Genomic change of chromosome 8 predicts the response to taxane-based neoadjuvant chemotherapy in node-positive breast cancer

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Abstract. This study was performed to investigate whether the response to neoadjuvant chemotherapy in ductal-type breast cancer could be predicted by different genomic alterations. Array-based comparative genomic hybridization (aCGH) was performed on samples from 15 patients who underwent neoadjuvant chemotherapy with epirubicin plus docetaxel (ED). Frozen tissue bank samples were retrospectively selected from 8 patients who demonstrated complete pathologic response (pCR) and from 7 patients resistant to neoadjuvant chemotherapy. We performed aCGH with 4,277 human bacterial artificial chromosome (BAC) clones, scanning the genome for DNA copy number changes. In a cluster dendrogram of aCGH data, responders showed changes clustered in S940, S984, S44, S98, S130, S115, S478, and 1150T, whereas non-responder group changes clustered in S1029, S209, S219, S660, S133, S323, and S670. Compared to responders, non-responders showed more complicated genomic alterations; the most common gains were located at chromosome 8q (71%), 13q (71%), and 20q (57%), with the smallest regions of genomic gain at 8q24.3, 8q24.22, 8q24.21, 8q22.1, 8q22.2, 8q22.3, 13q21.1, 20q13.2, and 20q13.33. The most frequently deleted regions were observed on chromosome 8p (71%) and 17p (57%), with the smallest regions of deletion at 8p23.3, 8p23.2, 8p23.1, 8p21.3, 8p21.2, and 17p13.3. The results of the current study suggest that aberrations in chromosome 8 may contribute to the resistance to taxane-based neoadjuvant chemotherapy in ductal-type breast cancer. Results of our study indicate that candidate gene identification through aCGH should be validated by specific gene analysis since the sites of chromosomal aberration are quite different among studies.

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Introduction

Breast cancer, the most common cancer in women worldwide, is a complex and intrinsically heterogeneous disease. Most patients with breast cancer tend to exhibit similar clinical features despite entirely different genetic backgrounds. While vast improvements have been made in diagnosis and treatment of this disease, clinical outcome of most patients have not dramatically improved. Due to the lack of reliable predictors for the response of specific treatment, many women at low risk of disease progression are unnecessarily subjected to aggressive systemic therapies, while those at high risk develop resistance to cytotoxic treatment in a rather short period even after systemic therapy. To establish efficient and more individualized therapy, better understanding for the biologic characteristics of breast cancer is a prerequisite step.

The introduction of multigene assay technologies using DNA microarrays has expanded our knowledge on genetic phenomena associated with breast cancer. However, the utility of gene expression studies has been limited by the identification of different candidate genes in different studies. Most multigene studies have concentrated on identifying high-risk patients who are more likely to have recurrent breast cancer after standard systemic treatment. Breast cancer progression is due to the accumulation of multiple genomic alterations that lead to growth advantages and clonal expansion, and these genomic alterations appear to be associated with the prognosis of individual patients (1). Such genetic alterations are important steps in the development of human malignancies, and may contribute both to disease progression and to responses to specific treatments. After the introduction of DNA microarray technology, several studies were performed to integrate gene expression profiles with the outcomes of specific cytotoxic treatments. Until now, no study has identified gene expression profiles that explain responses to specific chemotherapy.

Array-based comparative genomic hybridization (aCGH) offers a unique opportunity to identify those genes related to tumor development and progression (2). The routine use of aCGH profiles has been proposed to classify a disease into clinically relevant subgroups, with implications for the prognosis and treatment of breast cancer (3-6). Several attempts have been made to correlate patterns of genomic change

detected by aCGH with breast cancer prognosis (3,6-21). aCGH profiles have been successfully applied to identify new oncogenes and tumor suppressor genes that may be promising therapeutic targets (22,23). In the present study, we performed aCGH with frozen core needle biopsy samples of primary breast cancer and profiled the genetic changes associated with responses to docetaxel-based neoadjuvant chemotherapy.

Materials and methods

Study design and selection of patients. Patients with locally advanced breast cancer (i.e., primary cancers >4 cm, or clinically evident axillary metastases) diagnosed at Inje University Sanggye Paik Hospital between 2002 and 2007 were included in this study. Prior to neoadjuvant chemotherapy, we collected five or six core biopsies of the primary breast cancer in each patient. Three core biopsy specimens were immediately cryopreserved at -80˚C for later analysis. The remaining specimens were fixed in formalin for diagnostic analysis and immunohistochemical staining.

