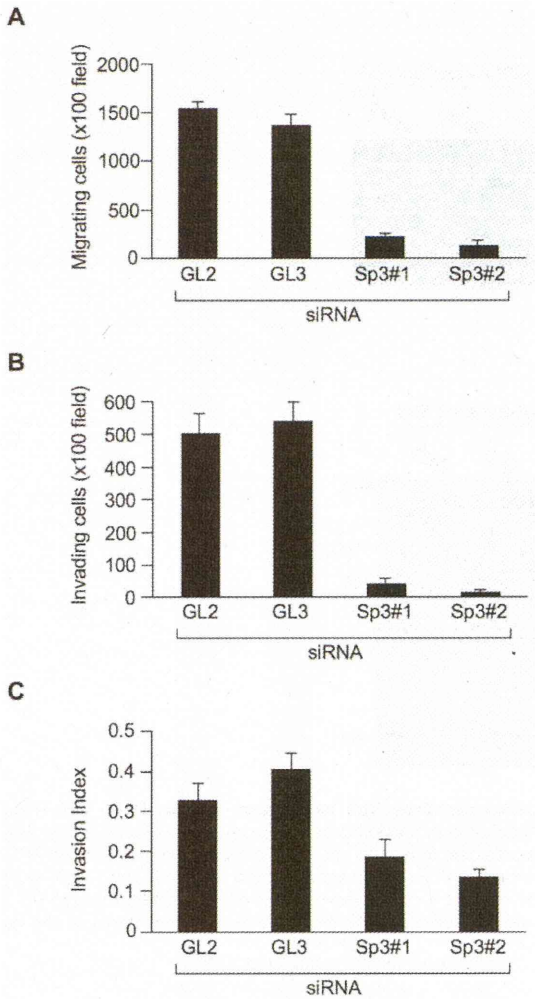

Figure 8. Inhibition of Sp3 expression reduces cell migration and invasiveness in U2OS cells. Numbers of cells migrating through the uncoated 8-micron membrane pores (A) and through the Matrigel-coated membranes (B) were counted in five randomly chosen fields at a magnification of ×100. (C) A cell invasion index was calculated as the ratio of the number of cells migrating through the matrigel to the number migrating through the uncoated membrane.
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which may be responsible for the reduction in promotor activity, based on the results of co-transfection experiments (Fig. S2). mRNA expression levels of *ELK1* and *ELK4* showed no significant differences among sarcoma cell lines irrespective of the AFAP1L1 expression level (Fig. S7C).

Sp3 is essential to the expression of AFAP1L1

Finally, siRNA was employed to investigate the role of Sp3 in AFAP1L1 transcription *in vivo*. In U2OS cells, siRNA targeting each of Sp1, Sp3, and Sp4 significantly reduced the expression of the targeted gene, but only the siRNA targeting Sp3 consistently reduced the expression of the *AFAP1L1* gene (Fig. 6A), which was confirmed by quantitative analyses (Fig. 6B). Specific reduction of *AFAP1L1* expression by siRNA against Sp3 was further confirmed at the protein level (Fig. 6C). These effects of siRNA against Sp3 were also confirmed in other cell lines (MG63 and SYO-1) at the mRNA level (Fig. S8A–D). This phenomenon was also observed in prostate cancer PC-3 cells (Fig. S9), indicating that the transcriptional role of Sp3 for the *AFAP1L1* gene is not restricted to sarcoma cells. To exclude the off-target effect of siRNA, a rescue experiment was carried out. Pre-induction of siRNA-resistant Sp3 using a lentivirus partially rescued *AFAP1L1* expression after Sp3 siRNA treatment (Fig. 7A–B), indicating that the reduction in AFAP1L1 expression cause by siRNA for Sp3 is due to a direct effect on the Sp3 gene.

Functional relevance of Sp3 to AFAP1L1

We have shown that the induction of AFAP1L1 expression increased cell motility and invasiveness in sarcoma cells [1]. Inhibition of Sp3 expression with siRNA also reduced the motility and invasiveness of U2OS cells, suggesting a functional link between Sp3 and AFAP1L1 (Fig. 8).

