RESEARCH COMMUNICATION

Real-Time Quantitative PCR Analysis of Amplified DNA on Chromosomes 4p15.2 and 6q23-24 from Formalin-Fixed, Paraffin-Embedded Breast Cancer Tissues

Sunanta Chariyalertsak[1], Wichai Purisa[1], Phongkitti Thisuphakorn[2], Anant Karalak[3], Tanett Pakeetoot[4], Songsak Petmitr[5]*

Abstract

This study was performed to detect amplification of DNA sequences on chromosomes 4p15.2 and 6q23-24, obtained from formalin-fixed, paraffin-embedded, breast-cancer tissues. The prognostic relevance of the amplification was also demonstrated. DNA from formalin-fixed, paraffin-embedded tumor and corresponding normal tissues of 53 patients with breast cancer was extracted and amplified by real-time quantitative PCR technique. Amplification of the DNA sequences on chromosomes 4p15.2 and 6q23-24 was detected in 23 (43%) and 32 (60%) cases, respectively. Thirty-six (68%) cases showed amplification on both or one of the chromosomes. These frequencies are similar to that obtained from fresh samples in our previous study. In addition, amplification of the DNA on chromosomes 4p15.2 and / or 6q23-24 was predominantly observed in tumors with invasive ductal carcinoma. The findings in this study demonstrate that DNA extracted from formalin-fixed, paraffin-embedded breast tumors can be used to determine amplification of DNA sequences on selective chromosomal regions. We also suggest that the amplified DNA on chromosomal regions 4p15.2 and 6q23-24 might be involved in the development and progression of breast cancer.

Key Words: Real-time PCR - amplified DNA - chromosome 4p15.2 - chromosome 6q23-24 - formalin-fixed, paraffin-embedded tissues - breast cancer

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Introduction

Breast cancer is the most common malignancy worldwide and the second leading cancer of women in Thailand (Parkin et al., 2002; Ferlay et., 2004). It is estimated that 5-10% of all breast cancers are caused by an inherited predisposition, whereas 90-95% are sporadic, among which cases occur randomly and are not genetically predetermined (Stratton, 1996; Claus et al., 1996). Germline mutations are identified in two breast cancer susceptibility genes, BRCA1 and BRCA2, which account for the majority of the hereditary form of the disease (Wooster et al., 1995; Welcsh et al., 2000). Several genes, located in specific regions of chromosomes that are frequently deleted or amplified in sporadic breast cancer, have been identified and well analyzed (Bieche et al., 1995; van Diest et al., 1997; Zhu et al., 1998; Janicke et al., 2001), but the critical sequence of events that leads to the development of such a cancer has not yet been established.

Several studies on the allelotyping of breast cancer have revealed various genomic abnormalities in these cancer cells. The most frequently found genetic alterations in primary breast cancers are amplifications of erbB-2, myc and cyclin D1 proto-oncogenes (Kallioniemi et al., 1992; Zhu et al., 1998; Steeg et al., 1998; Shimada et al., 2005). DNA copy number alterations have been determined in breast cancer using various methods. The studies are consistent, frequently reporting the same regions of gain (1q, 8q, 11q, 16p, 17q, 20q) and loss (1p, 6q, 8p, 11q, 13q, 16q) (Hermsen et al., 1998; Moore et al., 1999; Richard et al., 2000; Loveday et al., 2000; Seute et al., 2001; Pollack et al., 2002; Climent et al., 2002; Rennstam et al., 2003; Albertson, 2003; Naylor et al., 2005).