Clinicopathologic variables including tumor size, histologic grade, and hormone receptor status were determined before chemotherapy. We administered four cycles of neoadjuvant chemotherapy with epirubicin 75 mg/m^2 plus docetaxel 100 mg/m^2 at 3-week intervals, and assessed the clinical response after completion of the fourth cycle. As part of standard care, patients underwent chemotherapy for four cycles, unless clear evidence existed of progressive disease, defined as an increase in tumor size of more than 25%. After the completion of neoadjuvant chemotherapy, curative surgery was performed. Pathologic complete response (pCR) was defined as the disappearance of all invasive cancer in the breast after completion of neoadjuvant chemotherapy.

This study was conducted under the approval of the institutional review board of Inje University Sanggye Paik Hospital. Frozen tissue samples were selected retrospectively from a tissue bank belonging to 8 patients who demonstrated complete pathologic response (pCR) and 7 patients who were resistant to neoadjuvant chemotherapy.

Bacterial artificial chromosome (BAC) library. Bacterial artificial chromosome (BAC) clones were selected from Macrogen's proprietary BAC library (http://www.macrogen.com). Briefly, pECBAC1 (24) was restricted with *Hin*dIII, and size-selected *Hin*dIII-digested pooled male DNA was used to generate a BAC library. The vectors containing this library were transformed into and maintained in DH10B.

Construction of a BAC-mediated aCGH microarray. Clones were first selected bioinformatically to give an average genomic coverage of 1 Mb resolution. All of the clones were end-sequenced using Applied Biosystems 3700 sequencers, and their sequences were blasted and mapped to their chromosomal positions using the UCSC human genome database (http://www.genome.uscs.edu). Confirmation of locus specificity of the chosen clones was performed by removing multiple loci-binding clones and examining them individually under standard fluorescence *in situ* hybridization (FISH) conditions, as previously described (25). BAC clone DNA was prepared by conventional alkaline lysis and sonicated to generate fragments approximately 3 kb in length, before mixing with 50% dimethyl sulfoxide (DMSO) spotting buffer. Arrays were manufactured by Genomic Solutions using an OmniGrid 100 spotter. Each clone was represented on an array by duplicated spots, and each array was prescanned using a GenePix4200A scanner (Axon Instrument, Sunnyvale, CA, USA) for proper spot morphology. The array used in this study consisted of 4,362 human BAC clones.

DNA labeling for aCGH. Labeling and hybridization protocols used were as described by Pinkel *et al*, with some modification to the labeling procedure (23). Briefly, 2 μg of test and reference DNAs were digested overnight with Dpn II. After purification, 21 μl containing 800 ng of digested normal DNA (reference) or digested tumor DNA (test) and 20 μl BioPrime® Array CGH Genomic Labeling System Random primer solution (Invitrogen, Carlsbad, CA, USA) in water were combined and incubated for 5 min at 95˚C, and subsequently cooled on ice. After the addition of 5 μl 10x dNTP labeling mix (0.6 mM dCTP, 1.2 mM dATP, 1.2 mM dGTP, 1.2 mM dTTP), 3 μl 1 mM Cy3 or Cy5-dCTP (Perkin Elmer, Waitham, MA, USA), and 40 U BioPrime® Array CGH Genomic Labeling System Exo-Klenow fragment (Invitrogen), the mixture was gently mixed and incubated for 16 h at 37˚C. The addition of 5 μl BioPrime® Array CGH Genomic Labeling System Stop Buffer (Invitrogen) ended the reaction. After labeling, unincorporated fluorescent nucleotides were removed using a purification module (Invitrogen). In one tube, Cy3-labeled sample and Cy5-labeled reference DNAs were mixed together, and 100 μg of human Cot I DNA (Invitrogen), 30 μl 3 M sodium acetate, and 600 μl cold 100% ethanol were added to precipitate the DNA.

