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EPIDEMIOLOGY

Possible involvement of CCT5, RGS3, and YKT6 genes up-regulated in p53-mutated tumors in resistance to docetaxel in human breast cancers

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Abstract
Background Present study was aimed to investigate the relationship of p53 mutation status with response to docetaxel in breast cancers. In addition, attempts were made to identify the genes differentially expressed between p53-wild and p53-mutated breast tumors and to study their relationship with response to docetaxel.
Methods Mutational analysis of p53 was done in 50 breast tumor samples obtained from primary breast cancer patients ($n = 33$) and locally recurrent breast cancer patients ($n = 17$) before docetaxel therapy. Response to docetaxel was evaluated clinically. Gene expression profiling ($n = 2{,}412$) was conducted by adapter-tagged competitive-PCR in 186 tumor samples, which were also analyzed in their p53 mutational status in order to identify the differentially expressed genes according to p53 mutation status and their relationship with response to docetaxel.
Results Response rate of p53-mutated tumors (44%) was lower than that of p53-wild tumors (62%) though there was no statistical significance ($P = 0.23$). Of 2412 genes, mRNA expression of 13 genes was significantly different between p53-wild and p53-mutated tumors. Of these 13 genes, mRNA expression of CCT5, RGS3, and YKT6 was significantly up-regulated in p53-mutated tumors and associated with a low response rate to docetaxel. Treatment of MCF-7 cells with siRNA specific for CCT5, RGS3, or YKT6 resulted in a significant enhancement of docetaxel-induced apoptosis.
Conclusions CCT5, RGS3, and YKT6 mRNA expressions, which are up-regulated in p53-mutated breast tumors, might be implicated in resistance to docetaxel and clinically useful in identifying the subset of breast cancer patients who may or may not benefit from docetaxel treatment.

Keywords CCT5 · RGS3 · YKT6 · Docetaxel resistance · p53-Mutated tumors in human breast cancers

Abbreviations

ER	Estrogen receptor
RGS3	Regulator of G-protein signaling 3
CCT5	Chaperonin containing TCP1 Subunit 5 (epsilon)
YKT6	Soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) protein Ykt6
siRNA	Small interfering RNA
DHPLC	Denaturing high performance liquid chromatography
ATAC-PCR	Adapter-tagged competitive polymerase chain reaction

Introduction

There are many active anti-tumor drugs for the treatment of breast cancers, and, recently, an increasing

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number of breast cancer patients have been treated with chemotherapy in the neoadjuvant, adjuvant, and metastatic settings. It is obvious that chemotherapy has improved the survival in the adjuvant setting [1, 2], increased the feasibility of breast conserving surgery in the neoadjuvant setting [3, 4], and relieved the symptoms and prolonged the survival in the metastatic setting [5]. One of the most important problems in chemotherapy is inability to identify which drug will benefit a particular patient before the start of chemotherapy. It is well established that estrogen receptor (ER) is clinically very useful in the prediction of response to hormonal therapy but, unfortunately, no clinically useful predictive factor for any chemotherapy has been established yet, whereas a lots of preliminary and unconfirmed observations have been reported [6, 7]. It seems to be very important to develop such predictive factors in order to improve the efficiency of chemotherapy.

Docetaxel, which belongs to taxanes, is one of the most active anti-tumor drugs for breast cancer, and is often used in the neoadjuvant, adjuvant, and metastatic settings. The response rate to docetaxel is approximately 50% in the first line chemotherapy for metastatic diseases, and it decreases to be 20–30% in the second or third line chemotherapy [8, 9]. Therefore, it is of vital importance to select the patients who are very likely to respond to docetaxel in order to eliminate the unnecessary treatment. Several markers have been proposed as predictive factors for docetaxel response which include B-cell CLL/lymphoma 2 (Bcl-2) [10, 11], cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4) [12], β-tubulin [13], breast cancer 2, early onset (BRCA2) [14], human epithelial growth factor receptor type 2 (HER2) [15], and tau [16]. Furthermore, recently, Chang et al. [17] have demonstrated that expression profile of the 92 genes selected using the Affymetrix microarrays is useful in the prediction of a response to docetaxel in the neoadjuvant setting. We have also shown that the 85 genes selected using adapter-tagged competitive-PCR (ATAC-PCR) can predict a response to docetaxel in the neoadjuvant setting with a high accuracy [18]. However, clinical significant of these proposed predictive factors and diagnostic systems have yet to be established.

It has been suggested from the in vitro studies that docetaxel induces apoptosis of cancer cells through the p53-independent pathway, but p53 status may influence cell-cycle progression following mitotic arrest. Thus, it seems to be interesting to study the relationship between p53 mutation status and response to docetaxel in human breast cancers. A few reports have been available which studies the p53 status by immunohistochemistry and its relationship with response to docetaxel [7, 19]. Although these studies failed to demonstrate a significant association between p53 immunohistochemical status and response to docetaxel, it is still possible that p53 mutation status might show a significant association because immunohistochemistry can not detect all the p53 mutations, and location of p53 mutations might also be important as has been suggested from the study on the impact of location of p53 mutations on doxorubicin resistance, i.e., p53 mutations in the zinc finger domains, but not in the other regions, are associated with doxorubicin resistance [20, 21]. Thus, it is very important to investigate the p53 mutation status as well as its association with a response to docetaxel. In addition, it seems to be interesting to find out the genes differentially expressed between p53-mutated and p53-wild breast tumors and to investigate the significance of expression of such genes as a predictor of response to docetaxel.

Therefore, in the present study, firstly, we studied the relationship between the p53 mutation status and a response to docetaxel in the neoadjuvant setting, and, secondly, we selected the genes which were differentially expressed between p53-mutated and p53-wild breast tumors by gene expression profiling (ATAC-PCR), and studied the association of expression of such genes with a response to docetaxel.

Experimental procedures

Tissue specimens

Fifty female patients with primary invasive (n = 33) or locally recurrent (n = 17) breast cancers who were scheduled to be treated with docetaxel were recruited in this study. Tumor samples were obtained from primary breast tumors or locally recurrent lesions by incisional biopsy or vacuum-associated core needle biopsy, and, then, docetaxel (60 mg/m^2 i.v. every 3 weeks) was given to the patients with primary breast tumors at four cycles before surgery and to those with recurrent tumors until disease progression. No patients had been treated previously with taxanes before the recruitment in this study. Informed consent as to the study was obtained from all patients.

Tumor samples were also obtained for mutational analysis of p53 and for identification of the differentially expressed genes between p53-wild and p53-mutated tumors from the other 136 breast cancer patients (96 primary breast cancer patients and 40 locally

recurrent breast cancer patients) who were treated during the period of 1998–2000. Tumor samples were snap-frozen in liquid nitrogen and stored at –80°C until use. Total RNA and genomic DNA were extracted from frozen tissues using TRIZOL reagent (Molecular Research Center, Cincinnati, OH) and the standard phenol/chloroform and ethanol precipitation–extraction procedure, respectively.

Evaluation of response to docetaxel

Chemotherapeutic response was evaluated after four cycles of treatment or at the time when disease progressed according to the WHO clinical criteria: complete response (CR), disappearance of all known disease; partial response (PR), 50% or more decrease in tumor size; no change (NC), less than 50% decrease or less than 25% increase in tumor size; and progressive disease (PD), 25% or more increase in tumor size or appearance of new lesions. Patients showing CR or PR were considered as responders, and those showing NC or PD were considered as non-responders.

Sequence-based analysis of p53 status

Tumor samples obtained from the 50 patients before docetaxel therapy and from the other 136 patients were subjected to mutational analysis of p53. We sequenced the entire open reading frames of p53 (exons 2–11). PCR amplifications were performed using the TAKARA Ex Taq System (Takara, Shiga, Japan) according to the manufacture's protocol. Primers were designed according to the p53 sequence gene bank (Accession No. AY838896) and the respective sequences of primers are given in Table 1. Mutation screening was performed using denaturing high performance liquid chromatography (DHPLC) as described [22, 23]. A WAVE DNA-Fragment Analysis System (Transgenomic Inc. Omaha, NE) was used. All DHPLC positive signals were confirmed by conventional method. Sequencing reactions were performed using the Applied Biosystems Dye-Terminator Kit and analyzed on an ABI Prism 310 DNA sequencer (Applied Biosystems, Foster City, CA).

ATAC-PCR analysis and real-time quantitative PCR

The ATAC-PCR analysis of 2,412 genes mRNA expression was performed on tumor samples according to the method previously described [24]. The protocols and information on the genes analyzed by ATAC-PCR in the present study are available at http://genome.mc.pref.osaka.jp. Real-time PCR reactions were carried out using SYBER Green (Applied Biosystems). Levels of β-glucuronidase mRNA were quantified as an internal standard. The PCR products were detected using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers were designed using the ABI Primer Express software to cross an exon/exon boundary to minimize the chance that a signal was from contaminating DNA. PCR reactions performed in duplicate for each sample and the mean value at which the PCR product crossed the threshold (Ct) was calculated.

Table 1 Oligonucleotide primer pairs used for mutation analysis, covering the coding region of p53

Exon	Primer name	Nucleotide sequence (5′–3′)	Tm°C
2–3	p53-exon2–3F	tcc tct tgc agc agc cag act gc	66
	p53-exon2–3R	aac cct tgt cct tac cag aac gtt g	
4	p53-exon4F	tgg tcc tct gac tgc tct ttt c	64.5
	p53-exon4R	aag tct cat gga agc cag cc	
5	p53-exon5F	ctc ttc ctg cag tac tcc cct gc	66
	p53-exon5R	gcc cca gct gct cac cat cgc ta	
6	p53-exon6F	gat tgc tct tag gtc tgg ccc ctc	66
	p53-exon6R	ggc cac tga caa cca ccc tta acc	
7	p53-exon7F	gtg ttg tct cct agg ttg gct ctg	66
	p53-exon7R	caa gtg gct cct gac ctg gag tc	
8	p53-exon8F	acc tga ttt cct tac tgc ctc tgg c	66
	p53-exon8R	gtc ctg ctt gct tac ctc gct tag t	
9	p53-exon9F	gcc tct ttc cta gca ctg ccc aac	66
	p53-exon9R	ccc aag act tag tac ctg aag ggt g	
10	p53-exon10F	tgt tgc tgc aga tcc gtg ggc gt	66
	p53-exon10R	gag gtc act cac ctg gag tga gc	
11	p53-exon11F	tgt gat gtc atc tct cct ccc tgc	66
	p53-exon11R	ggc tgt cag tgg gga aca aga agt	

Tm°C: Annealing temperature

Immunohistochemistry

For immunohisotchemical detection of chaperonin containing TCP1, subunit 5 (CCT5), regulator of G-protein signaling 3 (RGS3), and soluble *N*-etylmaleimide-sensitive-factor attachment protein receptor (SNARE) protein Ykt6 (YKT6), paraffin sections were deparaffinized with xylene, and rehydrated using graded ethanol. The sections were incubated in 3% hydrogen peroxide and methanol for 10 min to inactivate endogenous peroxidase. Then, the sections were boiled for 3 min in 10 mM citrate buffer and cooled for 15 min at room temperature for three times to expose antigenic epitopes. Reagents were used as supplied in the Ready-To-Use VECTASTAIN® *Elite* ABC Kit (Vector Laboratories, Burlingame, CA). Sections were incubated for 30 min with goat polyclonal anti-RGS3 (sc-9304; Santa Cruzu Biotechnology, Santa Cruz, CA), (1:1000), rabbit polyclonal anti-TCP-1ϵ (CTA-230; Stress Gen, B.C., Canada), (1:1000) and goat polyclonal anti-v-SNARE Ykt6p (sc-10835; Santa Cruzu Biotechnology), (1:100) at room temperature. Detection was then completed with incubation with a 3,3′-diaminobenzidine solution (Vector Laboratories) diluted in distilled water for 10 min. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted.

