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ER-activating ability of breast cancer stromal fibroblasts is regulated independently of alteration of *TP53* and *PTEN* tumor suppressor genes

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ABSTRACT

Carcinoma-associated fibroblasts (CAFs) are associated with tumor progression and metastasis, and are able to activate estrogen receptor (ER) in breast cancer. We established a stable transformant of a human breast cancer cell line to detect CAF-specific ER-activating ability, and found that this CAF ability varied among tumors. Some studies have reported a high frequency of alterations among tumor suppressor genes in stromal cells, but do not generally agree as to the frequency. Moreover, the activation mechanism of CAF-induced estrogen signals, including the effects of these gene aberrations, is not fully understood. We investigated the relevance of tumor suppressor gene aberrations and ER-activating ability in CAFs derived from 20 breast cancer patients. Although CAF-specific ER-activating abilities varied among individual cases, all CAFs maintained wild-type alleles for *TP53* and *PTEN*. Also, copy number aberrations in these genes were not observed in any CAFs. Our results suggest that the ER-activating ability of the CAFs is regulated independently of aberrations in these genes; and that other mechanisms of tumor–stromal interaction may affect activation of estrogen signals in breast cancer.

1. Introduction

Carcinoma-associated fibroblasts (CAFs) in proximity to epithelial tumor cells have been associated with tumor-promoting roles in various human carcinomas. In the human prostate cancer model, CAFs have been grown with initiated nontumorigenic epithelial cells, stimulated tumor growth and altered histology of epithelial cells [1]. Significant evidence also shows that CAFs regulate tumor angiogenesis in neuroblastoma and prostate cancer [2,3]. Secreted factors, cytokines and cell surface proteins of CAFs are also associated with metastasis in colon and other tumors [4–6]. However, the activation mechanism of CAFs during tumor development is not yet fully understood.

In breast cancer, CAFs are similarly associated with tumor growth, metastasis and poor clinical outcome, and enhance tumor angiogenesis in comingled breast cancer cells [7,8]. Over two thirds of breast cancers express estrogen receptors (ERs), which can be mediated by two distinct types of signaling, often referred to as the genomic pathway, and the non-genomic or non-genotropic pathways. Therefore ERα expression is predictive of response to endocrine therapy to reduce estrogen stimulation for proliferation. While selective ER modulators (SERMs), including tamoxifen, have been used as first-line hormonal therapy for postmenopausal patients for many years, aromatase inhibitors (AIs) including letrozole, anastrozole and exemestane have shown benefit by minimizing risk of early relapse in advanced disease; AIs are potent inhibitors of aromatase activity that locally converts androgens into estrogens in a variety of tissues including muscle, connective tissue, skin and liver [9,10]. The CAFs in the vicinity of breast cancer tissues are known to express aromatase [11,12], and are target of AIs, which have now largely replaced tamoxifen as first-line therapy in the postmenopausal breast cancer [13,14].

High mutation frequencies for *TP53* and/or *PTEN* were described in CAFs of breast cancer tissue [15,16]. In addition, loss of heterozygosity (LOH) in *TP53*, *PTEN* and other loci was reported in CAFs [15–17]; such mutations and CAF-specific LOH were associated with lymph node metastasis in sporadic breast cancer [16]. For an *in vivo* model of prostate cancer, Hill et al. found that the selective mutation of *p53* in reactive stroma accelerates spontaneous tumor progression [18]. Although these results suggest that

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stromal mutations affect tumor growth and progression, further research has revealed that such mutations are rare events in stromal fibroblasts; this question is still controversial [19–21].

Previously, we reported establishing a stable transformant of a human breast cancer cell line to detect CAF-specific ER-activating ability in the co-culture by transfection with the estrogen-responsive element–GFP. This system is a useful tool for analyzing local ER-related signals and tumor–stromal interactions [22] (Fig. 1). We reported that the ability of CAFs to activate ERs and sensitivity to AIs varied among tumors, and that the analysis of CAF characteristics in an individual breast cancer is essential to prediction of hormone therapy efficacy [22]. However, the mechanism underlying regulation of ER-activating ability in CAFs, including the effects of genomic instability, remains unknown. In this study, we focused on aberrations of the tumor suppressor genes *TP53* and *PTEN* in CAFs of breast cancer, and clarified their relevance to clinicopathological features and ER-activating ability in CAFs.