Array hybridization, imaging, and data analysis. Labeled DNA pellets were resuspended in 40 μl hybridization solution containing 50% formamide, 10% dextran sulfate, 2 x SSC, 4% SDS, and 200 μg yeast tRNA. The hybridization solution was denatured for 10 min at 70˚C and incubated subsequently for 1 h at 37˚C to allow blocking of repetitive sequences. Hybridizations were performed in slide chambers for 48 h at 37˚C. After post-hybridization washes, arrays were rinsed, dried by centrifugation, and scanned, generating two 16-bit TIFF image files for each array, using a GenePix4200A two-color fluorescent scanner (Axon Instrument).

Validation of genomic changes by fluorescence in situ hybridization (FISH). To confirm specific gains and losses of BAC clones observed in the aCGH analyses, FISH was performed using individual BAC clones as probes on isolated nuclei from tumor sections. The gene loci examined corresponded to overrepresented or deleted BAC clones, using appropriate centromeric probes as controls. FISH was performed at selected loci (WISP1 and NDRG1, 8q24.22) from 15 tumors to validate the copy number changes identified by aCGH. Target probe labeled with Cy3 was mixed with the same quantity of 2 BAC clones (Macrogen, Seoul, Korea) of no. 359 and no. 21 on 8q24.22 region. Control probe labeled with FITC was made by the same method with 4 BAC clones (Macrogen) of no. 7443, no. 7444, 149_F07 and 28_B06 on 8q11.1 region. Target probe size is ~202.6 kb and control probe size is ~211.6 kb. For HER2 gene, PathVysion (Vysis

Table I. Clinicopathological characteristics of 15 patients with node-positive invasive ductal carcinoma.

	HG	NG	ER	PR	HER2	Ki67LI	Size (cm)
SD1	3	1	+	+	+	30	10
SD2	3	1	-	-	-	30	6
SD3	3	1	-	-	-	50	7
SD4	2	2	-	-	-	5	7
SD5	2	2	+	+	-	30	15
SD6	2	2	+	+	-	20	5
SD7	3	1	-	-	-	90	7
pCR1	3	1	+	+	+	30	4
pCR2	3	1	+	-	+	30	4
pCR3	2	2	+	+	+	5	3
pCR4	3	1	-	-	+	20	3
pCR5	3	1	+	+	+	40	3
pCR6	2	2	+	+	-	10	4
pCR7	2	2	+	+	-	10	5
pCR8	3	1	+	-	-	20	7

HG, Bloom-Richardson's histologic grade; NG, Black's nuclear grade; ER, estrogen receptor; PR, progesterone receptor; Ki67LI, Ki67 labeling index; SD, stable disease; pCR, pathological complete response.

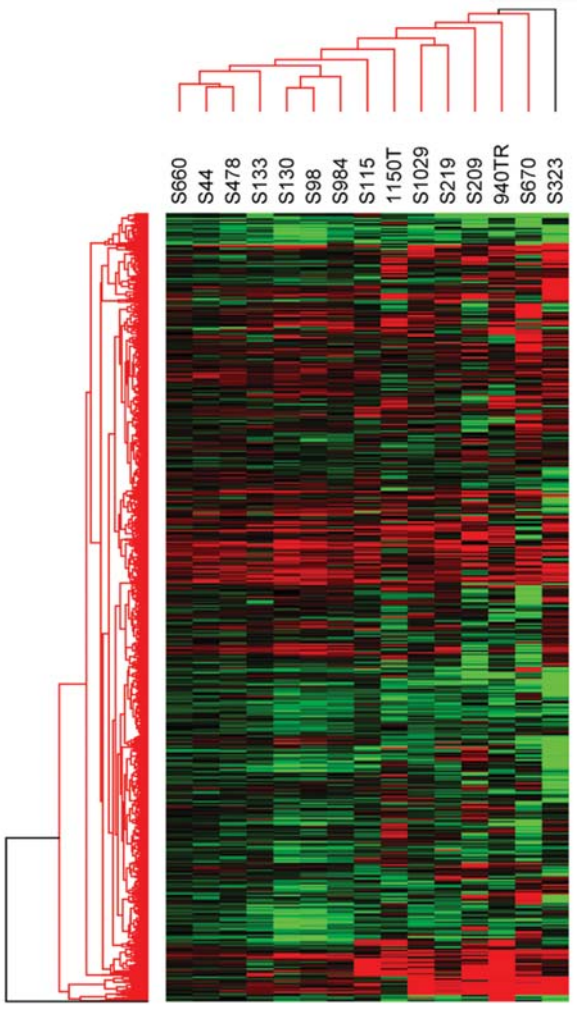

Figure 1. Cluster dendrogram of array CGH data from 15 node-positive invasive ductal carcinomas by unsupervised clustering. The samples from the response and non-response group reveal distinct cluster formation (response group: S44, S478, S130, S98, S984, S115, 1150T; no response group: S660, S133, S1029, S219, S209, S670, S323).