Cell cycle analysis

The human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (Sigma, ST. Louis, MO) supplemented with 10% fetal bovine serum (Gibco BRL, Carlsbad, CA) and incubated at 37°C in 5% CO_2. Cell cycle distribution was analyzed using a FACScan apparatus (BD biosciences, Franklin Lakes, NJ). MCF-7 cells were treated with various concentrations of docetaxel, for 24 h or 48 h. Cells were then trypsinized, washed with PBS twice and resuspended after removal of trypsin of DNA dye containing 50 μg/ml propidium iodide (Sigma), 0.1% sodium citrate (Wako, Osaka, Japan), 0.1% NP-40 (Wako) and 4 mM EDTA (Sigma). Cells were incubated at 4°C for 12 h, and then analyzed on a FACScan using CellQuest (BD bioscience) acquisition software. List mode data were acquired on a minimum of 2×10^4 single cells. The percentage of cells in different cell cycle phases was calculated using ModFit LT for Mac (BD bioscience).

Small interference RNA (siRNA) transfection

For the siRNA studies, a smart pool of double-stranded siRNA against RGS3, CCT5, or YKT6 as well as non-functioning siRNA were obtained from Dharmacon Tech (Lafayette, CO) and used according to the manufacturer's instructions. MCF-7 cells were seeded in six-well plates or 4-well Lab-Tek Chamber slides (Naloge Nunc International, Rochester, NY) and allowed to adhere overnight until 70–80% confluent. The next day, cells were transfected with siRNA using the oligofectamine protocol (Invitrogen, Carlsbad, CA) as described [25]. The final concentration for the siRNAs was 50 nM. Twenty-four hours after transfection the cells were treated with 10 nM docetaxel (LKT Laboratories, St. Paul, MN). After 24 h of treatment, the adherent cells were collected for either Western blot analysis, or another assays.

Western blot analysis

At 48 h after siRNA transfection, cells were harvested and subjected to protein immunoblot analysis. Cell were washed once with cold PBS and collected by scraping in lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% SDS, 0.12% Triton X, 1 mM EDTA, and protease inhibitor cocktail (Sigma)) and cleared by centrifugation. Protein concentration was determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of total protein were then separated using SDS-PAGE, electro-transferred onto Hybond™-P membranes (Amersham, Bucks, UK). Membranes were incubated with specific antibodies recognizing CCT5 (1:2,000), RGS3 ($1:2 \times 10^4$), and YKT6 (1:5,000). The results were visualized with chemiluminescence using ECL Plus™ detection reagents (Amersham). Band intensities were determined by a scanning laser densitometer.

Apoptosis assay

Apoptotic cells were confirmed with the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI), in accordance with the manufacturer's instructions. Cells were grown on chamber slides. The next day, cells were transfected with siRNAs. At 24 h after transfection, cells were treated with 10 nM docetaxel for 24 h. Slides with adherent cells were fixed in 4% paraformaldehyde for 25 min at 4°C and permiabilized with 0.2% Triton X-100 for 5 min at room temperature. Free 3′ ends of fragmented DNA were enzymatically labeled with the TdT-mediated dUTP nick end labeling (TUNEL) reaction mixture for 60 min at 37°C

in a humidified chamber. Labeled DNA fragments were monitored by fluorescence microscopy. Ten fields were randomly counted for each sample.

Results

Relationship between p53 mutation and response to docetaxel

Of 50 tumors obtained before docetaxel therapy, 16 were found to have somatic mutations including 7 missense mutations, 3 nonsense mutations, and 6 frameshift mutations. Relationship between p53 mutation status and response to docetaxel is shown in Table 2. Response rate of tumors with mutated p53 (44%) was lower than tumors with wild p53 (62%) though there was no statistical significance ($P = 0.23$). Of 16 tumors with p53 mutation, 9 tumors had mutations in the L2/L3 regions. Response rate of tumors with p53 mutation in these regions were lower, though not statistically significant, than the other tumors (33% vs. 61%).

Identification of differentially expressed genes between p53-wild tumors and p53-mutated tumors

In total, 186 tumor samples were analyzed in the p53 mutational status (Table 3) as well as the 2,412 genes mRNA expression profile. These tumor samples were divided into the first set ($n = 90$) and the second set ($n = 96$). The first set consisted of 67 p53-wild tumors and 23 p53-mutated tumors and the second set consisted of 74 p53-wild tumors and 22 p53-mutated tumors. Differentially expressed genes between p53-wild tumors and p53-mutated tumors were selected by permutation test with a statistical significance of $P < 0.05$ in each set, and, finally, 13 genes were found to be differentially expressed with a statistical significance ($P < 0.05$) in both sets (Table 4).

Table 2 Relationship between p53 mutation status and response to docetaxel

	Responders	Non-responders	
p53 mutation status			
p53-mutated tumors	7	9	
p53-wild tumors	21	13	$P = 0.23$
p53 mutation status			
p53-mutated (L2/L3) tumors	3	6	
Other tumors[a]	25	16	$P = 0.13$

[a]Including p53-wild tumors and p53-mutated (outside L2/L3) tumors

Relationship of mRNA levels of differentially expressed 13 genes between p53-wild tumors and p53-mutated tumors with response to docetaxel

The mRNA expression levels of the 13 genes differentially expressed between p53-wild tumors and p53-mutated tumors were further analyzed by a real-time PCR assay which is considered to be currently the most reliable method for quantification of mRNA levels, and their relationship with response to docetaxel was evaluated. Finally, of these 13 genes, three genes including chaperonin containing TCP1, subunit 5 (CCT5), regulator of G-protein signaling 3 (RGS3), and soluble *N*-etylmaleimide-sensitive-factor attachment protein receptor (SNARE) protein Ykt6 (YKT6) were found to be differentially expressed between responders and non-responders with a statistical significance (Fig. 1).

Expression of CCT5, RGS3, and YKT6 in breast cancers

Immunohistochemical staining of CCT5, RGS3, and YKT6 using the representative tumor samples revealed that all of them were predominantly localized in tumor cells. From the viewpoint of subcellular localization, YKT6 was exclusively localized in the cytoplasm of tumor cells, and CCT5 and RGS3 were predominantly localized in the nucleus with a concomitant cytoplasmic staining of various intensities (Fig. 2).

Effect of docetaxel on cell cycle

Effect of various concentrations of docetaxel on MCF-7 cell cycles was studied by flow cytometry (Fig. 3). There was no significant difference in flow cytometric pattern at 1 nM or lower concentrations but the percentage of cells in G_0/G_1 phase apparently decreased and that in the sub-2N apparently increased at 5 nM or higher concentrations, suggesting the presence of apoptosis induced by docetaxel. From these results, we chose 10 nM docetaxel for the further investigation.

Influence of knock-down of CCT5, RGS3, and YKT6 mRNA by siRNA on sensitivity to docetaxel in MCF-7 cells

For the purpose of validating the involvement of CCT5, RGS3, and YKT6 in resistance to docetaxel, we next studied the influence of knock-down of the endogenous CCT5, RGS3, and YKT6 expression by siRNA on sensitivity to docetaxel in MCF-7 cells. CCT5, RGS3, or YKT6-specific siRNAs were transfected into MCF-7

Table 3 Summary of p53 mutations found in 186 breast tumors

Type of variation	Exon	Nucleotide change	Protein change	Frequency
Missense mutation	2–3	1174 g to c	11 Glu to Gln	1
	4	12256 t to c	111 Leu to Pro	1
	6	13341 t to g	194 Leu to Arg	1
		13374 a to c	205 Tyr to Ser	1
		13405 t to g	215 Ser to Arg	1
		13418 t to a	220 Tyr to Asn	3
	7	14061 g to t	245 Gly to Val	1
		14069 c to t	248 Arg to Try	2
		14070 g to a	248 Arg to Gln	4
		14097 t to a	257 Leu to Gln	1
	10	17593 g to c	339 Glu to Gln	1
		17657 g to c	360 Gly to Val	1
	11	18673 c to t	392 Ser to Leu	1
Stop codon	4	12108 g to t	62 Glu to Stop	1
	5	13227 c to g	183 Ser to Stop	1
	6	13346 c to t	196 Arg to Stop	1
	10	17602 c to t	342 Arg to Stop	5
Deletion out-of-frame	5	1 bp deletion		1
		4 bp deletions		1
		20 bp deletions		1
	6	1 bp deletion		1
		2 bp deletions		1
		14 bp deletions		1
	7	1 bp deletion		1
		2 bp deletions		1
	8	1 bp deletion		1
		10 bp deletions		1
		20 bp deletions		1
		26 bp deletions		2
Insertion out-of-frame	4	1 bp insertion		3
	8	1 bp insertion		1
		17 bp insertions		1
Polymorphism	4	12032 g to a	32 Pro to Pro	2
		12139 g to c	72 Arg to Pro	3

cells and CCT5, RGS3, or YKT6 protein levels were determined by Western blotting. CCT5-, RGS3-, and YKT6-specific siRNA suppressed the CCT5, RGS3, and YKT6 protein levels by 90%, 40%, and 60%, respectively, in MCF-7 cells (Fig. 4). The inhibitory effect of each siRNA was considered to be specific because transfection with non-specific siRNA did not significantly alter the expression of each gene. CCT5 protein levels, but not RGS3 and YKT6 proteins, showed a significant increase as compared with non-specific siRNA transfected cells after the treatment with docetaxel.

Table 4 List of 13 genes differentially expressed between tumors with wild p53 and mutated p53

Clone ID	PRI	Definition	Expression in p53-mutated tumors
GS6451	AB028641	SRY (sex determining region Y)-box 11	↑
GS5078	AL050337	Interferon gamma receptor 1	↓
GS4467	X06323	Mitochondrial ribosomal protein L3	↑
GS7542	AL121961	Stromal membrane-associated protein 1	↓
GS3966	BC002971	Chaperonin containing TCP1, subunit 5 (epsilon)	↑
GS3957	AA193183	SNARE protein Ykt6	↑
GS6282	BC003667	40s ribosomal protein S27 isoform	↓
GS6864	BC000382	Interleukin enhancer binding factor 2	↓
GS6703	BC002460	Programmed cell death 9	↓
GS5014	U27655	Regulator of G-protein signaling 3	↑
GS3885	BC000672	Guanine nucleotide binding protein, beta polypeptide2-like1	↓
GS4530	BC003162	Lamin A/C	↑
GS6473	BC001929	Hypothetical protein MGC2376	↓

PRI: GenBank Accession No.