2. Materials and methods

2.1. Cells and culture conditions

The human breast cancer cell line ERE–GFP-E10, a MCF7 clone stably transfected with the d2E-green fluorescent protein (GFP) vector carrying the ptk-estrogen-responsive element (ERE) insert, was isolated and described previously [22]. ERE–GFP-E10 was maintained in RPMI-1640 medium (Sigma–Aldrich Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS).

Human breast cancer tissues were obtained by surgery at the Saitama Cancer Center Hospital (Saitama, Japan) after informed consent was obtained from the patients. The Saitama Cancer Center Ethics Committee approved this study. We have previously described the isolation procedure of intratumoral stromal cells and the characterization of CAFs obtained from individual breast cancer patients [22]. Isolated primary CAFs were maintained in modified minimum essential medium (MEM)-Alpha (Invitrogen, Carlsbad, CA) with 10% FBS. The ER-activating ability of CAFs was detected with GFP signals of ERE–GFP-E10 co-cultured with CAFs after pre-culture in phenol red-free RPMI 1640 with dextran-coated and charcoal-treated 10% FCS (DCC–FCS); ability to activate ER had been previously evaluated by the individual value of the ratio of GFP-positive cells for CAFs from 20 breast cancers [22].

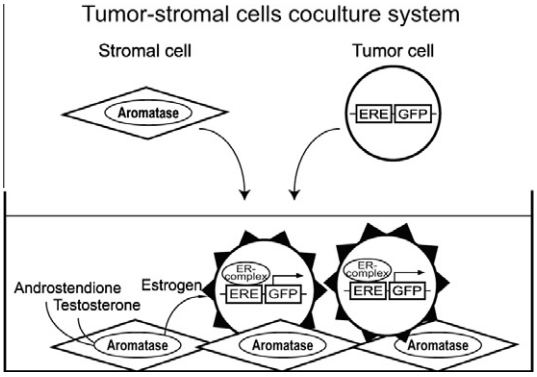

Fig. 1. Many breast cancer stromal fibroblasts can activate estrogen receptors (ER) and accelerate breast cancer proliferation and progression via the ER. Our established system detects ER-activating ability of CAFs by GFP signals of this human breast cancer cell line, and is useful for analysis of the local estrogen signaling pathway. ER: estrogen receptor; ERE: estrogen-responsive element.

2.2. Nucleic acid preparation

For genomic DNA isolation, a QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan) was used for CAFs from breast cancers and peripheral blood leukocytes from healthy volunteer, according to the manufacturer's instructions. DNA concentration and purity were determined by Nanodrop® ND-1000 Spectrophotometer (Laboratory & Medical Supplies, Tokyo, Japan), and then stored at −20 °C until analysis.

2.3. PCR amplification

The three fragments of *TP53* (corresponding to exon 4, exons 5–6 and exons 7–8) were amplified from genomic DNA extracted from CAFs for each case by polymerase chain reaction (PCR). The amplification conditions for exons 5–8 of *TP53* have been described in our previous report [23], with minor modifications. The primers for exon 4 were synthesized according to the IARC protocols of *TP53* direct sequencing (http://www-p53.iarc.fr/p53sequencing.html). Primer sequences of all sets are described in Additional file 1. The primers used for exons 1–9 of *PTEN* have been reported previously [24–26] (Additional file 1). PCR amplification was carried out in a total volume of 20 μl, consisting of 50 ng of DNA, 1× PrimeSTAR Buffer (Takara Bio Inc., Shiga, Japan), 200 μM dNTPs, 200 nM of each PCR primer, and 0.5 U of PrimeSTAR® HS DNA Polymerase (Takara Bio Inc.). PCR amplification was performed for 30–35 cycles of denaturation at 98 °C for 10 s, annealing at 55–63 °C for 5 s, and extension at 72 °C for 30 s. Annealing temperatures for each primer were shown in Additional file 1. Real-time RT-PCR was performed using a LightCycler® Carousel-Based System (Roche Diagnostics GmbH Mannheim, Germany) to analyze relative amounts of *CYP19A1* (Aromatae) mRNA; the averaged value of 12 samples in this standard curve method was used as cut-off value. The *CYP19A1* expression status of CAFs, including all 67 in our previous report [22], will be described elsewhere.