From our previous study (Pakeetoot et al., 2007), we used arbitrarily primed polymerase chain reaction (AP-PCR) to detect genomic changes in DNA extracted from normal and cancerous breast tissues obtained from surgery. The D15 arbitrary primer showed the presence of a DNA fragment highly amplified in more than half of all tumor samples from the patients analyzed. After cloning and sequencing this amplified fragment, two different DNA sequences were found, while BLAST

*[1]Divisions of Research, [2]Surgery and [3]Pathology, National Cancer Institute, Bangkok [4]Department of Medical Science, Faculty of Science, Rungsit University, Pathumthanee, [5]Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand *For Correspondence: e-mail: tmspm@mahidol.ac.th*

analysis revealed that they mapped to chromosomes 4p15.2 and 6q23-24. AP-PCR-derived SCAR markers were used to detect specific alterations in breast cancer, and the amplification was quantitatively determined by real-time PCR; the results indicated that, of the 30 patients with breast cancer, DNA sequence amplification was detected on chromosomes 4p15.2 and 6q23-24 in 18 (60%) and 15 (50%) cases, respectively. Twenty-three (77%) cases showed amplification on both or one of the chromosomes.

With growing interest in the genomic characteristics of many human tumors and a rapid increase in the availability of genomic tests for either clinical or research purposes, the amount of genomic DNA available from biological samples may limit the practicality of genomic analysis. Formalin-fixed paraffin-embedded (FFPE) tissues, comprising the most common form of human tissue samples archives, have been used for decades. Therefore, this study was performed to evaluate possibility of detecting amplification of the DNA sequences on chromosomes 4p15.2 and 6q23-24 from FFPE breast-tumor specimens. Where available, we used primary tumor and normal tissue from the same patient. DNA was extracted from these samples and used for detection. Real-time quantitative PCR was chosen to detect DNA sequence amplification. Furthermore, findings of the amplified DNA sequences that correlated with clinicopathological parameters and disease-free survival of patients with breast cancer were also demonstrated.

Materials and Methods

Subjects and tissue samples

Patients with primary breast cancer were studied, from whom two paraffin blocks, one containing breast cancer tissue and the other containing normal tissue, were selected from the National Cancer Institute archives for the years 2002-2003. All breast tissues had been fixed in 10% neutral buffered formalin and embedded in paraffin under standard conditions. None of the patients had received radiation or chemotherapy before surgery. Hematoxylin-eosin-stained sample sections from each tumor block were examined microscopically to confirm the presence of > 80% cancer cells. Paired normal tissues from the same patient were used as controls and showed histologically normal features.

Table 1. Clinicopathological Parameters and Amplification of the DNA Sequence on Chromosome 4p15.2 in Primary Breast Cancer

Parameter	Amplification of DNA sequence 4p15.2 + n (%)	4p15.2 - n (%)	P value
Age			0.277
< 50	15 (50.0)	15 (50.0)	
≥ 50	8 (34.8)	15 (65.2)	
Histological type			0.276
Intraductal carcinoma	1 (20.0)	4 (80.0)	
Invasive ductal carcinoma	22 (45.8)	26 (54.2)	
Tumor size (cm)			0.508
< 3	18 (46.2)	21 (53.8)	
≥ 3	5 (35.7)	9 (64.3)	
Lymph node metastasis			0.239
Positive	16 (50.0)	16 (50.0)	
Negative	7 (33.3)	14 (66.7)	
Distant metastasis			0.405
Positive	2 (28.6)	5 (71.4)	
Negative	21 (45.7)	25 (54.3)	

DNA preparation

FFPE breast samples (primary tumors and normal tissues) were cut by microtome. Three 10-μm- thick serial sections were placed in a 1.5-ml tube, 1.2 ml xylene added and the samples vortexed. The tissue was pelleted by centrifugation at room temperature for 5 min at 14,000 rpm. The supernatant was removed and the pellets were washed with 1.2 ml of 100% ethanol and vortexed. Again, the sample was centrifuged at room temperature for 5 min at 14,000 rpm. The ethanol wash step was repeated once more. The samples were then re-suspended in 600 μl of Cell Lysis Solution and 3 μl of Proteinase K Solution was added (Bio-Rad). Samples were incubated overnight at 55˚C with gentle shaking. The next day, 3 μl of RNase A Solution (Bio-Rad) was added and the sample was incubated for 15 min at room temperature. The DNA was isolated using the AquaPure DNA Isolation Kit (Bio-Rad) as described by the manufacturer. Seven of the 60 cases were discarded because of poor DNA yield.