Discussion

In the present study, we have found that Sp3 plays a critical role in the transcription of *AFAP1L1*, a gene associated with the metastasis of soft tissue spindle cell sarcomas [1]. Based on structural similarity, AFAP1, AFAP1L1 and AFAP1L2 belong to a family of new adaptor proteins. They all contain two pleckstrin homology domains flanking a serine/threonine-rich region, two Src homology (SH) 2-binding motifs and one or two SH3-binding motifs [1] [12] [13]. AFAP1, also known as AFAP-110, the most intensively investigated member of the family, is reported to have an intrinsic ability to alter actin filament integrity and may function as an adaptor protein by linking the Src family and/or other signaling proteins to actin filaments [13]. AFAP1L2, also termed XB130, has been cloned as an adaptor protein and Src kinase substrate and phosphorylated by RET/PTC, a genetically rearranged, constitutively active, thyroid- specific tyrosine kinase [14]. In contrast to AFAP1 and AFAP1L2, little is known about AFAP1L1. A recent study revealed that AFAP1L1 interacted with the SH3 domain of cortactin, an F-actin-binding protein [15] Although we had previously reported that AFAP1L1 was associated with the progression of sarcomas, how it functions in the invasiveness of tumor cells remains ill defined.

Sp3 is a member of the Sp/Kruppel-like factor (KLF) family. The Sp/KLF family recognizes GC/GT boxes and interacts with DNA through three zinc finger motifs [16]. Eight members of the Sp family, Sp1-8, have been reported. Sp1 was the first transcription factor identified and cloned among Sp family members [17] and has been intensively investigated. Since the DNA-binding domains of Sp1 and Sp3 share 90% homology in DNA sequence, they bind to the same DNA-binding site with similar affinity [16]. In spite of extensive studies on the Sp proteins, the difference in binding properties between Sp1 and Sp3 remains largely unknown. Notably, one study shows that promoters containing multiple adjacent Sp-binding sites form significantly more stable Sp3-DNA complexes than those with single Sp-binding sites, and as a consequence, Sp3 efficiently displaces Sp1 from preformed Sp1-DNA complexes from such sites [18]. Therefore, in *AFAP1L1*'s promoter region, the Sp3-SBS1 complexes might be more more stable than the Sp1-SBS1 complexes, because SBS1 contains two overlapping consensus Sp-binding sequences. The Sp3 protein has four isoforms; two long isoforms and two short isoforms [5]. All of them are derived from alternative translational start sites. The two long isoforms can act as transcriptional activators in certain settings, but the significance of the two small isoforms as transcriptional activators or inhibitors remains to be elucidated [5]. While investigating the

role of Sp3 and Ets in the *AFAP1L1* promoter's activity, we found that forced expression of ELK1, an Ets transcription factor, induced up-regulation of the two short isoforms of Sp3 and resulted in decreased *AFAP1L1* promoter activity (Fig. S7B). As forced expression of a short isoform (si-1) reduced the *AFAP1L1* promoter activity induced by endogenous factors (Fig. S2), si-1 may have a negative effect on the transcription of *AFAP1L1*.

Sp1 and Sp3 have been shown to be expressed ubiquitously and reported to regulate basal and constitutive expression of genes both in normal and cancerous tissues [19]. Several reports have referred to a correlation between Sp1 and Sp3 and tumor development, growth and metastasis. Sp1 is reported to be overexpressed and regulate vascular endothelial growth factor (VEGF) in gastric and shown to be linked to a poor prognosis [20]. Up-regulation of Sp1 expression has been also observed in thyroid [21] and colorectal cancer [22]. Sp3 enhances the growth of pancreatic cancer cells by suppressing p27 expression through interaction with GC-rich promoter elements [23]. In breast cancer, Sp3 accelerates tumor cell growth by acting as a repressor of TGF signaling [24]. A recent report demonstrated the expression of Sp3 to be an independent prognostic factor for the poor survival of head and neck cancer patients [25]. Of note, in the web database ONCOMINE (http://www.oncomine.org), upregulation of Sp3 expression in soft tissue sarcomas compared to normal connective tissue has been confirmed [26] [27].

Because the cause of sarcoma patients' death is uncurable distant metastasis in most cases, methods of both predicting and treating metastasis are urgently needed. Our findings may provide new insight regarding this clinical difficulty. Considering that Sp3 is expressed at higher levels in soft tissue sarcomas and transactivates the *AFAP1L1* gene, targeting Sp3 could be a powerful approach to treating advanced soft tissue sarcomas.

