disease-free (*C*) and overall (*D*) survival of 10 patients received tamoxifen therapy after surgery according to ERRα immunoreactivity (Kaplan-Meier method). ERRα immunoreactivity was also associated with an increased risk of recurrence (*C*) and worse prognosis (*D*) in the group of patients who received tamoxifen therapy. *P*s were not calculated, because no patient had a recurrence or died in the group of ERRα-negative breast cancer patients. *ERR*, estrogen-related receptor α.

Table 4 *Univariate and multivariate analyses of disease-free survival in 102 breast cancer patients examined*

| Variable | *P* | | Relative risk (95% CI[a]) |
|---|---|---|---|
| | Univariate | Multivariate | |
| Lymph node status ($pN_3$-$pN_0$)[b] | **<0.0001**[c] | **0.0015** | 2.593 (1.441–4.666) |
| Tumor size (75–7 mm)[b] | **<0.0001**[c] | 0.3306 | |
| EST (−/+, ++) | **0.0035**[c] | 0.0613 | |
| ERRα immunoreactivity (positive/negative) | **0.0097**[c] | **0.0215** | 1.953 (1.116–3.149) |
| c-myc (positive/negative) | 0.0581 | | |
| Adjuvant chemotherapy (no/yes) | 0.1305 | | |
| Ki-67 LI (≥10/<10) | 0.1795 | | |
| HER2 status (positive/negative) | 0.2713 | | |
| Histological grade (3/1, 2) | 0.2911 | | |
| ERα status (positive/negative) | 0.4363 | | |

[a] CI, confidence interval; EST, estrogen sulfotransferase; ERRα, estrogen-related receptor α; HER2, human epidermal growth factor receptor 2; LI, labeling index; ERα, estrogen receptor α.

[b] Data were evaluated as continuous variables in the uni- and multivariate analyses. All other data were evaluated as dichotomized variables.

[c] Data were considered significant in the univariate analyses, and were examined in the multivariate analyses.

various estrogen responsive genes in breast cancer cells, and these genes include not only activators of cell growth such as cyclin D1 (37) or c-myc (38) but also relatively good prognostic markers such as pS2 (29) or PR (39). ERRs display significant constitutive transcriptional activity (7, 9, 40). Therefore, poor clinical outcome in ERRα-positive breast cancer patients may be partly caused by constitutive modulation of the expression of estrogen-responsive genes, although we could not directly demonstrate such hypothesis from our present data, because of the lack of mechanistic examinations and the relatively limited number of cases examined in this study. Additional examinations are required to clarify the detailed mechanism of ERRα action in the breast cancer tissues.

Table 5 *Univariate and multivariate analyses of overall survival in 102 breast cancer patients examined*

| Variable | *P* | | Relative risk (95% CI[a]) |
|---|---|---|---|
| | Univariate | Multivariate | |
| Lymph node status ($pN_3$-$pN_0$)[b] | **<0.0001**[c] | **0.0085** | 2.414 (1.252–4.653) |
| Tumor size (75–7 mm)[b] | **0.0002**[c] | 0.2675 | |
| ERRα immunoreactivity (positive/negative) | **0.0053**[c] | **0.0118** | 5.076 (1.217–21.173) |
| EST (−/+, ++) | **0.0065**[c] | **0.0382** | 4.101 (1.027–19.705) |
| HER2 status (positive/negative) | **0.0175**[c] | 0.4669 | |
| Adjuvant chemotherapy (no/yes) | **0.0233**[c] | 0.0635 | |
| Histological grade (3/1, 2) | **0.0310**[c] | 0.1458 | |
| Ki-67 LI (≥10/<10) | 0.1818 | | |
| c-myc (positive/negative) | 0.2697 | | |
| ERα status (positive/negative) | 0.7646 | | |

[a] CI, confidence interval; ERRα, estrogen-related receptor α; EST, estrogen sulfotransferase; HER2, human epidermal growth factor receptor 2; LI, labeling index; ERα, estrogen receptor α.

[b] Data were evaluated as continuous variables in the uni- and multivariate analyses. All other data were evaluated as dichotomized variables.

[c] Data were considered significant in the univariate analyses, and were examined in the multivariate analyses.