Design of sequence characterized amplified region (SCAR) primers

Specific primers were designed according to nucleotide sequences of two different clones obtained from our previous study, using the GeneFisher program (http://www.genefisher.com): 5' TTG CAT CAT TTT TCC CTC TG 3' (forward primer); 5' CAC GTG GAC ATC TCA AGA CC 3' (reverse primer) for amplification of the DNA sequence on chromosome 4p15.2, and 5'- CCA TAG GGA CGA AGG CAT TA 3' (forward primer); 5'-5' CTC CCA AAG TGC TGG GAT TA -3' (reverse primer) for amplification of that on chromosome 6q23-24.

Table 2. Clinicopathological Parameters and Amplification of the DNA Sequence on Chromosome 6q23-24 in Primary Breast Cancer

Parameter	Amplification of 6q23-24 + n (%)	DNA sequence 6q23-242 - n (%)	P value
Age			0.294
< 50	20 (66.7)	10 (33.3)	
≥ 50	12 (52.2)	15 (47.8)	
Histological type			0.054
Intraductal carcinoma	1 (20.0)	4 (80.0)	
Invasive ductal carcinoma	31 (64.6)	17 (35.4)	
Tumor size (cm)			0.778
< 3	24 (61.5)	15 (38.5)	
≥ 3	8 (57.1)	6 (42.9)	
Lymph node metastasis			0.344
Positive	21 (65.6)	11 (34.4)	
Negative	11 (52.4)	10 (47.6)	
Distant metastasis			0.147
Positive	6 (85.7)	1 (14.3)	
Negative	26 (65.5)	20 (43.5)	

Table 3. Clinicopathological Parameters and Amplification of the DNA Sequence on Chromosome 4p15.2 and/or 6q23-24 in Primary Breast Cancer

Parameter	Amplification of 4p15.2/6q23-24 + n (%)	DNA sequence 4p15.2/6q23-24 - n (%)	P value
Age			0.345
< 50	22 (73.3)	8 (26.7)	
≥ 50	14 (60.9)	9 (39.1)	
Histological type			0.015
Intraductal carcinoma	1 (20.0)	4 (80.0)	
Invasive ductal carcinoma	35 (72.9)	13 (27.1)	
Tumor size (cm)			0.749
< 3	26 (66.7)	13 (33.3)	
≥ 3	10 (71.4)	4 (28.6)	
Lymph node metastasis			0.180
Positive	24 (75.0)	8 (25.0)	
Negative	12 (57.1)	9 (42.9)	
Distant metastasis			0.288
Positive	6 (85.7)	1 (14.3)	
Negative	30 (65.2)	16 (34.8)	

Real-time quantitative PCR

Amplification of the DNA sequences was detected by real-time PCR with the designed specific SCAR primers (Bioservice, Bangkok, Thailand). We used β-globin as a reference gene. The PCR was performed in a total volume of 20 µl in each LightCycler glass capillary, containing 18 µl of LightCycler mastermix: 8.8 µl water; 3.2 µl MgCl2 (4 mM); 2 µl forward primer (0.5 µM); 2 µl reverse primer (0.5 µM); and 2 µl LightCycler (Fast Start DNA Master SYBR Green I; Roche Diagnostics), and 2 µl genomic DNA (40 ng). The PCR condition consisted of an initial denaturation step at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 10 sec, and 72˚C for 1 min. Thermal cycling and fluorescent monitoring were performed using a LightCycler analyzer (Roche Diagnostics). The point at which the PCR product is first detected above a fixed threshold, termed the cycle threshold (Ct), was determined for each sample. The DNA content of tumor and normal tissue preparations was normalized using β-globin within the PCR reaction. The relative concentrations were determined employing Ct values, which are equivalent to the cycle number at which the PCR product is first detected above a fixed threshold (Figure 1). The Ct values obtained from the analyzer were then exported to Excel (Microsoft) for further analysis. Calculation of the DNA copy number was performed utilizing the delta-delta-Ct method (Livak et al., 2001). Samples were run in duplicate. A sample with a value ≥ 1.5-fold was interpreted as having amplification (+) and any sample with a value < 1.5-fold was interpreted as having no amplification (-).