Supporting Information

Figure S1 Expression of exogenous Sp1 or Sp3 protein in 293T cells. 293T cells were transfected with each plasmid, as described in Materials and Methods, and the expression of the Sp1 or Sp3 protein was analyzed 24 h later. pRC/Sp3 lacks N-terminal part of the *Sp3* gene as described in *Experimental Procedures*. β-tubulin was used as an internal control. Single and double asterisks indicate the long and short forms of the Sp3 protein, respectively.
(TIF)

Figure S2 Isoform-dependent activity of Sp3 on *AFAP1L1* promoter. The luciferase reporter assay was performed as described in Fig. 3B. Reporter plasmids were co-transfected with either an empty, Sp1 or Sp3 expression vector. Error bars indicate the standard deviations.
(TIF)

Figure S3 Binding of Sp transcription factors to the wild-type, but not mutated Sp-binding site *in vitro*. Nuclear extracts were prepared from U2OS cells and used for EMSA with radiolabeled SBS1WT (*lane a–d*) or SBS1MUT (*lanes e–h*). A supershifted assay was performed with anti-Sp1 (*lane c* and *g*) or anti-Sp3 (*lane d* and *h*) antibody. Open and closed arrowheads indicate an Sp3-OND and Sp1-OND complex.
(TIF)

Figure S4 EMSA using nuclear extracts from cells expressing the *AFAP1L1* gene very weakly (SYO-1) and strongly (MG63). Nuclear extracts were prepared from SYO-1 and MG63 cells, and EMSA was performed as described in Figure 4. Open and closed arrowheads indicate Sp3-OND and Sp1-OND complex, respectively. Single and double asterisks indicate bands supershifted by the addition of Sp1 or Sp3 antibody, respectively.
(TIF)

Figure S5 The effect of mithramycin in MG63 cells. RNA was extracted from MG63 cells treated with mithramycin A at the indicated dose or DMSO for 48 h, and subjected to RT-PCR. The *β-actin* gene was used as a control.
(TIF)

Figure S6 Western blot analyses of AFAP1L1, Sp1 and Sp3 in sarcoma cell lines. Total cell lysate (A) or nuclear extract (B) was prepared from each cell line and used for Western blotting. β-tubulin and acetylated H3K9 were used as the internal control for total cell lysate and nuclear extract, respectively. Single and double asterisks indicate the long and short forms of the Sp3 protein, respectively.
(TIF)

Figure S7 The effect of Ets transcription factors on the expression of *AFAP1L1*. (A) The effect of Ets transcription factors on luciferase activity. Luciferase assays were performed in U2OS cells 48 h after the co-transfection of various expression vectors containing an Ets transcription factor with PGV-(−224). (B) The effect of ELK1 on the expression of Sp3. 293T cells were transfected with indicated plasmids and proteins were analyzed at 24 h by Western blotting. β-tubulin was used as an internal control. Single and double asterisks indicate the long and short forms of Sp3, respectively. DN-Ets represents dominant negative Ets. (C) Expression of ELK family gene in sarcoma cells. RNA was extracted from cells and RT-PCR was performed.
(TIF)

Figure S8 Down-regulation of *AFAP1L1* expression by siRNA targeting the *Sp3* gene in SYO-1 and MG63 cells. (A) and (D) The specificity of siRNA. SYO-1 (A) and MG63 (D) cells were treated with siRNA targeting *Sp1*, *Sp3*, or *Sp4* for 48 h, and the expression of these genes as well as the *AFAP1L1* gene was analyzed by PCR. Two different siRNAs targeting the *Sp1* and *Sp3* genes were designed and used. *β-actin* was used as a control. (B) and (E) Down-regulation of *AFAP1L1* expression by siRNA targeting the *Sp3* gene at the mRNA level. SYO-1 (B) and MG63 (E) cells were treated with siRNAs targeting each gene for 48 h and the expression of *AFAP1L1* was analyzed by qPCR and indicated as fold changes relative to that in untreated cells. (C) and (F) Down-regulation of AFAP1L1 expression by siRNA targeting the *Sp3* gene at the protein level. SYO-1 (C) and MG63 (F) cells were treated with siRNAs targeting each gene for 72 h and proteins were extracted and used for Western blotting. β-actin was used as a control.
(TIF)

Figure S9 Down-regulation of Sp3 expression causes down-regulation of AFAP1L1 expression in prostate cancer cells. (A) The specificity of siRNA. PC-3 cells were treated with siRNA targeting *Sp1*, *Sp3*, or *Sp4* for 48 h, and the expression of these genes as well as the *AFAP1L1* gene was analyzed by PCR. Two different siRNAs targeting the *Sp1* or *Sp3* gene were designed and used. *β-actin* was used as a control. (B) Down-regulation of AFAP1L1 expression by siRNA targeting the *Sp3* gene at the protein level. PC-3 cells were treated with siRNA targeting each gene for 72 h and proteins were extracted and used for Western blotting. β-tubulin was used as a control. Single and double asterisks indicate the long and short forms of Sp3, respectively.
(TIF)

Table S1 Sequences for primers and other oligonucleotides used in this study.
(XLS)

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Author Contributions

Conceived and designed the experiments: YK HN EN OO JT. Performed the experiments: YK MF ST TK RT SN TA. Analyzed the data: YK JT. Contributed reagents/materials/analysis tools: TK YN JT. Wrote the paper: YK JT.