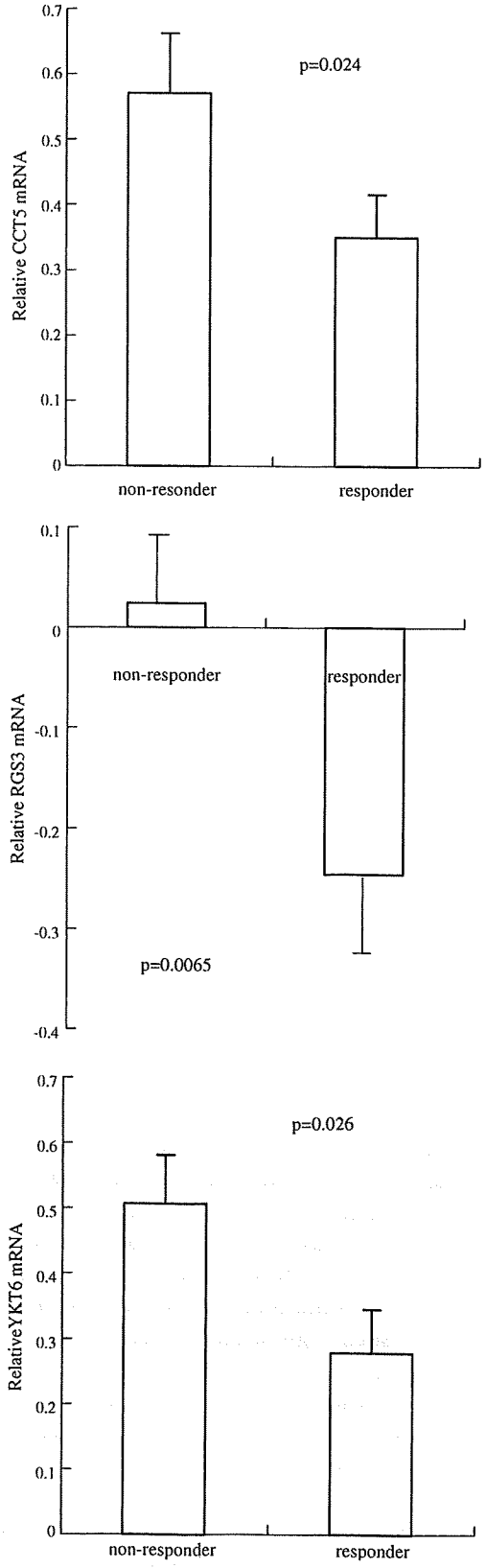

Fig. 1 Relationship of mRNA expression of CCT5, RGS3, or YKT6 with response to docetaxel. Bars; mean ± SE

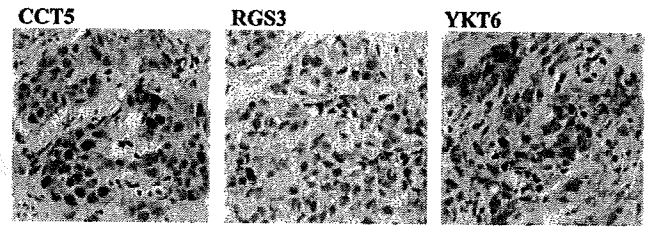

Fig. 2 Immunohistochemical study on CCT5, RGS3, and YKT6 in breast cancers (×200)

Effect of siRNA treatment on sensitivity to docetaxel was evaluated by apoptotic index determined by TUNNEL assay in MCF-7 cells. As shown in Fig. 5, transfection of CCT5-, RGS3-, and YKT6-specific siRNA showed a significant increase (6.6%, 8.6 %, and 8.1%, respectively) in apoptotic index after docetaxel treatment.

Discussion

Since p53 plays an important role in the induction of apoptosis and several in vitro studies have suggested that p53-apoptotic pathway might be involved in the anti-tumor activity of docetaxel, the relationship between p53 mutation status and response to docetaxel therapy has been investigated in the clinical settings by several investigators [7, 19]. These studies, however, failed to show a significant association between p53

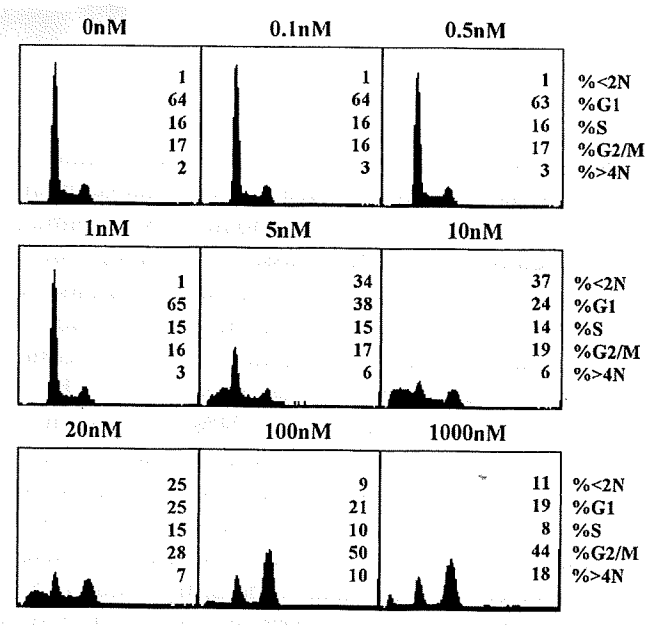

Fig. 3 Influence of docetaxel on cell cycle. MCF-7 cells were treated with various concentrations of docetaxel for 24 h and analyzed by flow cytometry. The percentages of cells in each phase of the cell cycle (sub 2N, G_0/G_1, S and G_2/M) are shown

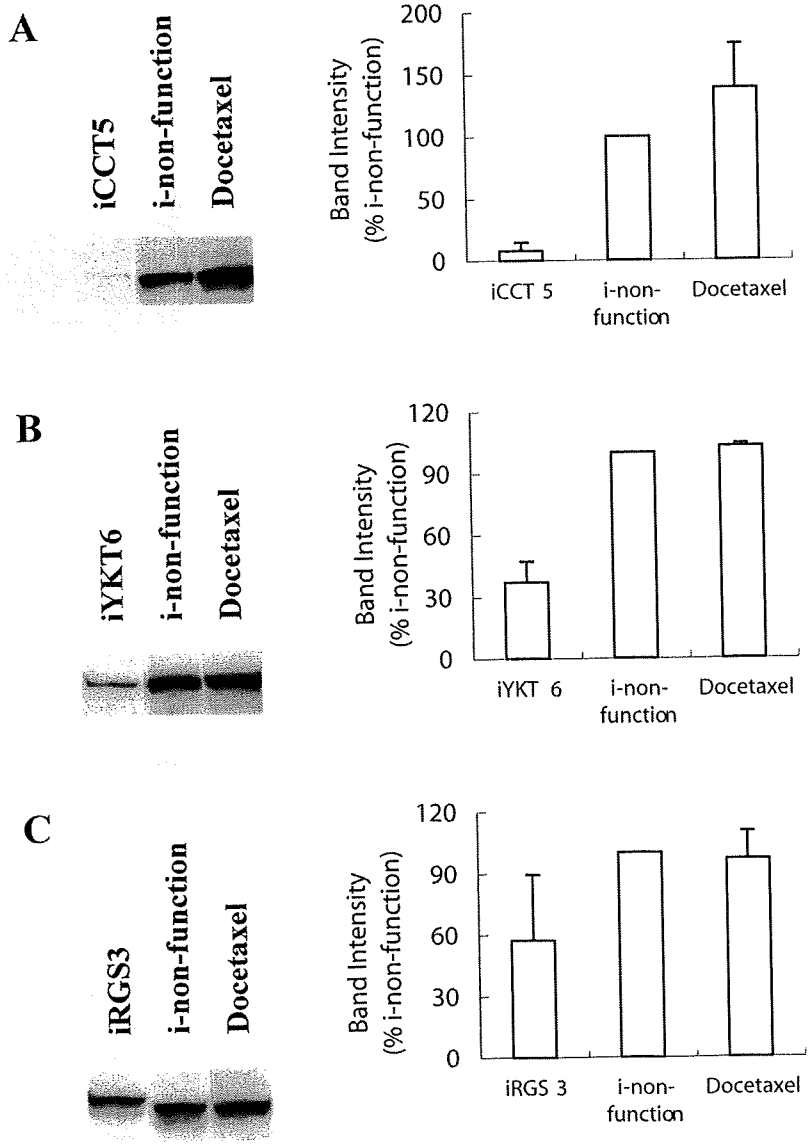

Fig. 4 Influence of siRNA transfection on CCT5, YKT6, and RGS3 protein levels in MCF-7 cells. Cells were transfected with siCCT5, siYKT6 or siRGS3, and CCT5 (panels A), YKT6 (panels B), or RGS3 (panels C) protein level was determined by Western blot analysis with treatment of 10 nM docetaxel for 24 h. Bars; mean ± SD of triplicate determinations

mutation status and response to docetaxel. In all of these studies, p53 mutation status was evaluated by immunohistochemistry, which is a less reliable method than mutational analysis of genomic DNA. Immunohistochemistry can detect missense mutation but not the other types of mutations including nonsense and frameshift mutations. Thus, in the present study, we have analyzed the p53 mutation status of genomic DNA in entire coding regions (exons 2–11). The frequency of p53 mutations was 24% (45/186) in our study, which is in accordance with the most published reports [26, 27]. Of these 45 mutations, as many as 26 mutations (58%) were nonsense mutations (8) or frameshift mutations (18), both of which are unable to be detected by immunohistochemistry. Although, in most studies, mutation analysis of p53 is limited to the hotspots (exons 5–8), we analyzed the entire coding exons 2–11 in the present study. Of the 45 mutations, 14 (31%) were located outside of the exons 5–8, indicating that almost 1/3 of mutations would have been overlooked if we had limited the analysis to the exons 5–8. These results clearly demonstrate that analysis of the entire coding exons is very important to elucidate the mutation status of p53. We have found that p53-wild breast tumors show a slightly higher response rate (62%) than p53-mutated breast tumors though there was no statistical significance. Since breast tumors with p53 mutations in the L2/L3 loop domains have been reported to show a strong resistance to doxorubicin, we have also studied the relationship between p53 mutations in L2/L3 and response to docetaxel. Nine breast tumors (56%) were found to have mutations in L2/L3. Their response rate (33%) was lower than that of the other breast tumors (61%) though, again, statistically

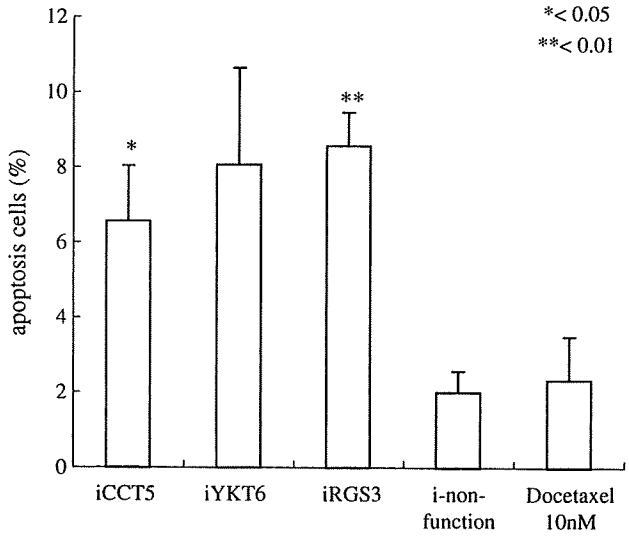

Fig. 5 Influence of CCT5, YKT6, and RGS3-specific siRNA on docetaxel-induced apoptosis in MCF-7 cells. MCF-7 cells were transfected with iCCT5, iYKT6, or iRGS3 alone and treated with 10 nM docetaxel for 24 h. Cells were then analyzed in apoptosis using the TUNEL assay. The percentages of apoptotic cells in each sample are shown. Bars: mean ± SD of triplicate determinations (P; * < 0.05, and ** < 0.01)

not significant. Our present observations seem to suggest that p53 mutations might play a certain role in resistance to docetaxel though a significant association has failed to be demonstrated probably due to the limited number of breast tumors (n = 50) analyzed in the present study.