Inc., Downers Grove, IL, USA) was applied. Four-μm-thick sections from paraffin blocks were deparaffinized, dehydrated, immersed in 0.2 N HCl for 20 min at room temperature and incubated in 1 mol/l NaSCN for 35 min at 80˚C. Slides were immersed in pepsin solution (Vysis) for 20 min at 37˚C, and tissues were fixed by 10% neutral buffered formalin. Dual probes for hybridization was performed according to the manufacturer's protocol. The probes and target DNA were simultaneously denatured. Ten microliters of the probe mixture was applied to slides, which were incubated in a humidified atmosphere with Hybrite (Vysis) for 16 h at 37˚C in the dark. Slides were immersed in 0.4x SSC/0.3% NP-40 for 20 min at room temperature, and then in 2x SSC/0.1% NP-40 for 5 min at 73˚C. After drying, nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and antifade compound (P-phenylenediamine). The marker (orange) and control (green) signal ratio was calculated and twice as many marker signals than control signals were considered as the criteria for gene amplification.

Data processing and analysis. The average fluorescence ratio of the two replicate spots for each clone was calculated. Clones with missing values (81 clones) and sex chromosomes (286 clones) were excluded; 3,991 different BAC clones were used in the final analysis. The log2-transformed fluorescent ratios were calculated from background-subtracted mean intensity values. These ratios were used to perform print-tip normalization before copy number calculations. The purpose of normalization is to adjust for effects that arise from variation between the red and green dyes or from scanner settings for the two fluors. R and G indicate the background-corrected Cy5 and Cy3 intensities for each spot, respectively. Normalization is usually applied to the log ratios of Cy3 and Cy5, which will be written as M = logR - logG. The log intensity of each spot is written as A = (logR + logG)/2. The M-value is normalized by subtracting from it the corresponding value of the print-tip group LOWESS curve. The normalized log ratios N are the

Table II. Most significant genomic aberrations in no response group in 15 node-positive invasive ductal carcinomas.

Gain	Representative genes	Frequency (%)	P-value
8q22.1		71	0.0468
8q22.2	KCNS2, STK3	71	0.0468
8q22.3		71	0.0468
8q24.12	MRPL13, MTBP, SNTB1	71	0.0468
8q24.21	DDEF1, DDEF1IT1	71	0.0468
8q24.21		1	0.0468
8q24.22	TG	71	0.0468
8q24.22	WISP1, NDRG1	71	0.0468
8q24.22	WISP1, NDRG1	71	0.0468
8q24.3		71	0.0468
8q24.3	LYPD2, LYNX1, LY6D, GML, LOC646338	71	0.0468
8q24.3	CYHR1, KIFC2, FOXH1, PPP1R16A, GPT, MFSD3, RECQL4, LRRC24, MGC70857, KIAA1688	71	0.0468
8q24.3	CYHR1, KIFC2, FOXH1, PPP1R16A, GPT, MFSD3, RECQL4, LRRC24, MGC70857, KIAA1688	71	0.0468
13q21.1	FLJ40296, LOC729233, LOC729240, LOC729246, LOC729250	71	0.0081
20q13.2		57	0.0295
20q13.33		43	0.0295
Loss			
8p23.3	LOC389607, ERICH1	71	0.0081
8p23.3	C8orf68	57	0.0295
8p23.3		1	0.0468
8p23.2	CSMD1	71	0.0468
8p23.1	MSRA	57	0.0295
8p21.3	DOK2, XPO7	57	0.0295
8p21.2	DOCK5, GNRH1, KCTD9, CDCA2	71	0.0081
17p13.3	RPH3AL	57	0.0295

residuals from the print-tip group LOWESS regression, i.e., N = M - lowess (A), where lowess (A) is the LOWESS curve as a function of A for the ith print-tip group. Each LOWESS curve is constructed by performing a series of local regressions, one for each point in the scatterplot.