Genes to Cells

Aberrant DNA methylation status of DNA repair genes in breast cancer treated with neoadjuvant chemotherapy

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Dysregulation of homologous recombination (HR) DNA repair has been implicated in breast carcinogenesis and chemosensitivity. Here, we investigated the methylation status of sixteen HR genes and analyzed their association with tumor subtypes and responses to neoadjuvant chemotherapy. Core specimens were obtained before neoadjuvant chemotherapy from sixty cases of primary breast cancer of the following four subgroups: luminal breast cancer (LBC) with pathological complete response (pCR), LBC with stable disease, triple-negative breast cancer (TNBC) with pCR and TNBC with poor response. The aberrant DNA methylation status of the following HR related-genes was analyzed using bisulfite-pyrosequencing: BRCA1, BRCA2, BARD1, MDC1, RNF8, RNF168, UBC13, ABRA1, PALB2, RAD50, RAD51, RAD51C, MRE11, NBS1, CtIP and ATM. Among the genes analyzed, only the incidence of BRCA1 and RNF8 methylation was significantly higher in TNBC than that in LBC. Whereas the incidence of BRCA1 methylation was tended to be higher in pCR cases than in poor-response cases in TNBC, that of RNF8 was significantly lower in pCR cases than in poor-response cases. Our results indicate that the methylation status of HR genes was not generally associated with TNBC subtype or chemosensitivity although hypermethylation of BRCA1 is associated with TNBC subtype and may impact chemosensitivity.

Introduction

Neoplastic transformation of a subset of breast cancers is attributed to dysregulation of DNA repair. In addition to the well-known familial breast cancer genes BRCA1 and BRCA2, hereditary defects in genes required for homologous recombination (HR) DNA repair, such as PALB2/FANCN (Rahman *et al.* 2007), BACH1/BRIP1/FANCJ (Cantor *et al.* 2001; Litman *et al.* 2005), ATM (Renwick *et al.* 2006), NBS1 (Steffen *et al.* 2004), RAD50 (Heikkinen *et al.* 2006) and RAD51C (Meindl *et al.* 2010), have also been implicated in breast cancer susceptibility. The deficiency of HR is important not only for breast cancer susceptibility, but also as a possible target for chemotherapies exploiting synthetic lethality. Inhibitors of poly (ADP-ribose) polymerase and platinum-based agents exhibit significant clinical activity in breast and ovarian cancers caused by BRCA1 or BRCA2 germline mutations in which HR is abrogated (Byrski *et al.* 2009; Audeh *et al.* 2010; Tutt *et al.* 2010). The HR deficiency may also sensitize cells to other DNA damage-inducing agents. For example, basal-like breast cancer, a subtype associated with BRCA1 dysfunction, generally responds to chemotherapy. Sporadic breast cancer with low BRCA1 mRNA expression is reported to be sensitive to anthracycline-based chemotherapy (Margeli *et al.* 2010). *In vitro*, functional deficiency of the genes required

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for the HR pathway sensitizes cells to DNA damage-inducing agents. Thus, it has been suggested that an assessment of HR competence could be valuable in predicting the sensitivity of cancer cells to chemotherapy or specific DNA-damaging agents (Livingston & Silver 2008). However, the contribution of the HR failure caused by genes other than BRCA1 and BRCA2 to the chemosensitivity has not been clarified.