We attempted to select the genes, which were differentially expressed between p53-wild and p53-mutated breast tumors by ATAC-PCR since such genes were speculated to play some role in resistance to docetaxel. DNA microarrays have a wider coverage of genes than ATAC-PCR but genes with low expression are excluded from the analysis with DNA microarrays. On the other hand, ATAC-PCR can be used to measure expression of the genes with low expression. In addition, ATAC-PCR requires a very small amount of RNA and can tolerate RNA degradation to some extent because this technique only uses the 3′ end of cDNA or mRNA. Actually, we have successfully applied ATAC-PCR to the study on gene expression profiling of breast cancers [28], thyroid cancers [29], hepatocellular carcinomas [30], gastric cancers [31], and colon cancers. In the present study, we have applied ATAC-PCR to the identification of genes differentially expressed between p53-wild and p53-muated breast tumors, and have found that mRNA expression of the 13 genes is significantly different (Table 4). Then, we studied the relationship of the mRNA expression of these 13 genes with response to docetaxel, where a real-time PCR assay was employed instead of ATAC-PCR because a real-time PCR is currently considered to be the most reliable method for quantification of mRNA levels. Eventually, we found that mRNA levels of three genes, i.e., CCT5, RGS3, and YKT6, were significantly associated with resistance to docetaxel. For the purpose of confirming the involvement of these three genes in resistance to docetaxel, we conducted a study with siRNA designed for knocking down of these genes. We were able to shown that siRNA treatment for each of these genes resulted in enhancement of docetaxel-induced apoptosis in MCF-7 cells, indicating that these genes play a significant role in resistance to docetaxel though the precise mechanism of action of these genes still remains to be studied.

Little is known about CCT5, RGS3, and YKT6 in breast cancer biology, and, at least, their mRNA expression is unlikely to be regulated directly by p53 because promoter regions of these three genes seem to lack the typical consensus sequence for p53 binding. Rather, they are likely to represent downstream effectors modulated by functional induction of p53 in the orchestration of apoptosis and tumor suppression. CCT5 is a molecular chaperone and is a member of the chaperonin containing TCP1 complex (CCT), also known as the TCP1 ring complex (TRiC). Unfolded polypeptides enter the central cavity of this complex and are folded in an ATP-dependent manner. The complex folds the various proteins including the β-tubulin. Microtubule cytoskeleton perturbation induced by paclitaxel is reported to increase CCT5 expression in *Tetrahymena* cells [32]. We also found an increase in CCT5 protein after docetaxel treatment. An increased expression of CCT5 is seen in multidrug-resistant gastric carcinoma cells [33]. Taken together with our present observation that treatment with CCT5-specific siRNA resulted in enhancement of docetaxel-induced apoptosis, it is suggested that CCT5 is involved in resistance to docetaxel though its mechanism still remains to be studied. RGS3 is a member of regulators of G-protein signaling (RGS) family that accelerates the intrinsic GTPase activity of G-alpha subunit. RGS3 regulates cellular adhesive and migratory behaviors [34]. Recently, expression of RGS3 was shown to be up-regulated in glioma cells and to enhance both adhesion and migration [35]. RGS3 interacts with ERα transcriptional activities [36], raising the possibility that RGS3 might be involved in the growth of breast cancer. Role of RGS3 in resistance to chemotherapy including docetaxel has never been reported. YKT6 is one of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) recognition molecules implicated in vesicular transport between

secretory compartments. It is a membrane associated, isoprenylated protein that functions at the endoplasmic reticulum–Golgi transport step. YKT6 is expressed at high levels in brain neurons. However, recently, YKT6 has been showed to be expressed in breast cancers and associated with invasive phenotypes [37], suggesting a possible involvement of this molecule in the pathogenesis and progression of breast cancer. Role of YKT6 in resistance to chemotherapy has never been reported.

In conclusion, we have shown that CCT5, RGS3, and YKT6 mRNA expression are up-regulated in p53-mutated breast tumors and are associated with a resistance to docetaxel. We have been able to further substantiate the implication of these genes through studies with siRNA. Our present observation seems to suggest that these genes might be clinically useful in identifying the subset of breast cancer patients who may or may not benefit from docetaxel treatment. Furthermore, our results might provide a new insight into research for molecular mechanism of resistance to docetaxel.

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References

1. Piccart MJ, de Valeriola D, Dal Lago L et al (2005) Adjuvant chemotherapy in 2005: standards and beyond. Breast 14:439–445
2. Hudis C (2005) The best use of adjuvant chemotherapy: new drugs and new use of "old" drugs. Breast 14:570–575
3. Charfare H, Limongelli S, Purushotham AD (2005) Neoadjuvant chemotherapy in breast cancer. Br J Surg 92:14–23
4. Trudeau M, Sinclair SE, Clemons M, Breast Cancer Disease Site Group (2005) Neoadjuvant taxanes in the treatment of non-metastatic breast cancer: a systematic review. Cancer Treat Rev 31:283–302
5. O'Shaughnessy J (2005) Extending survival with chemotherapy in metastatic breast cancer. Oncologist 10(Suppl 3): 20–29
6. Mauriac L, Debled M, MacGrogan G (2005) When will more useful predictive factors be ready for use? Breast 14:617–623
7. Sjostrom J, Blomqvist C, Heikkila P et al (2000) Predictive value of p53, mdm-2, p21, and mib-1 for chemotherapy response in advanced breast cancer. Clin Cancer Res 6:3103–3110
8. Palmeri S, Vaglica M, Spada S et al (2005) Weekly docetaxel and gemcitabine as first-line treatment for metastatic breast cancer: results of a multicenter phase II study. Oncology 68(4–6):438–445
9. Figgitt DP, Wiseman LR (2000) Docetaxel: an update of its use in advanced breast cancer. Drugs 59:621–651
10. Sjostrom J, Blomqvist C, von Boguslawski K et al (2002) The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. Clin Cancer Res 8:811–816
11. Buchholz TA, Davis DW, McConkey DJ et al (2003) Chemotherapy-induced apoptosis and Bcl-2 levels correlate with breast cancer response to chemotherapy. Cancer J 9:33–41
12. Miyoshi Y, Taguchi T, Kim SJ et al (2005) Prediction of response to docetaxel by immunohistochemical analysis of CYP3A4 expression in human breast cancers. Breast Cancer 12:11–15
13. Shalli K, Brown I, Heys SD, Schofield AC (2005) Alterations of beta-tubulin isotypes in breast cancer cells resistant to docetaxel. FASEB J 19:1299–1301
14. Egawa C, Miyoshi Y, Takamura Y et al (2001) Decreased expression of BRCA2 mRNA predicts favorable response to docetaxel in breast cancer. Int J Cancer 95:255–259
15. Im SA, Kim SB, Lee MH et al (2005) Docetaxel plus epirubicin as first-line chemotherapy in MBC (KCSG 01-10-05): phase II trial and the predictive values of circulating HER2 extracellular domain and vascular endothelial growth factor. Oncol Rep 14:481–487
16. Rouzier R, Rajan R, Wagner P et al (2005) Microtubule-associated protein tau: a marker of paclitaxel sensitivity in breast cancer. Proc Natl Acad Sci USA 102:8315–8320
17. Chang JC, Wooten EC, Tsimelzon A et al (2005) Patterns of resistance and incomplete response to docetaxel by gene expression profiling in breast cancer patients. J Clin Oncol 23:1169–1177
18. Iwao-Koizumi K, Matoba R, Ueno N et al (2005) Prediction of docetaxel response in human breast cancer by gene expression profiling. J Clin Oncol 23:422–431
19. Learn PA, Yeh IT, McNutt M et al (2005) HER-2/neu expression as a predictor of response to neoadjuvant docetaxel in patients with operable breast carcinoma. Cancer 103:2252–2260
20. Bergh J, Norberg T, Sjogren S, Lindgren A, Holmberg L (1995) Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. Nat Med 1:1029–1034
21. Aas T, Borresen AL, Geisler S et al (1996) Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. Nat Med 2:811–814
22. Wurzburger RJ, Gupta R, Parnassa AP et al (2003) Use of GC clamps in DHPLC mutation scanning. Clin Med Res 1:111–118
23. Zhang L, Jia G, Li WM, Guo RF, Cui JT, Yang L, Lu YY (2004) Alteration of the ATM gene occurs in gastric cancer cell lines and primary tumors associated with cellular response to DNA damage. Mutat Res 557:41–51
24. Saito S, Matoba R, Kato K (2003) Adapter-tagged competitive PCR (ATAC-PCR) – a high-throughput quantitative PCR method for microarray validation. Methods 31:326–331
25. Elbashir SM, Harborth J, Weber K, Tuschl T et al (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26:199–213
26. Andersson J, Larsson L, Klaar S et al (2005) Worse survival for TP53 (p53)-mutated breast cancer patients receiving adjuvant CMF. Ann Oncol 16:743–748
27. Soussi T, Beroud C (2001) Assessing TP53 status in human tumours to evaluate clinical outcome. Nat Rev Cancer 1:233–240
28. Iwao K, Matoba R, Ueno N et al (2002) Molecular classification of primary breast tumors possessing distinct prognostic properties. Hum Mol Genet 11:199–206

29. Taniguchi K, Takano T, Miyauchi A et al (2005) Differentiation of follicular thyroid adenoma from carcinoma by means of gene expression profiling with adapter-tagged competitive polymerase chain reaction. Oncology 69:428–435
30. Kurokawa Y, Matoba R, Nakamori S et al (2004) PCR-array gene expression profiling of hepatocellular carcinoma. J Exp Clin Cancer Res 23:135–141
31. Motoori M, Takemasa I, Yano M et al (2005) Prediction of recurrence in advanced gastric cancer patients after curative resection by gene expression profiling. Int J Cancer 114:963–968
32. Casalou C, Cyrne L, Rosa MR, Soares H (2001) Microtubule cytoskeleton perturbation induced by taxol and colchicine affects chaperonin containing TCP-1 (CCT) subunit gene expression in *Tetrahymena* cells. Biochim Biophys Acta 1522:9–21
33. Ludwig A, Dietel M, Lage H (2002) Identification of differentially expressed genes in classical and atypical multidrug-resistant gastric carcinoma cells. Anticancer Res 22:3213–3221
34. Bowman EP, Campbell JJ, Druey KM, Scheschonka A, Kehrl JH, Butcher EC (1998) Regulation of chemotactic and proadhesive responses to chemoattractant receptors by RGS (regulator of G-protein signaling) family members. J Biol Chem 273:28040–28048
35. Tatenhorst L, Senner V, Puttmann S, Paulus W (2004) Regulators of G-protein signaling 3 and 4 (RGS3, RGS4) are associated with glioma cell motility. J Neuropathol Exp Neurol 63:210–222
36. Ikeda M, Hirokawa M, Satani N (2001) Molecular cloning and characterization of a steroid receptor-binding regulator of G-protein signaling protein cDNA. Gene 273:207–214
37. Kluger HM, Kluger Y, Gilmore-Hebert M et al (2004) cDNA microarray analysis of invasive and tumorigenic phenotypes in a breast cancer model. Lab Invest 84:320–331

Clinicopathologic Analysis of Breast Cancers with *PIK3CA* Mutations in Japanese Women

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Abstract

Purpose: Somatic mutations of *PIK3CA*, which encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase, have recently been shown to play an important role in the pathogenesis and progression of human breast cancers. In this study, the frequency of *PIK3CA* mutations and their relationship with clinicopathologic and biological variables were investigated in Japanese breast cancers.