2.4. DNA sequence

Mutation analysis of *TP53* and *PTEN* was performed by direct sequencing. The purified PCR products were directly sequenced with upstream or downstream primers (Additional file 1) using Big Dye® Terminators v1.1 Cycle Sequencing Kit and ABI PRISM® 310 Genetic Analyzer (Life Technologies Corporation, Rockville, MD). The obtained nucleotide sequences were compared with the reference sequence of *TP53* and *PTEN* (GenBank accession number X54156 and AF067844, respectively).

2.5. Copy number analysis

TaqMan® Copy Number Assays (Life Technologies Corporation) were used to analyze loss of heterozygosity (LOH) of *TP53* and *PTEN* genes. PCR was performed using an Applied Biosystems 7300 Real-time PCR system (Life Technologies Corporation), and TaqMan® Copy Number Assays for *TP53* (Hs05516623_cn) and *PTEN* (Hs05177393_cn) were purchased from Life Technologies Corporation. PCR was performed with TaqMan® Genotyping Master Mix (Life Technologies Corporation) according to the manufacturer's instructions. PCR amplification was carried out in a total volume of 10 μl; the reaction mixture comprised 10 ng of DNA, 1× TaqMan® Copy Number Assay and 1× TaqMan® Copy Number Reference Assay RNase P. PCR amplification was performed using following conditions: 50 °C for 2 min, 95 °C for 10 min; and then 45 cycles of 95 °C for 15 s and 60 °C for 1 min. All PCR was performed in duplicate for each sample. Data analysis was carried out using the software CopyCaller v1.0 (Life Technologies

Table 1
Estrogen receptor (ER)-activating ability of CAFs and clinicopathological features of breast cancer patients ($n = 20$).

		ER-activating ability	
		<10% ($n = 7$)	⩾10% ($n = 13$)
ER, *n*	Positive	3	10
	Negative	4	3
PgR, *n*	Positive	1	4
	Negative	6	9
Histology, *n*	Papillotubular Ca.	0	2
	Solid-tubular Ca.	4	5
	Scirrhous Ca.	1	5
	Special types	1	1
	Invasive micropapillary Ca.	1	0
Tumor size, *n*	T1	4	9
	T2	2	4
	T3	1	0
Lymph node metastasis, *n*	N0	3	4
	N1	4	7
	N2	0	1
	Unknown	0	1
Menopausal status, *n*	Premenopausal	2	7
	Postmenopausal	5	6
Aromatase mRNA. *n*	High expression	3	2
	Low expression	3	4
	Unknown	1	7

ER and PgR status was determined using the Allred scoring system or enzyme immunoassay (EIA).
Tumor Allred scores ⩾3, or EIA ⩾15 fmol/mg were considered to be positive specimens in this study.

Corporation). Peripheral blood leukocyte DNA from a healthy volunteer was used to calibrate each experiment.

3. Results

3.1. Patient population and ER-activating ability of CAFs

The ER-activating ability of CAFs and clinicopathological features of the patients included in the study are listed in Tables 1 and 2. ER-activating ability of CAFs was evaluated by co-culture system with ERE–GFP-E10 cells in the presence of testosterone, an aromatase substrate. The ratio of GFP^+ cells was evaluated. Among the 20 breast cancer patients, 7 (35%) showed low ER-activating ability of CAFs (GFP^+ E10 cells <10%) and 13 (65%) were high ER-activating ability of CAFs (GFP^+ E10 cells ⩾10%). Levels similar to estrogen-induced ER-activation were detected in 3 out of 13 high ER-activating patients.

3.2. TP53 and PTEN sequence analysis of CAFs

Many CAFs activate ER in tumor cells by genomic or non-genomic pathways through the estrogen or growth factors, which are produced by the CAF itself. Because CAFs are reportedly associated with high frequency of genetic aberration, CAF-specific ER-activating ability may be affected by genomic instability. To clarify whether the aberrant tumor suppressor genes in CAFs affect the ER-activation, we performed the mutation analysis of *TP53* and *PTEN* gene. The CAF-derived DNA was analyzed for *TP53* mutations in exons 4–8 that corresponded to the sequence-specific DNA-binding domain, and for *PTEN* mutations in exons 1 and 3–9. As in the previous report [25], we could not detect exon 2 of *PTEN*, despite using two different primer sets. As shown in Table 2,

Table 2
Mutation analysis of *TP53* and *PTEN* in CAFs.