The aberrant DNA methylation status of HR genes in breast cancer tissues has previously been analyzed for BRCA1, BRCA2, PALB2 and ATM. In particular, BRCA1 has been intensively studied (Dobrovic & Simpfendorfer 1997; Magdinier *et al.* 1998; Catteau *et al.* 1999; Esteller *et al.* 2000, 2001; Niwa *et al.* 2000; Rice *et al.* 2000; Alvarez *et al.* 2005; Matros *et al.* 2005; Wei *et al.* 2005, 2008; Turner *et al.* 2007; Chen *et al.* 2009; Xu *et al.* 2009; Branham *et al.* 2012; Cerne *et al.* 2012; Hsu *et al.* 2013; Xu *et al.* 2013). However, in most previous reports, the methylation status was analyzed with methylation-specific restriction enzyme-based assays (Dobrovic & Simpfendorfer 1997; Magdinier *et al.* 1998; Catteau *et al.* 1999; Niwa *et al.* 2000) or methylation-specific PCR (MSP) (Esteller *et al.* 2000, 2001; Rice *et al.* 2000; Alvarez *et al.* 2005; Matros *et al.* 2005; Wei *et al.* 2005, 2008; Turner *et al.* 2007; Chen *et al.* 2009; Xu *et al.* 2009; Hsu *et al.* 2013). The incidence of BRCA1 methylation in breast cancer in these studies varies, and it remains controversial whether aberrant DNA methylation of BRCA1 is preferentially present in basal-like or triple-negative breast cancer (TNBC). DNA methylation analysis can be a useful diagnostic biomarker, but it is important to consider detection methods. Pyrosequencing provides quantitative screening and allows one to set cutoff points, which makes accurate comparisons possible. Recently, BRCA1 methylation in ER α -positive breast cancer analyzed by bisulfite-pyrosequencing was reported (Cerne *et al.* 2012). However, subtype-specificity for the methylation status was not addressed. Moreover, whether tumor response to chemotherapy was associated with the methylation status of HR genes has not been previously addressed.

In this study, we investigated the methylation status of HR genes, including BRCA1 and BRCA2, in primary sporadic breast cancer patients who underwent neoadjuvant anthracycline-based chemotherapy followed by docetaxel by bisulfite-pyrosequencing. We included 15 cases each with pathological complete response (pCR) both in the luminal HER2-negative (LBC) group and in the TNBC group and compared the methylation status of HR genes with that in poor-responding cases. We demonstrate here that the incidence and quantity of methylation in the CpG island of sixteen HR genes in primary breast cancer and investigate their correlation with subtype and chemosensitivity.

Results

Methylation of selected HR markers in breast, colorectal and gastric cancer cell lines

We determined the levels of methylation of all genes in 5 breast cancer cell lines, 8 colorectal cancer cell lines and 11 gastric cancer cell lines and compared them with mixed peripheral blood lymphocytes (PBL) DNA obtained from two healthy male individuals (Table S1 in Supporting Information). Methylation was determined by bisulfite-pyrosequencing, which yields % methylated alleles in the studied DNA (examples in Fig. S1, Supporting Information). The criterion for the assessment of the methylation level of the genes was no methylation, 1–10%; moderate methylation, 11–40%; high methylation, 41–70%; and severe methylation, 71–100%. We found that HERC2, an imprinted gene, is moderately methylated in all the cells, including PBL. BRCC36 is an X-linked gene and is moderately or highly methylated in 6 cell lines. With the exception of the imprinted genes, the incidence of hypermethylation of HR genes was generally low in the cell lines (Table S1 in Supporting Information).

Aberrant DNA methylation status of HR genes in LBC and TNBC

Following sorting due to several limited conditions (Fig. S2 in Supporting Information), we selected 16 HR genes (BRCA1, BRCA2, BARD1, MDC1, RNF8, RNF168, UBC13, ABRA1, PALB2, RAD50, RAD51, RAD51C, MRE11, NBS1, CtIP and ATM) to be examined in breast cancer tissue samples. Clinicopathological characteristics of the patients are shown in Table 1. Of the 960 total PCR reactions, 787 (82.0%) reactions were successful (Table S2 in Supporting Information). The other reactions failed despite multiple re-examinations because of insufficient quantity or quality of the DNA samples from the limited amount of core needle biopsy specimens. We first compared the methylation level of the genes in LBC with that