Experimental Design: Mutational analysis of *PIK3CA* was done in 188 primary breast cancers of Japanese women. Relationship of these mutations with various clinicopathologic variables [histologic type, tumor size, histologic grade, lymph node status, estrogen receptor (ER)-α and progesterone receptor status, and prognosis], biological variables [phospho-AKT (pAKT) and HER2 expression determined by immunohistochemistry], and *p53* mutation status was studied.

Results: Missense mutations of *PIK3CA* were found in 44 of 158 invasive ductal carcinomas, 4 of 10 invasive lobular carcinomas, 1 of 4 mucinous carcinomas, 2 of 2 squamous carcinomas, and 2 of 2 apocrine carcinomas, but no mutation was found in 12 noninvasive ductal carcinomas. *PIK3CA*-mutated tumors were found to be more likely to be ER-α positive ($P < 0.05$) and pAKT positive ($P < 0.05$). There was no significant association between *PIK3CA* mutations and *p53* mutation status. *PIK3CA* mutations were significantly ($P < 0.05$) associated with a favorable prognosis, and multivariate analysis showed that *PIK3CA* mutation status was a significant ($P < 0.05$) prognostic factor independent of the other conventional prognostic factors.

Conclusions: The frequency of *PIK3CA* mutations in Japanese breast cancers is similar to that of Caucasian breast cancers. Association of *PIK3CA* mutations with positive pAKT and positive ER-α suggests that *PIK3CA* mutations might exert their effects through activation of the phosphatidylinositol 3-kinase/AKT/ER-α pathway. *PIK3CA* mutations seem to have a potential to be used as an indicator of favorable prognosis.

Phosphatidylinositol 3-kinase (PI3K) is an activator of AKT, which regulates many cellular processes implicated in tumorigenesis such as cell growth, cell survival, and cell migration (1–3). Actually, AKT has been shown to be frequently activated in various types of human tumors including breast cancers (4, 5), suggesting that the PI3K/AKT pathway plays an important role in the pathogenesis and progression of human breast cancers.

PI3K consists of heterodimers with catalytic subunits (p110α, p110β, or p110δ) and regulatory subunits (p85α, p85β, or p55δ; ref. 6). The catalytic subunits are composed of several modular domains: catalytic lipid kinase domain, helical domain, C2 domain, Ras-binding domain, and the NH_2-terminal domain that interacts with the regulatory subunits. It is well established that PI3K is activated by autocrine or paracrine stimulation of receptor tyrosine kinases. Recently, in addition to this mechanism, somatic mutations of *PIK3CA*, which encodes the p110α catalytic subunit, have been shown to play an important role in the activation of PI3K in various human cancers (7–10). A high frequency of *PIK3CA* mutations has been reported in colorectal cancers, ovarian cancers, lung cancers, and breast cancers (7, 11–20). A great majority of somatic mutations in *PIK3CA* are missense mutations clustering in exons 9 and 20, which encode a part of the helical and kinase domains, respectively (7, 11, 13). *In vitro* studies have shown that the most frequently observed *PIK3CA* mutations in human breast cancers [i.e., E545K (exon 9) and H1047R (exon 20)] are associated with an increased kinase activity (8, 9), indicating that the *PIK3CA* mutations actually activate the PI3K pathway and thus are thought to be implicated in the pathogenesis and progression of breast cancers.

PIK3CA mutations, because they are found in 20% to 40% of breast cancers (11–14, 17), are considered to be one of the

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most commonly observed genetic changes besides *p53* mutations and *HER2* amplification. Although many reports have been available on the clinicopathologic characteristics of breast cancers with *p53* mutations or *HER2* amplification (21, 22), only a few reports have been available thus far on the clinicopathologic characteristics of breast cancers with *PIK3CA* mutations (11, 23). Elucidation of the characteristics of breast cancers with *PIK3CA* mutations seems to be important for the execution of personalized medicine in future. In addition, all the studies reported until now on *PIK3CA* mutations dealt with Caucasian breast cancers. It seems to be interesting to compare the frequency of *PIK3CA* mutations between Japanese and Caucasian breast cancers because breast cancer incidence in Japanese women is much lower (one fourth) than that of Caucasian women and, thus, contribution of *PIK3CA* mutations to pathogenesis of breast cancers might be different between two ethnicities. Therefore, in the present study, we have analyzed somatic mutations of *PIK3CA* in Japanese breast cancers as well as their relationship with the various clinicopathologic variables including patient prognosis. To further characterize breast cancers with *PIK3CA* mutations, correlation of *PIK3CA* mutations with phospho-AKT (pAKT) expression, HER2 overexpression, or *p53* mutation status has also been studied.

Materials and Methods

Patients and surgical specimens. Tumor tissue samples were obtained from 188 primary breast cancer patients who underwent mastectomy or breast conserving surgery during the period from March 1998 to October 2002 at Osaka University Hospital. Tumor tissue samples were obtained from the surgical specimens and snap frozen in liquid nitrogen and kept at −80°C until use. Informed consent was obtained from each patient before surgery.

As adjuvant chemotherapy (Table 1), six cycles of CMF (cyclophosphamide 100 mg/d orally days 1-14 + methotrexate 40 mg/m^2 i.v. days 1 and 8 + 5-fluorouracil 600 mg/m^2 i.v. days 1 and 8 q4w) were given to 15 patients, four cycles of EC (epirubicin 60 mg/m^2 i.v. day 1 + cyclophosphamide 600 mg/m^2 i.v. day 1 q3w) were given to 12 patients, and four cycles of docetaxel 60 mg/m^2 i.v. day 1 q3w were given to 4 patients. One hundred patients were treated with adjuvant hormonal therapy [tamoxifen 20 mg/d (n = 77), tamoxifen 20 mg/d + goserelin 3.6 mg q4w (n = 23)]. Forty-seven patients were treated with combination of chemotherapy [CMF (n = 21), EC (n = 21), or other chemotherapies (n = 5)] and hormonal therapy [tamoxifen (n = 44), goserelin (n = 1), or tamoxifen + goserelin (n = 2)]. Ten patients received no adjuvant therapy. Duration of tamoxifen treatment was 5 years and that of goserelin treatment was 2 years in most cases. Indication for adjuvant treatment was decided essentially according to the St. Gallen recommendation (24).

Physical examination every 3 months for 2 years postoperatively and every 6 months thereafter, combined with blood test and chest X-ray examination every 6 months postoperatively, was done. The median follow-up period of these 188 patients was 64 months, ranging from 38 to 88 months. Forty-five patients developed recurrences (i.e., 16 developed bone metastases, 9 developed liver metastases, 5 developed brain metastases, 6 developed lung metastases, 4 developed soft tissue metastases, and 12 developed lymph node metastases). Ipsilateral breast recurrences after breast conserving surgery were not counted as recurrences.

***Mutational analysis of* PIK3CA.** PCR amplification was done with the primers previously described for exons 1, 2, 4, 7, 9, 13, 18, and 20 of *PIK3CA* (7). Sequencing of the PCR products was done using an ABI 3300 automated capillary sequencer. We obtained the sequence data of *PIK3CA* gene from GenBank (accession no. NM_006218). Genomic DNA from corresponding normal tissue was subjected to sequence analysis to confirm that the nucleotide substitutions detected in tumor tissues are somatic in nature as for samples when nucleotide changes were detected in tumor tissues.

Immunohistochemistry of pAKT, HER2, and phospho-S6 expression. The expression of pAKT, HER2, and phospho-S6 (pS6) was studied by immunohistochemistry. In brief, for pAKT and pS6, endogeneous peroxidases were quenched by incubating the sections for 10 min in 6% H_2O_2. After several washes in TBS-T, antigen retrieval was done by heating the samples in 10 mmol/L citrate buffer (pH 6.0) at 95°C for 40 min. After blocking serum (DAKO Diagnostics, Mississauga, Ontario, Canada) for 30 min, the samples were incubated with a polyclonal rabbit anti-pAKT (Ser^{473}) antibody (1:100 dilution; Cell Signaling Technologies, Beverly, MA) or with a polyclonal anti-phospho-S6 ribosomal protein (1:50 dilution; Cell Signaling Technologies) at 4°C overnight. We then used the LSAB+ System (DAKO), which involved incubation with streptavidin treatment followed by secondary antibody for signal amplification (pAKT), or an avidin-biotin method (pS6). The positive reaction of pAKT was scored into four grades according to the intensity of the staining (0, none; 1+, weakly positive; 2+, moderately positive; and 3+, strongly positive) according to the method previously reported (25, 26). 0 and 1+ recorded as negative and 2+ and 3+ recorded as positive (Fig. 1). As for the cutoff level of pS6, we have scored both staining intensity and the percent of positive cells according to Allred scoring (27). The proportion score varies from 0 to 5 [0 (none or negative), 1 (<1/100), 2 (1/100-1/10), 3 (1/10-1/3), 4 (1/3-2/3), and 5 (>2/3)] and intensity score is the average intensity of all the positive cells (0, negative; 1, weak; 2, intermediate; and 3, strong). We classified positive when the total score that was obtained by summing proportion score and intensity score was >3. HER2 score was determined according to the DAKO system scale (DAKO Diagnostics, Vienna, Austria): HER2 negative (0 and 1+) and HER2 positive (2+ and 3+).

***Mutational analysis of* p53.** For identify genomic abnormalities of *p53*, each exon-intron junction from exon 5 to exon 8 was screened using PCR-single-strand conformation polymorphism method or direct sequencing, following the method previously described (28). Nucleotide alterations detected by single-strand conformation polymorphism were determined by sequencing analysis.

Estrogen receptor and progesterone receptor assay. Estrogen receptor (ER) and progesterone receptor (PR) contents of breast cancer tissues were measured by means of enzyme immunoassay using the kit provided by Abbott Research Laboratories (Chicago, IL). The cutoff value was 5 fmol/mg protein for ER and PR in accordance with the manufacturer's instruction.

Statistics. The relationship between *PIK3CA* mutation status and clinicopathologic variables of breast tumors was analyzed by the χ^2 test or Kruskal-Wallis test. Relapse-free survival curves were calculated by the Kaplan-Meier method, and the log-rank test was used to evaluate the differences in relapse-free survival rates. Cox proportional hazards model was used to calculate the hazard ratio for each variable in the univariate and multivariate analyses. Statistical significance was defined as $P < 0.05$.

Results

Frequency and location of* PIK3CA *mutations. Mutational analysis of *PIK3CA* was done in 188 primary breast cancers and, finally, 54 missense mutations were identified in total (Fig. 2). Because all the mutations were not detected in the corresponding normal tissues, these mutations were confirmed as somatic mutations. Of these 54 mutations, 17 and 29 mutations clustered in exon 9 and exon 20, respectively.