CAF No.	GFP positive rate (%)	*TP53*	*PTEN*
1	4.6	wt	wt
2	27.3	wt	wt
3	27.5	wt	wt
4	2.8	wt	wt
5	9.3	wt	wt
6	1.0	wt	wt
7	40.5	wt	wt
8	38.8	wt	wt
9	31.0	wt	wt
10	38.2	wt	wt
11	5.0	wt	wt
12	28.5	wt	wt
13	9.3	wt	wt
14	13.1	wt	wt
15	28.5	wt	wt
16	14.5	wt	wt
17	23.7	wt	wt
18	12.5	wt	wt
19	9.3	wt	wt
20	16.2	wt	wt

wt, wild-type.

mutations were not found among the regions of these genes in all 20 CAF samples. We also examined the genotypes of ten SNP sites to evaluate LOH using this sequence analysis. Seven out of 10 SNP sites (rs55950612, rs56196266, rs1642786, rs1642787, rs1642788, rs1794288, rs35979531) showed homozygous genotypes in all 20 CAF cells (data not shown), and 3 SNP sites showed a heterozygous genotype in at least one sample (Table 3). These results have shown that LOH had not occurred at least in 12 CAF samples for *TP53* and in 1 sample for *PTEN*.

3.3. Copy number analysis of TP53 and PTEN

The copy number of the *TP53* and *PTEN* gene was evaluated by quantitative real-time PCR-based Copy Number Assays for determining LOH. Copy number of *TP53* ranged from 1.73 to 2.39 copies; copy number aberrations were not observed in any CAFs (Fig. 2A). Although the calculated copy number of *PTEN* had extended more widely than that of *TP53*, LOH at these loci was not detected in any CAFs (Fig. 2B; range: 1.62–3.41). Four cases out of the 20 CAFs examined were predicted to show three copies of *PTEN*, with copy numbers of 2.84, 2.85, 3.05 and 3.41 (Fig. 2B). In these cases, no clear correlation was found between the ER-activating ability and *PTEN* gene copy number in CAFs. These results were true of both premenopausal and postmenopausal patients (Fig. 2). In addition, correlation between gene copy number and ER protein expression in tumor specimens were not found (data not shown).

4. Discussion

In the present study, we investigated genomic alterations of *TP53* and *PTEN* genes in breast cancer CAFs, and for the first time, examined their correlation with ER-activating ability of CAFs and clinicopathological features of tumors. Although various ER-activating abilities were detected in individual CAFs, all CAFs tested in this study maintained wild-type alleles for the genes. In addition, in contrast to previous reports [15,16,27], none of these breast-cancer CAFs showed any evidence of LOH in these genes. This genomically stable phenotype in all 20 CAFs agrees with two previous reports that aberration of these genes in CAFs is a rare event [19,21]. Therefore, our results suggest that the ER-activating ability of the CAFs is regulated independently of

Table 3
The genotypes of three SNP sites in CAFs.

CAF No.	*TP53*		*PTEN*
	rs.1042522	rs.12951053	rs.1799734
1	GCG	TTC	TTATC
2	GCG/GGG	TTC	TTATC
3	GCG/GGG	TTC/TGC	TTATC
4	GCG/GGG	TTC/TGC	TTATC
5	GCG/GGG	TTC/TGC	TTATC
6	GCG/GGG	TTC	TTATC/ –
7	GCG	TTC	– / –
8	GCG	TTC	TTATC
9	GCG/GGG	TTC	TTATC
10	GCG/GGG	TTC/TGC	TTATC
11	GGG	TGC	TTATC
12	GCG/GGG	TTC/TGC	TTATC
13	GCG	TTC	TTATC
14	GCG/GGG	TTC/TGC	TTATC
15	GCG	TTC	TTATC
16	GCG/GGG	TTC/TGC	TTATC
17	GCG	TTC	TTATC
18	GCG	TTC	TTATC
19	GCG/GGG	TTC/TGC	TTATC
20	GCG/GGG	TTC/TGC	TTATC

rs, reference SNP clusters number in NCBI's dbSNP database.