Table 1 Clinicopathological characteristics

	LBC-pCR (n = 15)	LBC-SD (n = 15)	TNBC-pCR (n = 15)	TNBC-PR (n = 15)
Age, median (range)	46.7 (33–63)	51.2 (39–61)	50.1 (27–67)	55.3 (24–76)
Tumor stage				
T1	0	0	6	0
T2	11	10	5	11
T3	4	3	1	2
T4	0	2	3	2
Nodal involvement				
Negative	6	4	2	5
Positive	9	11	13	10
Distant metastasis				
Negative	15	15	15	15
Positive	0	0	0	0
Histological type				
Invasive ductal	15	15	14	13
Metaplastic	0	0	1	1
Apocrine	0	0	0	1
Estrogen receptor status				
Negative	0	0	15	15
Positive	15	15	0	0
Progesteron receptor status				
Negative	4	0	15	15
Positive	11	15	0	0
HER2 status				
Negative	15	15	15	15
Positive	0	0	0	0
Neoadjuvant Chemotherapy				
FEC	3	0	8	3
EC	12	15	7	10
DOC	15	15	15	12
Others	0	0	0	2*
Tumor response				
PD	0	0	0	1
SD	0	15	0	3
PR	0	0	0	11
pCR	15	0	15	0

*One case with six 21-day cycles of TC (docetaxel 75 mg/m^2 and cyclophosphamide 600 mg/m^2) and one case with six 28-day cycles of CMF (cyclophosphamide 200 mg/m^2, methotrexate 40 mg/m^2 and 5-FU 500 mg/m^2 on day 1 and 8).

in TNBC (Fig. 1). Of the 16 genes tested, hypermethylation of BRCA1 and RNF8 was statistically associated with TNBC. Whereas BRCA1 was hypermethylated (14–94%) in 7 of 30 cases of TNBC with a mean methylation level of 14.8 ± 25.4%, it was only moderately methylated (11%) in one of 29 cases of LBC with a mean methylation level of 4.3 ± 1.9% (Fig. 1 and Table S2 in Supporting Information). Although there no cases with high or severe methylation of RNF8 were observed, the incidence of moderate methylation of RNF8 was significantly higher (4/21 cases, 19.0%) in TNBC than that in LBC (0/24 cases, 0%). The mean methylation levels for RNF8 in TNBC and LBC were 6.4 ± 7.1% and 3.0 ± 2.7%, respectively.

The mean methylation level of the majority of HR genes (BRCA2, MDC1, RNF168, PALB2, RAD50, RAD51C, MRE11, CtIP and ATM) tended to be higher in TNBC than in LBC (Fig. 1 and Table S2 in Supporting Information), although there were no significant differences. We also examined the Ki-67 index as an indicator of cell proliferation. The Ki-67