PIK3CA *mutations and clinicopathologic characteristics of breast cancers.* The frequency of *PIK3CA* mutations according

Table 1. Regimens used in postoperative adjuvant chemotherapy and/or hormonal therapy for breast cancer patients

	Mutation (+)	Mutation (−)	Total	P
Chemotherapy	6	25	31	
EC (4 cycles)*	3	9	12	
CMF (6 cycles)†	3	12	15	0.55
TXT (4 cycles)‡	0	4	4	
Hormonal therapy	31	69	100	
Tamoxifen§	24	53	77	0.99
Tamoxifen + goserelin‖	7	16	23	
Chemotherapy + hormonal therapy	16	31	47	
CMF (6 cycles) + tamoxifen	8	13	21	
EC (4 cycles) + tamoxifen	5	13	18	
TXT + tamoxifen	1	2	3	0.92
EC (4 cycles) + goserelin	0	1	1	
EC (4 cycles) + tamoxifen + goserelin	1	1	2	
Others + tamoxifen	1	1	2	
No therapy	1	9	10	

*Epirubicin 60 mg/m^2 i.v. day 1 + cyclophosphamide 600 mg/m^2 i.v. day 1 q3w.
†Cyclophosphamide 100 mg/d orally days 1-14 + methotrexate 40 mg/m^2 i.v. days 1 and 8 + 5-fluorouracil 600 mg/m^2 i.v. days 1 and 8 q4w.
‡Docetaxel 60 mg/m^2 i.v. day 1 q3w.
§Tamoxifen 20 mg/d.
‖Goserelin 3.6 mg q4w.

to histologic types is shown in Table 2. *PIK3CA* mutations were found in 44 of 158 (28%) invasive ductal carcinomas, 4 of 10 (40%) invasive lobular carcinomas, 1 of 4 (25%) mucinous carcinomas, 2 of 2 (100%) squamous carcinomas, and 2 of 2 (100%) apocrine carcinomas, but no mutation was found in 12 noninvasive ductal carcinomas.

The relationship of *PIK3CA* mutations with clinicopathologic variables is shown in Table 3. The frequency of *PIK3CA* mutations in ER-positive tumors (34%) was significantly ($P < 0.05$) higher than that in ER-negative tumors (19%), and the frequency of *PIK3CA* mutations in PR-positive tumors (33%) tended ($P = 0.09$) to be higher than that in PR-negative tumors (22%). *PIK3CA* mutation status was not significantly associated with menopausal status, tumor size, lymph node status, or histologic grade.

Relationship of* PIK3CA *mutations with pAKT or HER2 expression and* p53 *mutation status. Because it is suggested that *PIK3CA* mutations activate AKT function through its phosphorylation, we investigated the relationship between *PIK3CA* mutations and expression of pAKT. The frequency of *PIK3CA* mutations was significantly ($P < 0.05$) higher in pAKT-positive tumors (66%) than pAKT-negative tumors (40%; Table 4). We also studied the relationship between *PIK3CA* mutations and HER2 expression or *p53* mutations (Table 4). HER2 expression and *p53* mutations were not significantly associated with *PIK3CA* mutations.

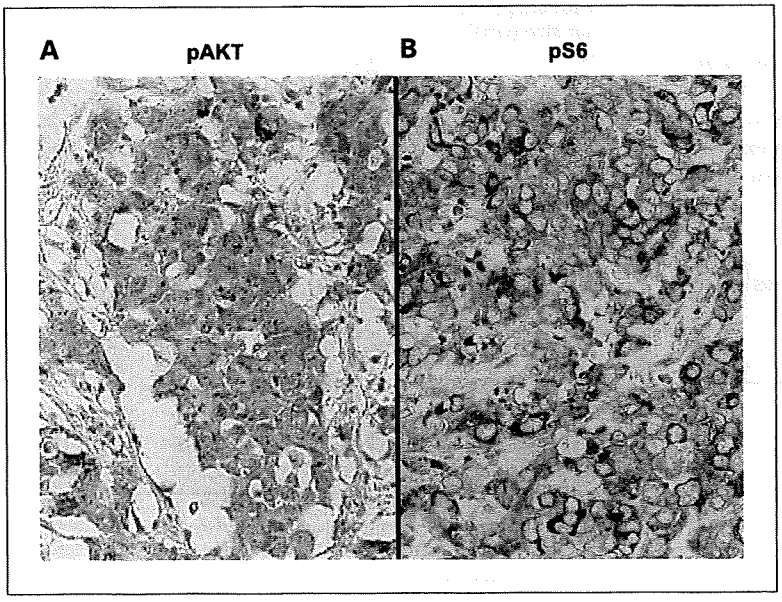

Fig. 1. Representative results of immunohistochemical staining of pAKT, pS6, and PTEN in breast cancer tissues. *A*, pAKT (×400); *B*, pS6 (×400).

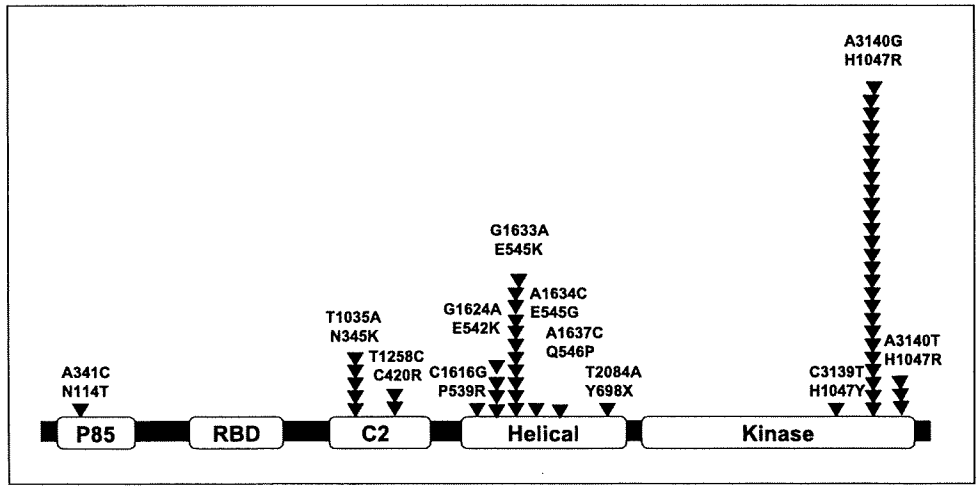

Fig. 2. Location of *PIK3CA* mutations in breast cancers. Arrowheads, location of missense mutations; boxes, functional domains.

Relationship of pAKT expression with pS6 expression. To further confirm the downstream activation of PI3K/AKT pathway induced by the *PIK3CA* mutations, we have investigated pS6, a downstream target molecule of pAKT by immunostaining. As shown in Table 5, the frequency of pS6-positive tumors was significantly ($P < 0.05$) higher in pAKT-positive tumors (76%) than in pAKT-negative tumors (51%).

PIK3CA *mutations and patient prognosis.* The relationship of *PIK3CA* mutations with patient prognosis was analyzed in 176 invasive carcinomas. The relapse-free survival rates of patients with *PIK3CA* mutations were significantly ($P < 0.05$) better than those of patients without them in the total patients (Fig. 3A) as well as in the subset of patients with ER-α-positive tumors (Fig. 3B). Univariate analysis showed that *PIK3CA* mutation status, tumor size, lymph node status, ER-α status, and PR status were significant ($P < 0.05$) prognostic factors, and multivariate analysis showed that *PIK3CA* mutation status, tumor size, lymph node status, and PR status were significant ($P < 0.05$) and mutually independent prognostic factors (Table 6).

Discussion

In the present study, we have identified *PIK3CA* mutations in 29% (54 of 188) of Japanese breast cancers including two novel missense mutations (N114T in exon 1 and Y698X in exon 13). Majority [83% (45 of 54)] of the mutations clustered in exon 9 and exon 20, helical and kinase domains, respectively. Not only the frequency but also the location of *PIK3CA* mutations is quite similar to that reported in Caucasian breast cancers (11). These results indicate that the contribution of *PIK3CA* mutations to pathogenesis and progression of breast cancers might be similar between two ethnicities.

No *PIK3CA* mutation was found in 12 noninvasive ductal carcinomas in the present study and Lee et al. reported *PIK3CA* mutations in only 2 of 15 (13%) noninvasive ductal carcinomas. These mutation frequencies in noninvasive ductal carcinomas seem to be slightly lower than those reported in invasive ductal carcinomas (20-40%). Because AKT stimulates tumor invasion by promoting the secretion of matrix metalloproteinases (29, 30) and the induction of epithelial-mesenchymal transition (31, 32), it is speculated that *PIK3CA* mutations

Table 2. *PIK3CA* mutations and histologic types of breast cancers

Histologic types	*PIK3CA* mutations (%)
Noninvasive ductal carcinoma	0 of 12 (0)
Invasive carcinoma	54 of 176 (31)
Invasive ductal carcinoma	44 of 158 (28)
Invasive lobular carcinoma	4 of 10 (40)
Mucinous carcinoma	1 of 4 (25)
Squamous cell carcinoma	2 of 2 (100)
Apocrine carcinoma	2 of 2 (100)

Table 3. *PIK3CA* mutations and clinicopathologic variables of breast cancers

	PIK3CA		*n*	*P*
	Mutant (%)	Wild (%)		
Menopausal status				
Premenopausal	29 (32)	63 (68)	92	
Postmenopausal	25 (26)	71 (74)	96	NS
Tumor size (cm)*				
>2	19 (31)	43 (69)	62	
≤2	35 (31)	79 (69)	114	NS
Lymph node metastasis*				
Negative	33 (31)	74 (69)	107	
Positive	21 (31)	46 (69)	67	NS
Unknown			2	
Histologic grade*				
1	9 (32)	19 (68)	28	
2	37 (33)	74 (67)	111	NS
3	3 (14)	19 (86)	22	
Unknown			15	
ER				
Positive	42 (34)	82 (66)	124	
Negative	12 (19)	52 (81)	64	0.03
PR				
Positive	38 (33)	76 (67)	114	
Negative	16 (22)	57 (78)	73	0.09
Unknown			1	

Abbreviation: NS, not significant.
*Noninvasive ductal carcinomas were excluded.

Table 4. Relationship between *PIK3CA* mutations and pAKT, HER2 expression, and *p53* mutations

	PIK3CA		*n*	*P*
	Mutant (%)	Wild (%)		
pAKT				
Positive	19 (66)	10 (34)	29	0.03
Negative	19 (40)	28 (60)	47	
HER2				
Positive	9 (38)	15 (62)	24	NS
Negative	39 (33)	80 (67)	119	
p53				
Mutant	9 (27)	24 (73)	33	NS
Wild	45 (29)	110 (71)	155	

might play a certain role in the progression from noninvasive to invasive ductal carcinomas through the activation of AKT.

The frequency of *PIK3CA* mutations in breast cancers with histologic types other than ductal carcinomas has rarely been reported (13). Although the number of tumors with histologic types other than invasive ductal carcinomas is small in the present study, we have been able to show that *PIK3CA* mutations are found in 4 of 10 invasive lobular carcinomas, 1 of 4 mucinous carcinomas, 2 of 2 squamous carcinomas, and 2 of 2 apocrine carcinomas. These results indicate that *PIK3CA* mutations are implicated in the pathogenesis and progression of not only ductal carcinomas but also other types of breast cancers. Recently, Buttitta et al. (33) have reported that the frequency of *PIK3CA* mutations is higher in invasive lobular carcinomas than in invasive ductal carcinomas, being consistent with our present observation that the frequency of *PIK3CA* mutations was 40% in invasive lobular carcinomas and 28% in invasive ductal carcinomas. One characteristic phenotype of invasive lobular carcinomas is its high ER positivity (34, 35). This phenotype of invasive lobular carcinomas seems to be explained, at least in part, by the higher frequency of *PIK3CA* mutations, which are associated with ER-α-positive breast cancers.