Statistical analysis

Clinicopathological features of patients with primary breast cancer -patient's age at initial diagnosis, histological type, tumor size, metastasis status - were correlated with amplification of the DNA sequences. Results were evaluated by chi-square test. Survival analysis was carried out with patients who were followed up for a period of 36 months, or until first relapse, after surgery. Patients who were lost to follow-up before 36 months after surgery were excluded. Disease-free survival distributions were calculated by Kaplan-Meier method and analyzed using the log-rank test. P values < 0.05 were considered statistically significant.

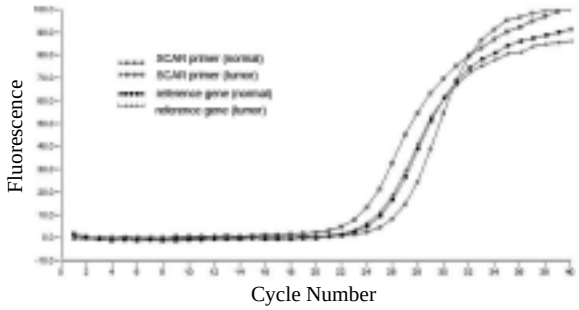

Figure 1. Real-time PCR SYBR Green I Fluorescence Record versus Cycle Number of SCAR primer (6q23-24) and Reference Gene (β globin) in Tumor DNA and Corresponding Normal DNA

Results

Real-time quantitative PCR findings indicated that, of the 53 patients with primary breast cancer, DNA

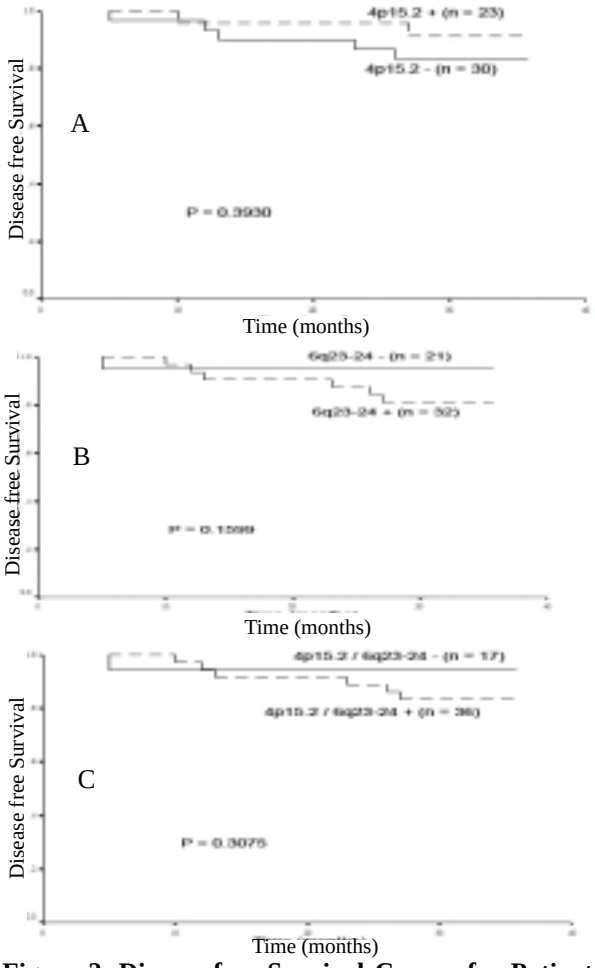

Figure 2. Disease-free Survival Curves for Patients with Breast Cancer According to Amplification of DNA on Chromosome 4p15.2 (A), 6q23-24 (B), and 4p15.2 and/or 6q23-24 (C)

sequence amplification on chromosomes 4p15.2 and 6q23-24 was determined in 23 (43%) and 32 (60%) cases, respectively. Thirty-six (68%) cases showed amplification on both or one of the chromosomes. A housekeeping gene (β globin) was amplified as an internal control.