Chromosomal aberrations were categorized as a gain when the normalized log2-transformed fluorescent ratio was higher than 0.25 and as a loss when this ratio was below -0.25. These two threshold values were chosen empirically by selecting a 3xSD value calculated from 30 normal male to normal female hybridization experiments. After calling loss, gain, and normal loci in the aCGH data, we performed χ^2 tests using the counts of abnormality (loss, gain) versus normal. We then determined the adjusted p-values (FDR <0.05) from the R package of Bioconductor. Macrogen's MAC viewer aCGH analysis software was used for quantification and image analysis of aCGH data.

Results

Patient characteristics. The clinicopathological characteristics of 15 patients and their tumors are summarized in Table I. All 15 patients received docetaxel-based neoadjuvant chemotherapy. Eight patients showed pCR and seven patients were resistant to neoadjuvant chemotherapy.

Genomic profiling. aCGH analysis was performed on 15 node-positive invasive ductal carcinoma core biopsies, which were all identified with high-resolution specific regions of gain and loss throughout the genome. A cluster dendrogram of aCGH data from the 15 biopsies was analyzed by unsupervised clustering (Fig. 1). The samples from the responder (pCR) and non-responder groups revealed distinct cluster formation. Responders clustered in S940, S984, S44, S98, S130, S115, S478, and 1150T, whereas non-responders clustered in S1029, S209, S219, S660, S133, S323, and S670. The eight patients of the responder group and the seven patients of the non-responder group were distinctly separated in the cluster dendrogram (Fig. 1).

All tumors showed genomic changes and the non-responders revealed more complicated changes than responders in a log2 ratio plot of chromosomal copy number alterations (Fig. 2). Twenty-four clones that mapped to 16 different chromosome loci in five different chromosome arms were

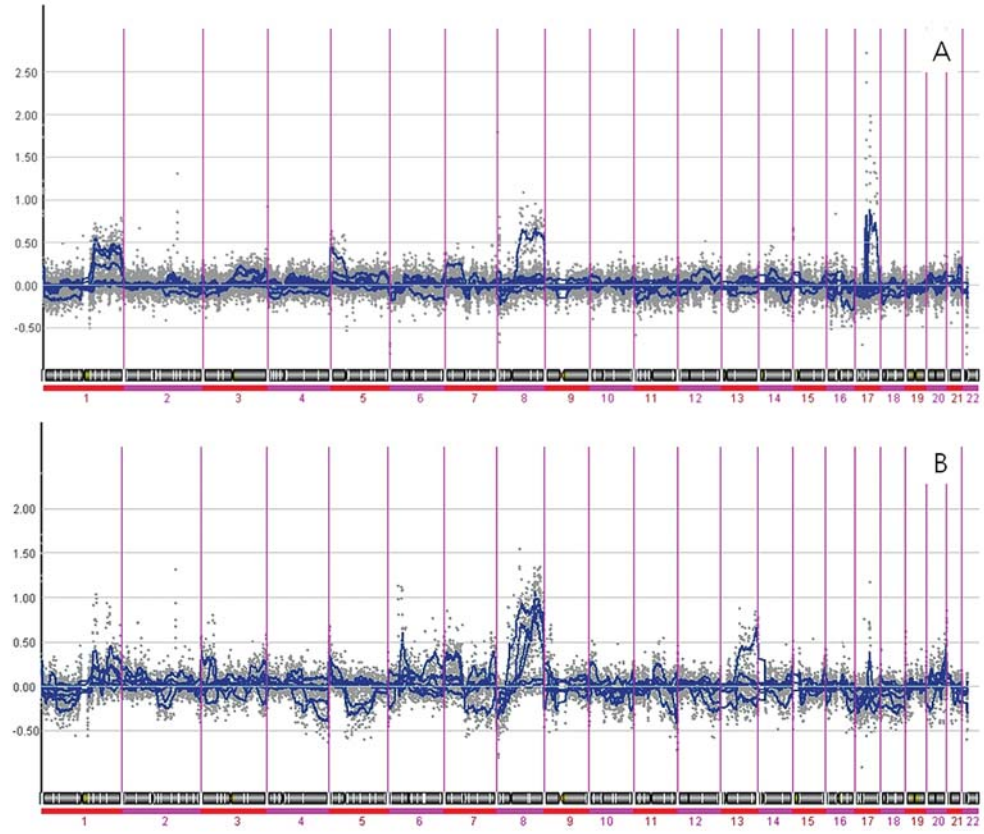

Figure 2. Log2 ratio plot of response group (A) and no response group (B).