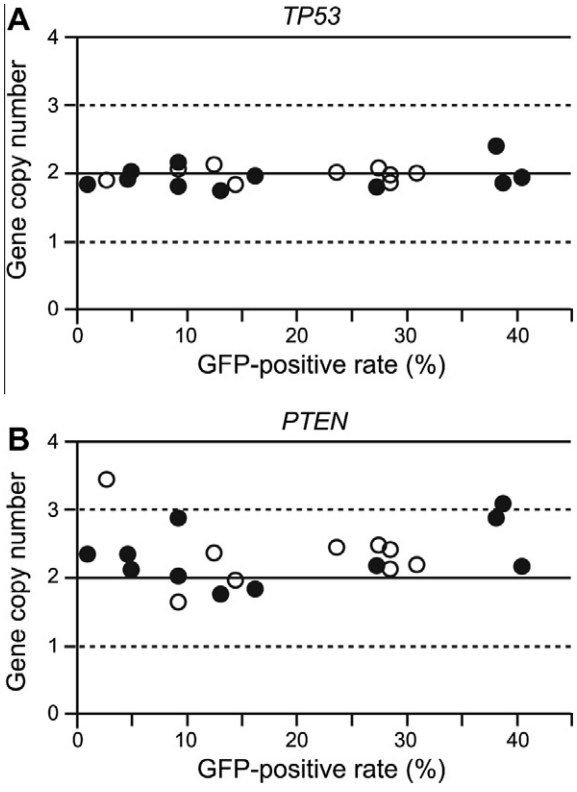

Fig. 2. Copy number analysis and GFP-inducing ability of CAFs. (A and B) Quantitative real-time PCR-based copy number assays for determining loss of heterozygosity (LOH) in CAFs show normal copy number variations of *TP53* (A) and *PTEN* (B). Open and closed circles indicated the menopausal status of premenopausal and postmenopausal patients, respectively.

aberrations in these tumor suppressor genes, and that the mutation or LOH of the genes is rare event.

High frequency mutations and LOH of stromal cells in the previous reports by Kurose et al. and by Patocs et al. have been investigated using DNA derived from microdissected formalin-fixed paraffin-embedded (FFPE) tissues [15,16]. Although obtaining LOH and mutation data from FFPE-derived DNA is a well-established method, low concentrations or low-yield template DNA for PCR might lead to false conclusions [28]. Consequently, the existence of frequent genetic alterations in CAFs is still controversial [19–21]. In this study, we investigated DNA from short-term cultured mammary fibroblasts, which were isolated from carcinoma-associated regions of tumor masses obtained from breast cancer patients. This isolation method can establish adipose stromal fibroblast cells from tumor masses [29], and avoids contamination by tumor cells and other effects.

In the present experiments, we showed that copy numbers were below the cut-off value at 4 copies, which indicates amplification, although the slight increase in copy number of *PTEN* gene was detected in four out of 20 CAFs. No relation was also found between the CAF-specific ER-activating ability and the gene copy number amplification. Somatic mutation and/or deletion of the *PTEN* tumor suppressor gene have been shown to play a crucial role in proliferation and cell survival [30,31]. While the copy number gain of *PTEN* gene in breast cancer does not significantly affect protein levels [32]. Therefore, it can be speculated that the PTEN in our CAFs is not the main regulating factor in activation of ER in tumor cells. However, two previous studies have reported genome-wide copy number analysis of breast CAF samples, and reported 2 CAFs to show genomic alterations in several loci of chromosomes [19,21]. Therefore, the effects of CAFs with low copy amplification of several genes remain to be elucidated.

As for the analysis of CAF-specific ER-activating ability, the detection system of the GFP-based ERE element had been established by our previous report (Fig. 1). We demonstrated that CAFs of postmenopausal patients did not always have high ER-activating ability. Furthermore, it was shown that GFP induction levels did not always correlate with expression of the aromatase gene in CAFs [22]. These results suggest that the ERE–GFP system is not only activated by the estrogen-dependent pathway, but also by estrogen-independent pathways, such as phosphorylation by growth factor-inducing signals. Nevertheless, our results demonstrated that *TP53* or *PTEN* mutation was not the main regulator in either pathway. In this study, we concluded that CAF-specific ER-activating ability is regulated independently from genetic aberrations; however, methylation patterns of several gene regions in tumor stroma have been shown to be distinctly different from normal breast tissue in one report [33]. Furthermore, other epigenetic modifiers of stromal fibroblasts, such as a microRNA that critically affects tumor suppressor function, have also been reported [34,35]. The functional contribution of ER-activation by CAFs in this microenvironment is still unclear; further studies are needed.

Authors' contributions

T.S. carried out the molecular biological studies and drafted the manuscript. H.O. measured aromatase transcripts with RT-PCR. H.O. and M.K. performed pathological analysis. H.T. collected and analyzed clinical data from breast cancer patients. S.H. reviewed all data, and contributed to the preparation of the manuscript. Y.Y. directed the overall project, and participated in the editing of final manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.035.

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