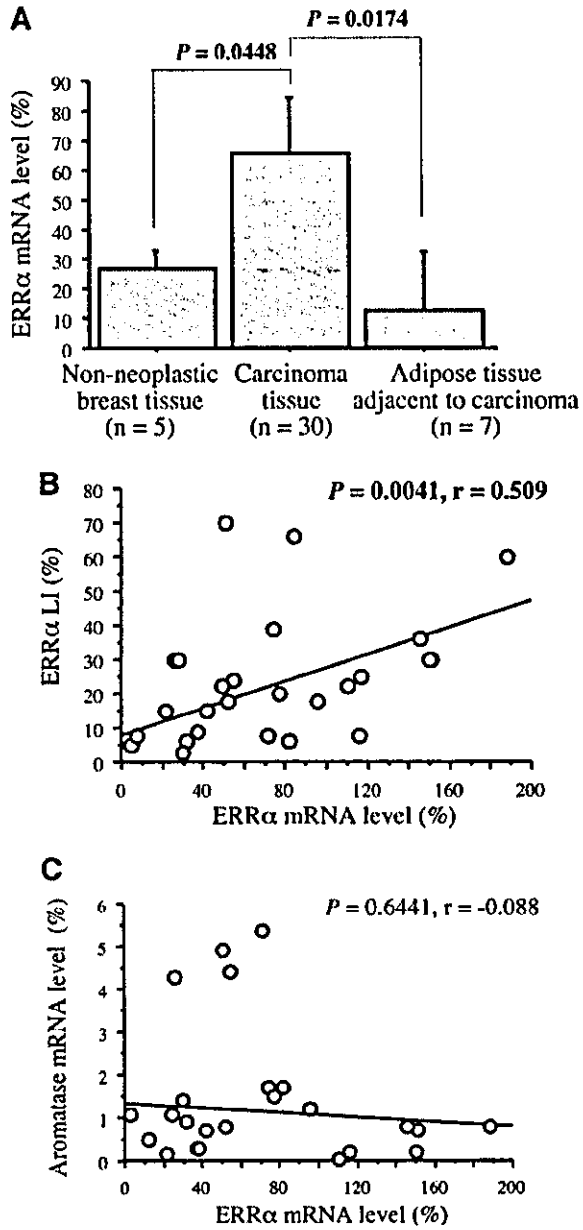


Fig. 3. Real-time reverse transcription-PCR for ERRα in the breast carcinoma. *A*, expression of ERRα mRNA was significantly higher in the breast carcinoma tissues (65.7 ± 9.0%, $n$ = 30) than in non-neoplastic breast tissues [25.4 ± 6.0% ($n$ = 5), $P$ = 0.0448 *versus* carcinoma tissues] or adipose tissues adjacent to the carcinoma [12.6 ± 7.3% ($n$ = 7), $P$ = 0.0174 *versus* carcinoma tissues]. Data represent the mean ± 95% confidence interval. The mRNA level of ERRα in each specimen was evaluated as a ratio (%) of the positive control tissue (human heart tissue = 100%). *B*, association between the mRNA level and relative immunoreactivity (labeling index) of ERRα in 30 cases of breast carcinoma tissues. Significant positive association was detected ($P$ = 0.0041, $r$ = 0.509). *C*, association between ERRα and aromatase mRNA levels in 30 breast carcinoma tissues. No significant correlation was detected ($P$ = 0.6441, $r$ = −0.088). Aromatase mRNA expression in each case was evaluated as a ratio (%) of that in the human placental tissue. *ERR*, estrogen-related receptor α.

ERRα immunoreactivity was also associated with poor prognosis in the group of breast cancer patients who received tamoxifen therapy, which suggests that ERRα status is a possible predictive marker for tamoxifen therapy, although the number of cases examined was limited in this study. Previous *in vitro* studies demonstrated that both tamoxifen and 4-hydroxytamoxifen did not bind to ERRα or did not have any effects on the transcriptional activity of ERRα, whereas these are high-affinity ligands for ERRβ or ERRγ (41, 42). Therefore, ERRα may constitutively function independently of tamoxifen and result in tamoxifen resistance in ERRα-positive breast cancer patients.

Aromatase is a key enzyme in *in situ* estrogen biosynthesis in breast cancer tissue, and aromatase inhibitors are currently used in breast cancer patients as an endocrine therapy as well as antiestrogens. Aromatase is markedly activated by SF1 through an SF1-binding element within the promoter region (43). However, SF1 is not expressed in breast carcinoma tissues (11, 44). Previously, Yang *et al.* (11) reported the induction of aromatase expression by ERRα through a SF1-binding element in breast fibroblast, suggesting the possible importance of ERRα as a regulator of aromatase expression in breast cancer. However, in our study, we did not find ERRα immunoreactivity in the intra-tumoral stromal cells or adipocytes adjacent to the carcinoma, although these cells are well-known to express aromatase (45). Previous *in vitro* studies have shown the regulation of aromatase transcription in breast fibroblasts and/or adipocytes by various factors, including cytokines (46), prostaglandin $E_2$ (47), liver receptor homologue-1 (44) and CCAAT/enhancer-binding protein β (48).

In summary, ERRα immunoreactivity was detected in carcinoma cells in 55% of breast cancer tissues and was associated with its mRNA level. Association between ERα and ERE-containing estrogen-responsive genes was markedly altered according to ERRα status in the breast cancer tissues. ERRα immunoreactivity was associated with poor prognosis of the patients, and similar tendency was also detected in the group who received tamoxifen therapy. These findings suggest that ERRα possibly modulates the expression of ERE-containing estrogen responsive genes, and ERRα immunoreactivity is a potent prognostic factor, including a possible predictive marker for tamoxifen resistance, in human breast carcinoma.

## ACKNOWLEDGMENTS

We thank Yoshiko Murakami, Chika Kaneko, and Yuko Hirakata (Department of Pathology, Tohoku University School of Medicine, respectively) for skillful technical assistance.

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