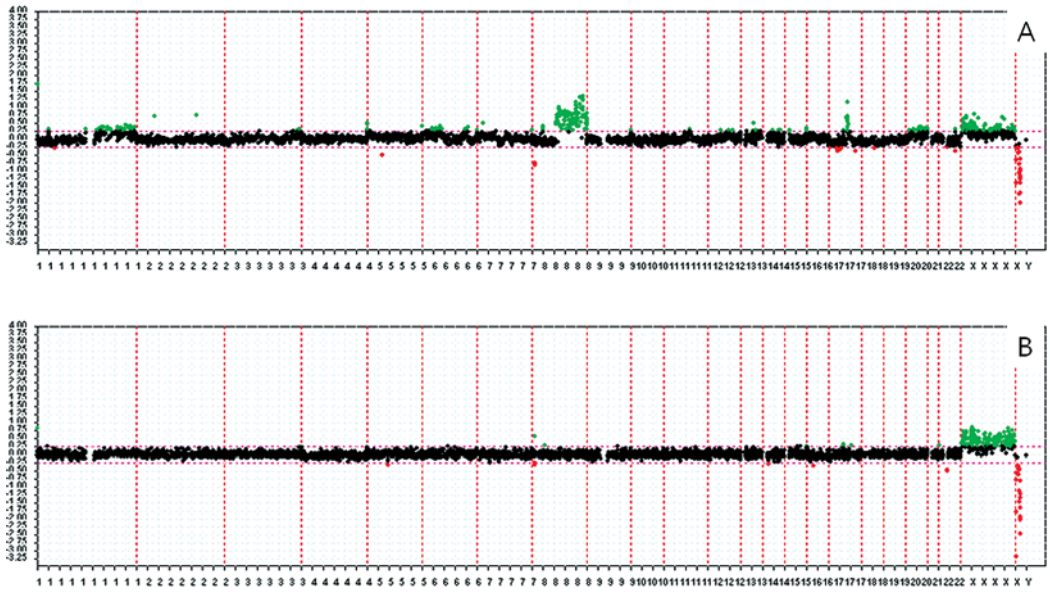

Figure 3. Scatter plot of no response group (A: S1029) and response group (B: S44). S1029 reveals chromosome 8q gain and 8p loss (log2 ratio mean 0.276, threshold: 0.25 - -0.25) and S44 reveals normal chromosome 8 (log 2 ratio mean -0.008, threshol: 0.25 - -0.25).

identified. The chromosomal regions with the most common genomic alterations (>40%) are shown in Table II. The most common gains corresponded to chromosome 8q (71%), 13q (71%), and 20q (57%), with the smallest identified regions of genomic gain at 8q24.3, 8q24.22, 8q24.21, 8q22.1, 8q22.2, 8q22.3, 13q21.1, 20q13.2, and 20q13.33. The region of 8q22.1-8q24.22 includes the genes *KCNS2*, *STK3*, *MRPL13*, *MTBP*, *SNTB1*, *DDEF1*, *DDEF1IT1*, *TG*, *WISP1*, *NDRG1*, *LYNX1*, *LY6D*, *GML*, *LOC646338*, *CYHR1*, *KIFC2*, *FOXH1*, *PPP1R16A*, *GPT*, *MFSD3*, *RECQL4*, *LRRC24*, *MGC70857*,