It has been reported that the hotspot mutations in *PIK3CA* actually enhance the lipid kinase activity as compared with wild type, leading to the increased phosphorylation of AKT and the resultant transformation of normal breast epithelial cells to tumor cells by *in vitro* and *in vivo* studies (8, 9). To examine whether the *PIK3CA* mutations found in the present study actually activate AKT through phosphorylation in human breast cancers, immunohistochemical study using anti-pAKT specific antibody was done. As expected, the frequency of *PIK3CA* mutations was significantly ($P < 0.05$) higher in pAKT-positive tumors (66%) than in pAKT-negative tumors (40%), suggesting that activation of AKT through phosphorylation by *PIK3CA* mutations actually occurs in human breast cancers. Furthermore, to confirm whether the pAKT activates downstream targets, the relationship between pAKT and phosphorylation of S6, one of the target molecules phosphorylated by pAKT signaling, was investigated by immunostaining. The positive correlation between pAKT and pS6 might suggest a downstream activation of this signal transduction induced by *PIK3CA* mutations.

The PI3K/AKT pathway regulates the various important cell functions implicated in tumorigenesis including cell growth, cell survival, and cell migration. In the present study, we have found that *PIK3CA* mutations are significantly higher in ER-α-positive tumors than in ER-α-negative tumors. Saal et al. (11) also reported a significant association between *PIK3CA* mutations and ER-α tumors. A positive association between pAKT and ER-α was also shown by an immunohistochemical study in breast cancers (36). Recently, it has been shown that AKT phosphorylates Ser^{167} of ER-α and enhances the transcriptional activity of ER-α (37). Thus, it is speculated that, in tumor cells with *PIK3CA* mutations, the PI3K/AKT/ER-α pathway might be activated, resulting in the preferential growth of ER-α-positive tumors.

Because the effect of *PIK3CA* mutations on patient prognosis has rarely been studied, we have investigated the prognostic significance of *PIK3CA* mutations in the present study. *PIK3CA* mutations activate AKT through phosphorylation and the pAKT expression has been reported to be associated with poor prognosis (38). The reason for such an association is considered to be attributable to resistance of pAKT-positive tumors to adjuvant tamoxifen, being based on the findings that prognosis of pAKT-positive tumors is poorer than that of pAKT-negative tumors in the ER-α-positive group treated with adjuvant tamoxifen but not in that treated without adjuvant tamoxifen. Thus, we assumed that tumors with *PIK3CA* mutations would be associated with poor prognosis in the present study where almost all patients (93%) with ER-α-positive tumors had been treated with tamoxifen. Until now, only two reports have been available on the relationship between *PIK3CA* mutations and prognosis. Li et al. (23) reported a significant association of *PIK3CA* mutations with poor prognosis, but Saal et al. (11) failed to confirm such an association. In the present study, we have obtained an unexpected result that tumors with *PIK3CA* mutations are significantly associated with a favorable prognosis in the total patients as well as in the subset of patients with ER-α-positive tumors.

Our result that *PIK3CA* mutations are associated with a favorable prognosis seems to be inconsistent with the fact that pAKT-positive tumors are associated with poor prognosis (38). In *PIK3CA*-mutated tumors, the PI3K/AKT pathway is probably the principal pathway for carcinogenesis and progression. However, in pAKT-positive tumors, because AKT is activated not only by *PIK3CA* mutations but also by various growth factors, other pathways (e.g., extracellular signal–regulated kinase/mitogen-activated protein kinase pathway) are very likely to be activated in addition to the PI3K/AKT pathway. Therefore, it is not surprising that the biological behaviors of the *PIK3CA*-mutated tumors and pAKT-positive tumors are different. Very recently, it has been shown that breast cancer cells with *PIK3CA* mutations are more likely to respond to tamoxifen than those without them as opposed to the findings that pAKT is associated with a resistance to

Table 5. Relationship between pAKT expression and pS6 expression

	pS6		*n*	*P*
	Positive (%)	Negative (%)		
pAKT				
Positive	22 (76)	7 (24)	29	0.03
Negative	24 (51)	23 (49)	47	

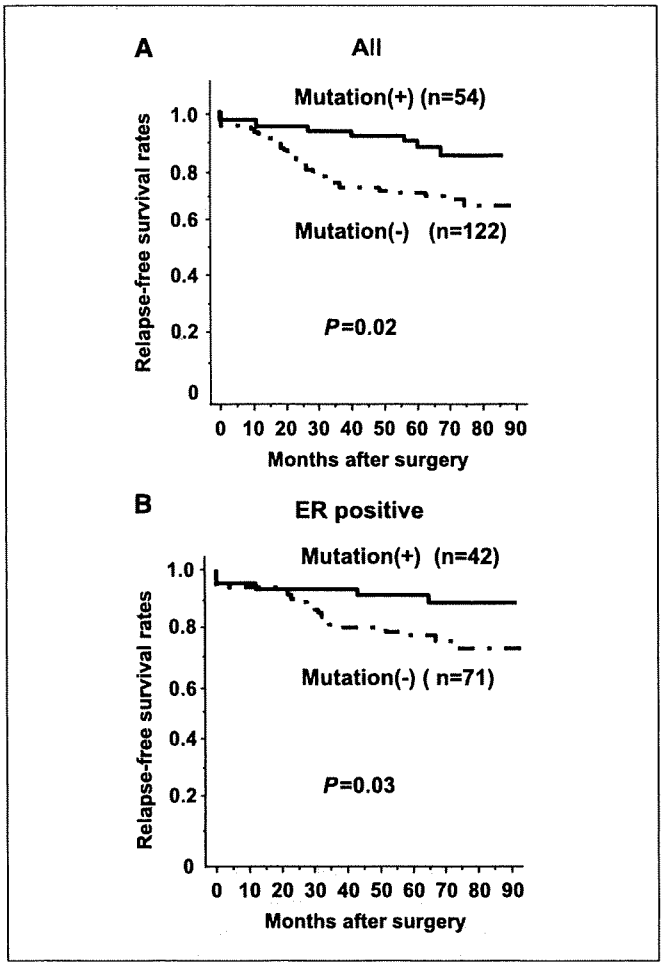

Fig. 3. Relapse-free survival rates of total patients with breast cancers (*A*) and patients with ER-α-positive breast cancers (*B*).

tamoxifen (39). We speculate that growth of tumor cells with *PIK3CA* mutations is highly dependent on estrogens due to the activation of the PI3K/AKT/ER-α pathway, and such cells are more likely to be growth inhibited by tamoxifen, and thus that *PIK3CA*-mutated tumors are associated with a favorable prognosis in patients treated with tamoxifen. Interestingly, recently, Yamashita et al. (37) have reported that phosphorylation of Ser^{167}, which is induced by pAKT, is associated with a good response to hormonal therapy including tamoxifen. Ideally, the effect of *PIK3CA* mutations on prognosis would better be analyzed in ER-positive breast cancer patients treated separately with and without tamoxifen to clarify whether *PIK3CA* mutation status would serve as a prognostic factor or as a predictive factor for response to tamoxifen. However, because almost all ER-positive breast cancer patients had been treated with tamoxifen, such an analysis was unable to be done in the present study.

Because both *PIK3CA* mutations and loss of phosphatase and tensin homologue (PTEN) function are thought to activate PI3K pathway, it is speculated that *PIK3CA* mutations and loss of PTEN expression are mutually exclusive. Consistent with this speculation, Saal et al. (11) have reported a negative association between *PIK3CA* mutations and loss of PTEN expression. Singh et al. (40) reported that *PIK3CA* mutations and *p53* mutations were mutually exclusive. Inconsistent with their report, however, we have found no association between *p53* mutations and *PIK3CA* mutations. The reason for these discrepancies is currently unknown; the relatively small number of tumors analyzed in these reports and the present study prevents from drawing a conclusion about the correlation of *PIK3CA* mutations with PTEN expression or *p53* mutations, and seems to indicate a necessity of further studies.

Because HER2 overexpression activates the PI3K/AKT pathway, tumors with *HER2* amplification are speculated not to require a further activation of this pathway by *PIK3CA* mutations. However, Saal et al. (11) reported a significant positive association between HER2 overexpression and *PIK3CA* mutations, suggesting that more than one input activating the PI3K/AKT pathway might be necessary for carcinogenesis of breast cancer. In the present study, we have failed to show a significant association between HER2 overexpression and *PIK3CA* mutations. Because immunohistochemistry is not an accurate method for determination of *HER2* amplification and only a limited number of breast cancers were analyzed in HER2 overexpression, our result needs to be interpreted with caution and should be confirmed by fluorescence *in situ* hybridization analysis of *HER2* amplification using a larger number of tumors.

In conclusion, we have identified somatic missense mutations of *PIK3CA* in 54 of 188 (29%) Japanese breast cancers. Majority (83%) of the mutations clustered in exon 9 and exon 20, helical and kinase domains, respectively. *PIK3CA* mutations were significantly associated with ER-α-positive tumors, or pAKT-positive tumors. Patients with *PIK3CA*-mutated tumors showed a significantly more favorable prognosis than those with *PIK3CA*-nonmutated tumors. It is currently unknown whether *PIK3CA* mutation status serves as a prognostic factor or as a predictive factor of response to tamoxifen. Our preliminary results need to be confirmed by a future study including a larger number of patients with a longer follow-up period.

Table 6. Univariate and multivariate analyses of various prognostic factors

	Univariate		Multivariate	
	HR* (95% CI)	P	HR* (95% CI)	P
PIK3CA mutation	2.36 (1.10-5.06)	0.03	2.34 (1.08-5.08)	0.03
Tumor size (cm)	6.25 (2.33-16.7)	0.0004	4.35 (1.54-12.5)	0.006
Lymph node status	3.03 (1.69-5.56)	0.0002	2.17 (1.18-4.17)	0.01
Histologic grade	1.27 (0.53-3.03)	0.59		
ER status	2.01 (1.13-3.59)	0.02	0.71 (0.33-1.53)	0.38
PR status	2.94 (1.61-5.35)	0.0004	2.96 (1.35-6.50)	0.007

NOTE: Noninvasive ductal carcinomas were excluded.
Abbreviation: CI, confidence interval.
*Hazard ratio of mutation negative against positive, large tumor (2.0 cm <) against small tumor (≤2.0 cm), lymph node positive against negative, histologic grade 2 + 3 against grade 1, ER-negative against ER-positive, and PR-negative against PR-positive.