An investigation of the relationship of the amplified DNA sequences with the available clinicopathological data of the tumors analyzed found no relation between DNA sequence amplification on chromosome 4p15.2 and patient's age at diagnosis, histological types, tumor size, lymph-node metastasis, or distant metastasis (Table 1). The frequency of amplified DNA on chromosome 6q23-24 was relatively low in intraductal carcinoma tumors (P=0.054). No significant difference was found between DNA sequence amplification on chromosome 6q23-24 and other clinicopathological parameters (Table 2). Interestingly, when the findings for DNA sequence amplification on these two chromosomes were combined, amplified DNA on one or both chromosomes was detected frequently in tumors with invasive ductal carcinoma, with statistically significant difference (P=0.015). However, there was no significant correlation between these amplifications and other clinical features (Table 3). Disease-free survival analysis also demonstrated no relationship between DNA sequence amplification on either chromosome 4p15.2 or 6q23-24 and disease-free survival (Figure 2).

Discussion

A great limitation of cancer-related studies has been the lack of prospective collection of DNA specimens, to allow description of the correlation between alteration in genomic DNA and clinical outcome. FFPE tissues represent the largest source of archival biological material available for the genomic investigation of human cancers. Therefore, several investigators have demonstrated the feasibility and reliability of genotyping or detection of amplification using DNA extracted from FFPE samples (Rae et al., 2003, Schneider et al., 2006, Aviel-Ronen et al., 2006, Ghazani et al., 2006). In this study, we showed that DNA extracted from FFPE tissue could be used to detect amplified DNA sequences on chromosomes 4p15.2 and 6q23-24.

DNA isolated from FFPE specimens are highly fragmented because of the cross-linkage effect of formalin. Therefore, the present study tried to design specific primers that produced the shortest possible PCR products, and found that primers used to generate a 200 bp fragment amplicon were available for detection. Amplification was determined by real-time quantitative PCR technique, which could eliminate all distortions of amplification efficiency arising from fixation artifacts or sample impurities (Livak et al., 2001; Lehmann et al., 2001). The present analysis demonstrated similar DNA amplification frequencies to those obtained using fresh samples in our previous study.

Several reports have noted DNA amplification or gain on chromosomes 4p and 6q in various malignancies, including breast (Richard et al., 2000; Rodriguez et al., 2000; Naylor et al., 2005), pancreas (Solinas-Toldo et al., 1996; Wallrapp et al., 1997), prostate (Kasahara et al., 2002), and uterine corpus carcinomas (Micci et al., 2004). DNA gain at 4p16.1 was detected in 51% of breast tumors (Naylor et al., 2005). Gains of 4p14-qter were demonstrated to be late events in midgut carcinoid tumor progression (Kytola et al., 2001). A gain at chromosomal region 6q was highly significantly correlated with mitotic and apoptotic activity in breast carcinomas (Buerger et al., 2000). A gain of 6q seems to be more common among breast carcinomas with mutated TP53 than those with wild-type TP53 (Kleivi et al., 2005). In addition, DNA gain in the 6q21-22 region was frequently found in invasive ductal breast carcinoma (Rodriguez et al., 2000), and a gain on chromosome 6q23-q26 occurred at a significantly higher percentage of poorly differentiated breast carcinomas (Richard et al., 2000). In accord with previous findings, our data show that 6q is a frequent target of DNA amplification in breast cancer.