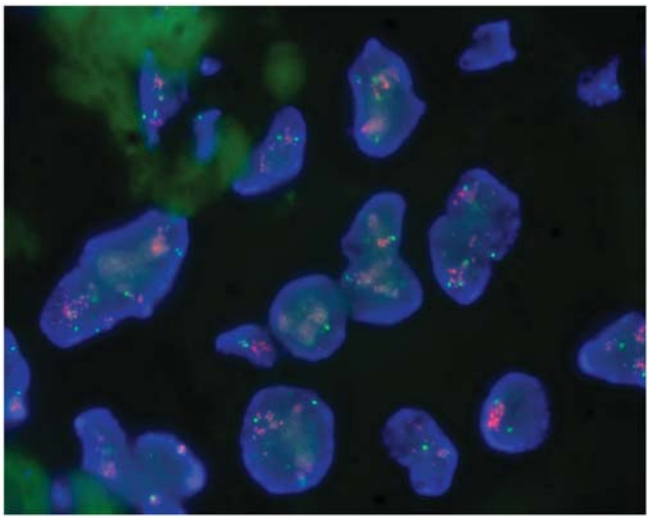

Figure 4. Amplification of WISP1 and NDRG1 was observed in the cases of 8q24.22 gain in aCGH. (Red signal: WISP1 and NDRG1; green signal: control, 8q11.1)

and *KIAA1688*. The most frequently deleted regions were observed at chromosome 8p (71%) and 17p (57%), with the smallest regions of deletion at 8p23.3, 8p23.2, 8p23.1, 8p21.3, 8p21.2, and 17p13.3. The region of 8p21 includes the genes *DOK2*, *XPO7*, *DOCK5*, *GNRH1*, *KCTD9*, and *CDCA2*, and 8p23 includes *CSMD1*, *MSRA*, *LOC389607*, *ERICH1*, and *C8orf68* (Fig. 3).

FISH was performed on tumors to test selected genes. *WISP1* and *NDRG1* were chosen on the basis of aCGH results, and amplification of *WISP1* and *NDRG1* were observed in the cases with gains in 8q24.22 (Fig. 4). *HER2*-overexpressing breast cancers showed gains at chromosome 17q (Fig. 5). Results of aCGH on 17q21 coincided well with the *HER2* amplification confirmed by FISH in six patients. Of these patients, five responded to epirubicin plus docetaxel (ED) neoadjuvant chemotherapy.

Discussion

In the current study, most of the non-responders showed chromosomal gains in the 8q region compared to responders. Gains in 8q as well as 20q are known to be common in invasive ductal carcinomas and have been regarded as a poor prognostic indicator. In our study, patients with 8q gains responded poorly to ED neoadjuvant chemotherapy. Gains on 8q are known to be associated with higher proliferation rates, and this association is partly explained by c-*myc*-driven proliferation (26,27). In our study, the most common gains were observed at 8q24.3, 8q24.22, 8q24.21, 8q22.1, 8q22.2, 8q22.3, 13q21.1, 20q13.2, and 20q13.33. The 8q22-24 region is close to that of c-*myc*, which is located at 8q24.1, but c-*myc* amplification was not observed in either group in our study. In a gene expression analysis of breast cancers, only two of eight tumors with c-*myc* amplification had increased expression of its mRNA (28). Another aCGH study also reported the absence of c-*myc* amplification (29), indicating that c-*myc* might not be the only target in the 8q24 amplicon in breast cancers. An association between poor response to neoadjuvant chemotherapy and 8q gain might be due to genes other than c-*myc*.

Candidate genes for gains in 8q22-24 are *DDEF1*, *CCND1*, and *WISP1*. The role of *WISP1* in breast cancer, however, is controversial. One study reported that *WISP1* protein expression was reduced in advanced breast cancer compared to early stage disease (30). In contrast, other investigators reported that *WISP1* protein expression was associated with an aggressive phenotype in breast cancer (31). Amplification of *WISP1* was observed in the cases with gains in 8q24.22 and it was validated by FISH analysis. Clarifying the role of *WISP1* in breast cancer will require a further validation study. *DDEF1* amplification has been reported to be associated with high-grade melanoma, but a biological role of *DDEF1* in breast cancer has not been reported (32).

Other studies combining CGH and expression profiles identified two candidate genes in the case of the 8q11-12 amplicon, *FLJ14229* and *SPFH2*(*C8org2*) (33-35). However, we found no gains in 8q11-12 in the current study. The three studies were conducted on breast cancer tissues from Western women. An ethnic difference might exist in chromosomal aberration patterns of breast cancer. In our data, other putative genes responsible for genomic aberrations and implicated in breast cancer progression include *AIB1*, which is located on 20q12, and *CCND3* and *P21/WAF* on 6p21 (36). Gains in 20q13 were also demonstrated in our study. Amplification at 20q13 together with gains on 8p11-12 and 11q13-14 was correlated with poor prognosis in another study (6). Gains of 8q together with loss of 8p may represent the aggressive genotype of invasive breast cancer, although specific loci of chromosomal alterations differ among studies.