References

1. Osaki M, Oshimura M, Ito H. PI3K-AKT pathway: its functions and alterations in human cancer. Apoptosis 2004;9:667–76.
2. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, Gonzalez-Baron M. PI3K/Akt signaling pathway and cancer. Cancer Treat Rev 2004;30: 193–204.
3. Stephens L, Williams R, Hawkins P. Phosphoinositide 3-kinases as drug targets in cancer. Curr Opin Pharmacol 2005;5:357–65.
4. Bellacosa A, de Feo D, Godwin AK, et al. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J Cancer 1995;64:280–5.
5. Cheng JQ, Ruggeri B, Klein WM, et al. Amplication of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Prc Natl Acad Sci U S A 1996;93:3636–41.
6. Vanhaesebroeck B, Waterfield MD. Signaling by distinct classes of phosphoinositide 3-kinases. Exp Cell Res 1999;253:239–54.
7. Samuels Y, Wang Z, Bardelli A, et al. High frequency of mutations of the *PIK3CA* gene in human cancers. Science 2004;304:554.
8. Ikenoue T, Kanai F, Hikiba Y, et al. Functional analysis of *PIK3CA* gene mutations in human colorectal cancer. Cancer Res 2005;65:4562–7.
9. Samuels Y, Diaz LA, Jr., Schmidt-Kittler O, et al. Mutant *PIK3CA* promotes cell growth and invasion of human cancer cells. Cancer Cell 2005;7:561–73.
10. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proc Natl Acad Sci U S A 2005;102:802–7.
11. Saal LH, Holm K, Maurer M, et al. *PIK3CA* Mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res 2005; 65:2554–9.
12. Levine DA, Bogomolniy F, Yee CJ, et al. Frequent mutation of the *PIK3CA* gene in ovarian and breast cancers. Clin Cancer Res 2005;11:2875–8.
13. Campbell IG, Russell SE, Choong DY, et al. Mutation of the *PIK3CA* gene in ovarian and breast cancer. Cancer Res 2004;64:7678–81.
14. Bachman KE, Argani P, Samuels Y, et al. The *PIK3CA* gene is mutated with high frequency in human breast cancers. Cancer Biol Ther 2004;3:772–5.
15. Broderick DK, Di C, Parrett TJ, et al. Mutations of *PIK3CA* in anaplastic oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. Cancer Res 2005;64:5048–50.
16. Wang Y, Helland A, Holm R, Kristensen GB, Borresen-Dale AL. *PIK3CA* mutations in advanced ovarian carcinomas. Hum Mutat 2005;25:322.
17. Lee JW, Soung YH, Kim SY, et al. *PIK3CA* gene is frequency mutated in breast carcinomas and hepatocellular carcinomas. Oncogene 2005;24:1477–80.
18. Velho S, Oliveira C, Ferreira A, et al. The prevalence of *PIK3CA* mutations in gastric and colon cancer. Eur J Cancer 2005;41:1649–54.
19. Li VSW, Wong CW, Chan TL, et al. Mutaitons of *PIK3CA* in gastric adenocarcinoma. BMC Cancer 2005;5:29.
20. Wu G, Xing M, Mambo E, et al. Somatic mutation and gain of copy number of *PIK3CA* in human breast cancer. Breast Cancer Res 2005;7:609–16.
21. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. Science 1987;235:177–82.
22. Kourea HP, Koutras AK, Scopa CD, et al. Expression of the cell cycle regulatory proteins p34cdc2, p21waf1, and p53 in node negative invasive ductal breast carcinoma. Mol Pathol 2003;56:328–35.
23. Li SY, Rong M, Grieu F, Iacopetta B. *PIK3CA* mutations in breast cancer are associated with poor outcome. Breast Cancer Res Treat 2006;96:91–5.
24. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thurlimann B, Senn HJ; Panel Members. Meeting highlights: International Expert Consensus on the Primary Therapy of Early Breast Cancer 2005. Ann Oncol 2005;16:1569–83.
25. Kucab JE, Lee C, Chen CS, et al. Celecoxib analogues disrupt Akt signaling, which is commonly activated in primary breast tumours. Breast Cancer Res 2005;7:796–807.
26. David O, Jett J, LeBeau H, et al. Phospho-Akt overexpression in non-small cell lung cancer confers significant stage-independent survival disadvantage. Clin Cancer Res 2004;10:6865–71.
27. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 1999;17:1474–81.
28. Miyoshi Y, Iwao K, Takahashi Y, Egawa C, Noguchi S. Acceleration of chromosomal instability by loss of BRCA1 expression and p53 abnormality in sporadic breast cancers. Cancer Lett 2000;159:211–6.
29. Park BK, Zeng X, Glazer RI. Akt1 induces extracellular matrix invasion and matrix metalloproteinase-2 activity in mouse mammary epithelial cells. Cancer Res 2001;61:7647–53.
30. Thant AA, Nawa A, Kikkawa F, et al. Fibronectin activates matrix metalloproteinase-9 secretion via the MEK1-MAPK and the PI3K-Akt pathways in ovarian cancer cells. Clin Exp Metastasis 2000;18: 423–8.
31. Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3 kinase/AKT pathways. Oncogene 2005; 24:7443–54.
32. Grille SJ, Bellacosa A, Upson J, et al. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer Res 2003;63: 2172–8.
33. Buttitta F, Felicioni L, Barassi F, et al. *PIK3CA* mutation and histological type in breast carcinoma: high frequency of mutations in lobular carcinoma. J Pathol 2006;208:350–5.
34. Middleton LP, Palacios DM, Bryant BR, Krebs P, Otis CN, Merino MJ. Pleomorphic lobular carcinoma: morphology, immunohistochemistry, and molecular analysis. Am J Surg Pathol 2000;24:1650–6.
35. Frolik D, Caduff R, Varga Z. Pleomorphic lobular carcinoma of the breast: its cell kinetics, expression of oncogenes, and tumor suppressor genes compared with invasive ductal carcinomas and classical infiltrating lobular carcinomas. Histopathology 2001;39: 503–13.
36. Kirkegaard T, Witton CJ, McGlynn LM, et al. AKT activation predicts outcome in breast cancer patients treated with tamoxifen. J Pathol 2005;207: 139–46.
37. Yamashita H, Nishio M, Kobayashi S, et al. Phosphorylation of estrogen receptor α serine 167 is predictive of response to endocrine therapy and increases postrelapse survival in metastatic breast cancer. Breast Cancer Res 2005;7:753–64.
38. Tokunaga E, Kimura Y, Oki E, et al. Akt is frequently activated in HER2/neu-positive breast cancers and associated with poor prognosis among hormone-treated patients. Int J Cancer 2006;118:284–9.
39. Whyte DB, Holbeck SL. Correlation of *PIK3CA* mutations with gene expression and drug sensitivity in NCI-60 cell lines. Biochem Biophys Res Commun 2006;340:469–75.
40. Singh B, Reddy PG, Goberdhan A, et al. p53 regulates cell survival by inhibiting PIK3CA in squamous cell carcinomas. Genes Dev 2002;16:984–93.

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Priority Report

mTOR Signal and Hypoxia-Inducible Factor-1α Regulate CD133 Expression in Cancer Cells

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Abstract

The underlying mechanism regulating the expression of the cancer stem cell/tumor-initiating cell marker CD133/prominin-1 in cancer cells remains largely unclear, although knowledge of this mechanism would likely provide important biological information regarding cancer stem cells. Here, we found that the inhibition of mTOR signaling up-regulated CD133 expression at both the mRNA and protein levels in a CD133-overexpressing cancer cell line. This effect was canceled by a rapamycin-competitor, tacrolimus, and was not modified by conventional cytotoxic drugs. We hypothesized that hypoxia-inducible factor-1α (HIF-1α), a downstream molecule in the mTOR signaling pathway, might regulate CD133 expression; we therefore investigated the relation between CD133 and HIF-1α. Hypoxic conditions up-regulated HIF-1α expression and inversely down-regulated CD133 expression at both the mRNA and protein levels. Similarly, the HIF-1α activator deferoxamine mesylate dose-dependently down-regulated CD133 expression, consistent with the effects of hypoxic conditions. Finally, the correlations between CD133 and the expressions of HIF-1α and HIF-1β were examined using clinical gastric cancer samples. A strong inverse correlation ($r = -0.68$) was observed between CD133 and HIF-1α, but not between CD133 and HIF-1β. In conclusion, these results indicate that HIF-1α down-regulates CD133 expression and suggest that mTOR signaling is involved in the expression of CD133 in cancer cells. Our findings provide a novel insight into the regulatory mechanisms of CD133 expression via mTOR signaling and HIF-1α in cancer cells and might lead to insights into the involvement of the mTOR signal and oxygen-sensitive intracellular pathways in the maintenance of stemness in cancer stem cells. [Cancer Res 2009;69(18):7160–4]

Introduction

The CD133/prominin-1 protein is a five-transmembrane molecule expressed on the cell surface that is widely regarded as a stem cell marker. Growing evidence indicates that CD133 can be used as a cell marker for cancer stem cells or tumor-initiating cells in colon cancer, prostate cancer, pancreatic cancer, hepatocellular carcinoma, neural tumors, and renal cancer (1). Strict regulatory mechanisms governing CD133 expression are thought to be deeply related to inherent cancer stemness; however, such mechanisms remain largely unclear, especially in cancer cells. In brain tumors, the Hedgehog (2), bone morphogenetic protein (3), and Notch (4) signaling pathways have been implicated in the control of CD133+ cancer stem cell function.

Some investigators have shown a relation between hypoxia and CD133 expression in brain tissue. The percentage of CD133-expressing cells was found to increase in a glioma cell line cultured under hypoxic conditions (5), and mouse fetal cortical precursors cultured under normoxic conditions exhibited a reduction in CD133(hi)CD24(lo) multipotent precursors and the failure of the remaining CD133(hi)CD24(lo) cells to generate glia (6). With the exception of these studies in brain tissue, however, data on the expression of CD133 and the involvement of hypoxia and other signaling pathways in cancer cells remains limited.

Several reports have indicated that mTOR is a positive regulator of hypoxia-inducible factor (HIF) expression and activity (7), and the inhibition of HIF-mediated gene expression is considered to be related to the antitumor activity of mTOR inhibitors in renal cell carcinoma (8). We found that mTOR signaling was involved in CD133 expression in gastric and colorectal cancer cells. Thus, we investigated the regulatory mechanism of CD133 in cancer cells.

Materials and Methods

Reagents. 5-Fluorouracil, irinotecan (CPT-11), and rapamycin were purchased from Sigma-Aldrich. Gemcitabine was provided by Eli Lilly. Tacrolimus (LKT Laboratories), LY294002 and wortmannin (Cell Signaling Technology), and deferoxamine mesylate (DFO; Sigma-Aldrich) were purchased from the indicated companies.

Cell cultures and hypoxic conditions. All of the 28 cell lines used in this study were maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), except for LoVo (F12; Nissui Pharmaceutical), WiDr, IM95, and HEK293 (DMEM; Nissui Pharmaceutical), and Huvec (Humedia; Kurabo). Hypoxic conditions (0.1% O_2) were achieved using the AnaeroPouch-Anaero (Mitsubishi Gas Chemical) with monitoring using an oxygen indicator.

Real-time reverse transcription-PCR. The methods were previously described (9). The primers used for the real-time reverse transcription-PCR (RT-PCR) were as follows: CD133, forward 5′-AGT GGC ATC GTG CAA ACC TG-3′ and reverse 5′-CTC CGA ATC CAT TCG ACG ATA GTA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPD), forward 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse 5′-ATG GTG GTG AAG ACG CCA GT-3′. GAPD was used to normalize the expression levels in the subsequent quantitative analyses.

Clinical samples. The mRNA expression levels of CD133, HIF-1α, and HIF-1β in gastric cancer specimens were obtained from previously published microarray data (9).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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