To date, DNA gains and losses have both been demonstrated at 4p and 6q. DNA losses in either chromosome 4p or 6q have frequently been found in small-cell lung (Lui et al., 2001), ovarian (Okada et al., 2002), and prostate carcinomas (Verhagen et al., 2002). Deletion of the 4p15-p16 region was found in metastatic squamous-cell carcinomas of the lung, and invasive ductal carcinomas of the breast (Richard et al., 2000). Loss of heterozygosity (LOH) at 4p15.1-15.3 has been shown to occur in breast (Shivapurkar et al., 1999), colorectal (Shivapurkar et al., 2001), invasive cervical (Sherwood et al., 2000), and head and neck squamous-cell carcinomas (Pershouse et al., 1997). Loss of 6q23-24 might be associated with some prostate cancers (Srikantan et al., 1999). Furthermore, LOH in the 6q23-24 region has been linked to the progression of breast and cervical cancers (Noviello et al., 1996; Mazurenko et al., 1999). One previous study indicated that invasive ductal breast carcinomas carried DNA gains on chromosome 6q21-qter, and losses on 4p15.2-p16 and 6q16-q24 (Richard et al., 2000). It is questionable whether all these regions carry tumor suppressor genes (TSGs) or oncogenes responsible for tumor progression, but both oncogenes and TSGs have been observed in these regions. For example, amplification of SLA/LP and STIM2 genes located at 4p15 was recently reported in glioblastoma multiforme tumors (Ruano et al., 2006). STIM2 codes for a transmembrane phosphoprotein whose structure is unrelated to that of any other known protein (Williams et al., 2001) and whose biological function has not been completely studied. SLIT2 has been mapped to chromosome 4p15.2 (Georgas et al., 1999). The SLIT2 gene is frequently inactivated in lung and breast cancer by promoter region hypermethylation and allele loss, and is an excellent candidate for the lung and breast tumor suppressor gene (Dallol et al., 2002). The proto-oncogene c-myb amplified in pancreatic cancer at 6q24 encodes a transcriptional activator protein with repeated helix-turn-helix DNA binding motifs (Wallrapp et al., 1997). c-myb is known to be activated as an oncogene through amplification in several tumor cells, including primary breast cancer (Zhou et al., 1989, Kauraniemi et al., 2000), some acute myelogenous leukemic cell lines (Pellicci et al., 1984), and colon cancer

(Greco et al., 1994). Data in the literature suggest that increased c-myb activity leads to selective precedence in tumor cell proliferation (Melani et al., 1991; Graf, 1992).

Interestingly, in addition to the high number of copy amplifications, other genetic alterations such as LOH, were also detectable in the c-myb locus. These alterations have been described in pancreatic cancer (Wallrapp et al., 1997), in leukemias and lymphomas (Barletta et al., 1987), and in breast cancer (Hall et al., 1989). It has been suggested that genetic imbalances of the c-myb locus, either amplification or deletion, might appear along with tumor progression or metastasis (Yokota et al., 1986). UROC28, another oncogene candidate, localized to chromosome 6q23-24, is up-regulated in prostate and breast cancers (An et al., 2000).

The identification of genetic events involved in the development of breast cancer not only helps us understand the mechanism of tumor progression, but may also lead to the discovery of genetic markers beneficial for early diagnostic and prognostic purposes, and management of consequent therapy. In this study, we found significantly higher frequencies of DNA amplification on chromosomes 4p15.2 and/or 6q23-24 among patients with invasive ductal carcinoma than among patients with non-invasive ductal carcinoma, indicating that patients bearing the amplified 4p15.2 and/or 6q23-24 sequences are likely to have more aggressive phenotype.

In conclusion, our findings show that real-time PCR has some potential in quantifying DNA amplification. Its ease of use and broad dynamic range make it a viable screening tool for DNA amplification in FFPE samples. This may be significant for the recovery of archived DNA for appropriate correlative studies from abundant trials where clinical outcomes are available, but no DNA was collected.

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