Our study showed that patterns of genomic alteration detected by aCGH differ between responders and non-

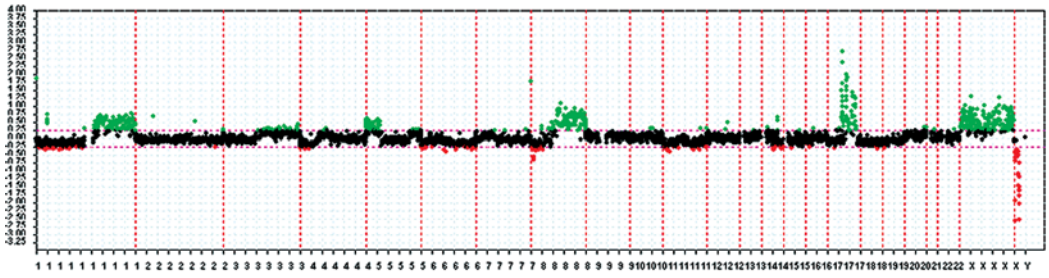

Figure 5. HER2 overexpressed breast cancer (threshold: 0.25 - -0.25) showed chromosome 17q amplification in aCGH.

responders to taxane-based chemotherapy. In our study, all the patients underwent four cycles of neoadjuvant ED chemotherapy. Thus, the therapeutic outcome could be influenced by the susceptibility of the tumors to anthracycline as well as to taxane. Amplification of topoisomerase II-α is a well-known predictor of anthracycline sensitivity, and this association has been confirmed in large-cohort clinical trials. We have reported an intimate association between topoisomerase II-α amplification, which is almost always coamplified with *HER2* and response to anthracycline-based neoadjuvant chemotherapy (37). We have also observed a close association between the amplification of HER2 and *c-myc* in breast cancer (38). In the current study, we observed a gain on 17q21 in four patients of the responder group, and topoisomerase II-α amplification by FISH coincided well with aCGH results. Aberrations in chromosomal sites other than 17q, such as 8q and 8p, might contribute to the resistance to docetaxel, although gains of 8q24 are common in ductal-type breast cancer.

In early breast cancer, patients with 11q deletion appeared to benefit from anthracycline-based adjuvant chemotherapy (4). However, we could not observe copy number aberration on 11q in the current study. A recent study reported that comparison of the pretreatment and the tumor specimens excised after neoadjuvant chemotherapy revealed genomic gains on 11p15.2-15.5 rather than 11q (39). The same investigators reported that loss of 13q31.1-32.2 were the only region to have significant difference between responders and non-responders to neoadjuvant chemotherapy with doxorubicin plus cyclophosphamide. In summary, the site of genomic aberration associated with response to neoadjuvant chemotherapy is different according to individual studies including our study. The three studies were performed on different patients regard to clinical characteristics and with different therapeutic regimens albeit all three contained anthracycline.

The goal of aCGH is to address relevant biological and clinical questions, such as the identification of genes responsible for carcinogenesis or cancer progression, and the prediction of clinical outcomes. Copy number aberrations are in many cases very large in area, making it difficult to determine how many genes are responsible for growth selection. Between 40 and 60% of the genes within an amplicon are believed to be overexpressed, but gene expression studies are needed to identify the genes responsible for any observed amplification. Validation study upon candidate genes identified by aCGH is mandatory since the genomic aberrations have found in quite different regions from studies to studies. However, specific chromosomal alterations can provide interesting clues to the identification of candidate genes, since these changes do not take place randomly in tumors, but represent preferential mutations of particular allelic forms.

In conclusion, the therapeutic response to taxane-based neoadjuvant chemotherapy apparently differed in accordance with gains on 8q and losses on 8p. Our results indicate that genomic alteration patterns can be integrated into the prediction of response to docetaxel-based chemotherapy, although our conclusions are limited by a small sample size. The discrepancies of chromosomal aberration foci with other studies such as 8q11-12 and 11q suggest a possible ethnic difference of genetic changes of breast cancer between Western and Korean women. Results of our study indicates that candidate gene identification through aCGH should be validated by specific gene analysis since the sites of chromosomal aberration are quite different among studies.

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