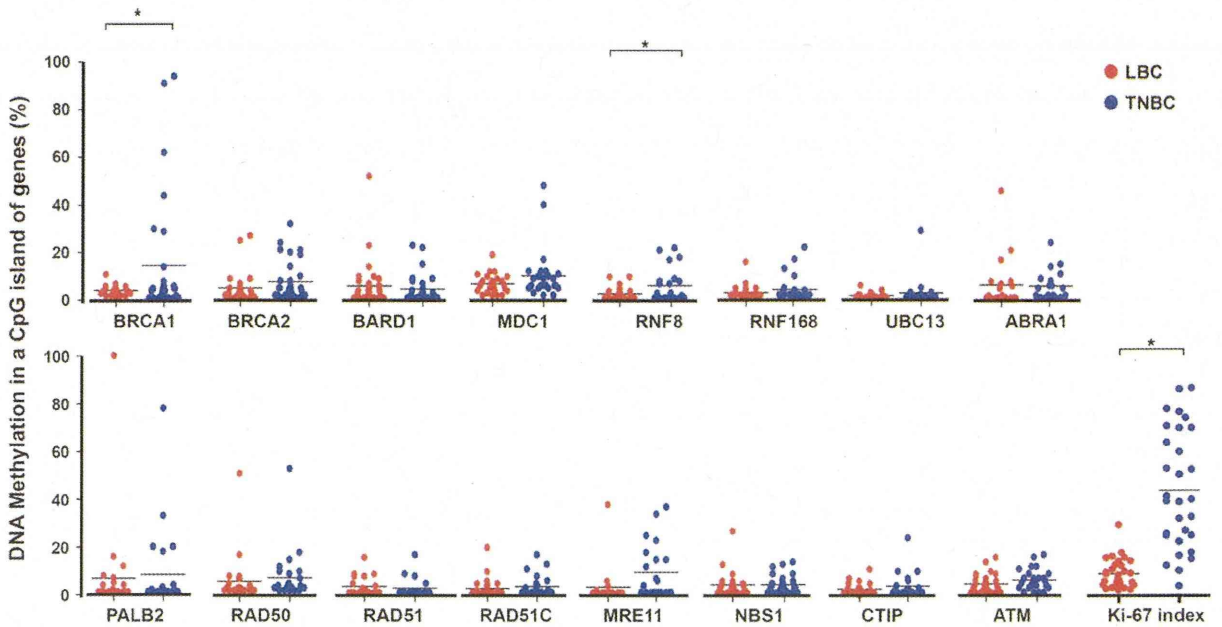

Figure 1 Aberrant DNA methylation status of homologous recombination (HR) genes in luminal breast cancer (LBC) and triple-negative breast cancer (TNBC). Red circles and blue circles indicate percent methylation of HR genes in each case of LBC and TNBC, respectively. The Ki-67 index, as determined by immunohistochemical analysis, is also indicated. Mean percentages are shown as horizontal bars. (*), statistical significance according to Student's *t*-test, BRCA1: $P = 0.03$, RNF8: $P = 0.04$, Ki-67: $P < 0.0001$.

index was significantly higher in the TNBC cases than in LBC (43.6 ± 24.6% vs. 9.0 ± 6.1%, $P < 0.001$) (Fig. 1 and Table S2 in Supporting Information).

Aberrant DNA methylation status of HR genes in pCR cases and poor-responder cases

We next analyzed the association of the methylation level with tumor response to neoadjuvant chemotherapy in TNBC and LBC (Fig. 2). BRCA1 methylation levels tended to be higher in pCR cases than in poor-responder patients in TNBC (Fig. 2B). This tendency was not observed in LBC (Fig. 2A). One metaplastic breast cancer case with severe BRCA1 methylation (94%) in TNBC achieved pCR (Table S2 in Supporting Information). PALB2 methylation levels also tended to be higher in pCR cases in TNBC (Fig. 2B) but not in LBC, with the exception of one case with 100% methylation (Fig. 2A). There were no genes that exhibited higher methylation levels in pCR cases than in SD or poor-response cases with statistical significance. Instead, RNF8 and ATM exhibited lower methylation levels in pCR cases than in poor-response cases (RNF8: 2.7 ± 2.8% vs. 13.7 ± 7.7%, ATM: 5.3 ± 5.0% vs. 8.8 ± 3.8%) in TNBC with statistical significance (RNF8: $P < 0.001$ and ATM: $P = 0.04$, respectively) (Fig. 2B, Table S2 in Supporting Information). The Ki-67 index tended to be higher in SD cases than in pCR cases in both LBC (10.4 ± 6.7% vs. 7.7 ± 5.3%, $P = 0.870$) and TNBC (50.6 ± 23.4% vs. 36.6 ± 24.5%, $P = 0.836$).

Combined hypermethylation of BRCA1, BRCA2, MDC1, ABRA1 and PALB2 with pCR in TNBC

Finally, we examined the individual genes and gene combinations for their association with tumor response by ROC curve analysis. No individual genes exhibited notable AUC values in LBC, in which BRCA2 exhibited the best sensitivity (53.8%), specificity (37.3%) and AUC (0.428) (Fig. 3A). On the other hand, BRCA1 exhibited a relatively high AUC value in TNBC with respect to sensitivity (66.7%), specificity (53.7%) and AUC (0.649) (Fig. 3A). We next analyzed the combinations of the genes in methylation panels that could increase the performance of these markers. Whereas a combination of all 16 genes exhibited a low AUC value (sensitivity: 60.0%, specificity: 40.0%, AUC: 0.489 in LBC, sensitivity:

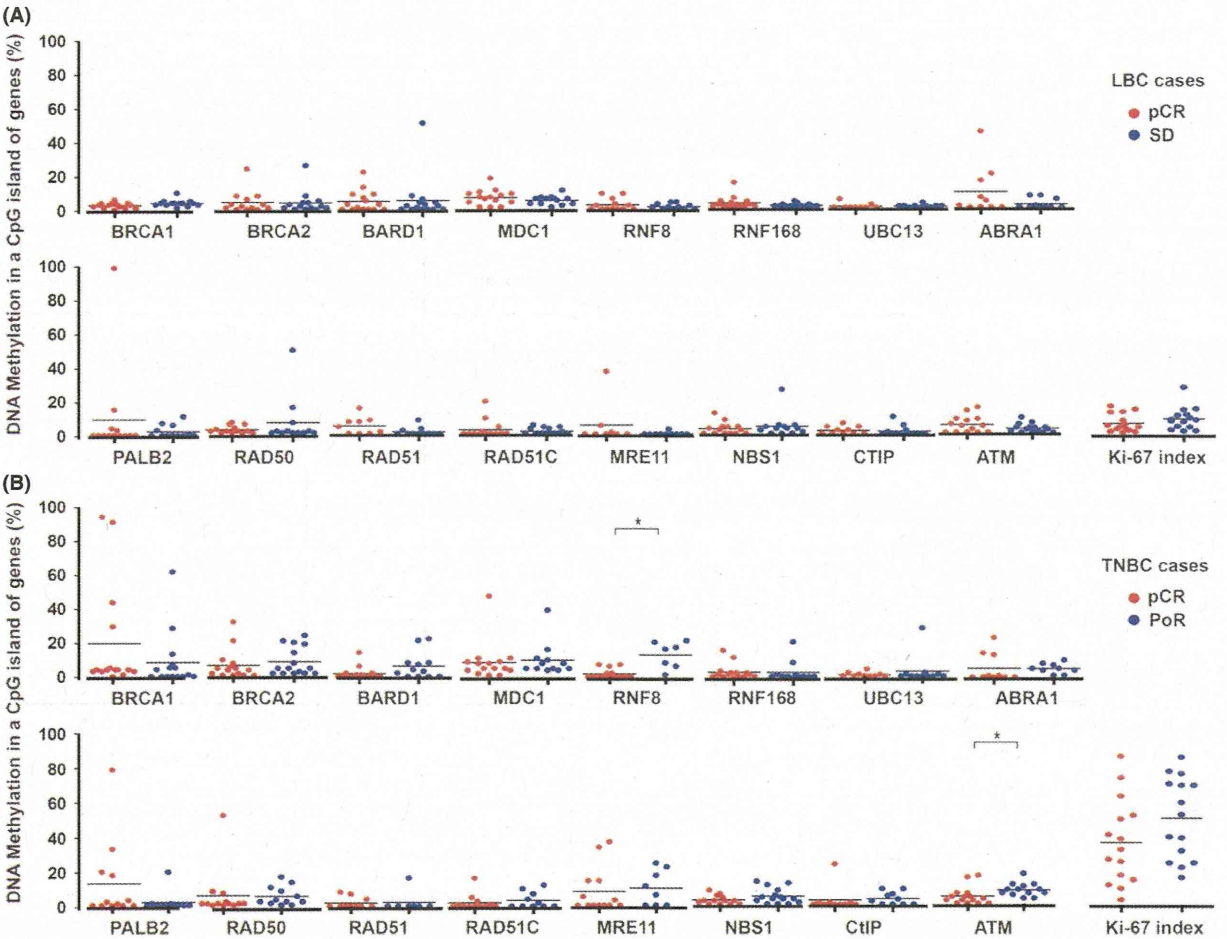

Figure 2 Aberrant DNA methylation status of homologous recombination (HR) genes in cases with pCR and poor response. Red circles and blue circles indicate percent methylation of HR genes in each case of pCR and SD in luminal breast cancer (LBC) (a) or in each case of pCR and poor responder (PoR) in triple-negative breast cancer (TNBC) (b), respectively. The Ki-67 index, as determined by immunohistochemical analysis, is also indicated. Mean percentages are shown as horizontal bars. (*), statistical significance according to Student *t*-test, RNF8: $P < 0.001$, ATM: $P = 0.004$.

73.3%, specificity: 53.3%, AUC: 0.588 in TNBC) (Fig. 3B), a 5-gene combination, BRCA1 + BRCA2 + MDC1 + ABRA1 + PALB2 demonstrated a substantially higher AUC value in TNBC (sensitivity: 73.3%, specificity: 55.6%, AUC: 0.655 in TNBC) (Fig. 3C). The AUC value of the 5-gene combination was rather low in LBC (sensitivity: 60.0%, specificity: 53.3%, AUC: 0.469) (Fig. 3C).

Discussion

In the current study, we investigated the aberrant DNA methylation status of sixteen HR genes in primary sporadic breast cancer cases for their correlation with tumor subtypes and responses to neoadjuvant chemotherapy. Whereas we could detect methylation of only a few of the HR genes in cultured cancer cell lines, we observed substantial methylation in some HR genes in the cancer tissues. There could be some limitation of our study using core needle biopsy specimens as research materials, for example, limited amount of the samples and molecular heterogeneity among different areas within each tumor. However, the core specimen represents tumor characteristics including chemosensitivity in majority of cases in clinical practice.

The correlation between the methylation status of BRCA1 and breast cancer subtype has long been investigated but remains controversial. Using methylation-specific restriction enzyme-based techniques, BRCA1

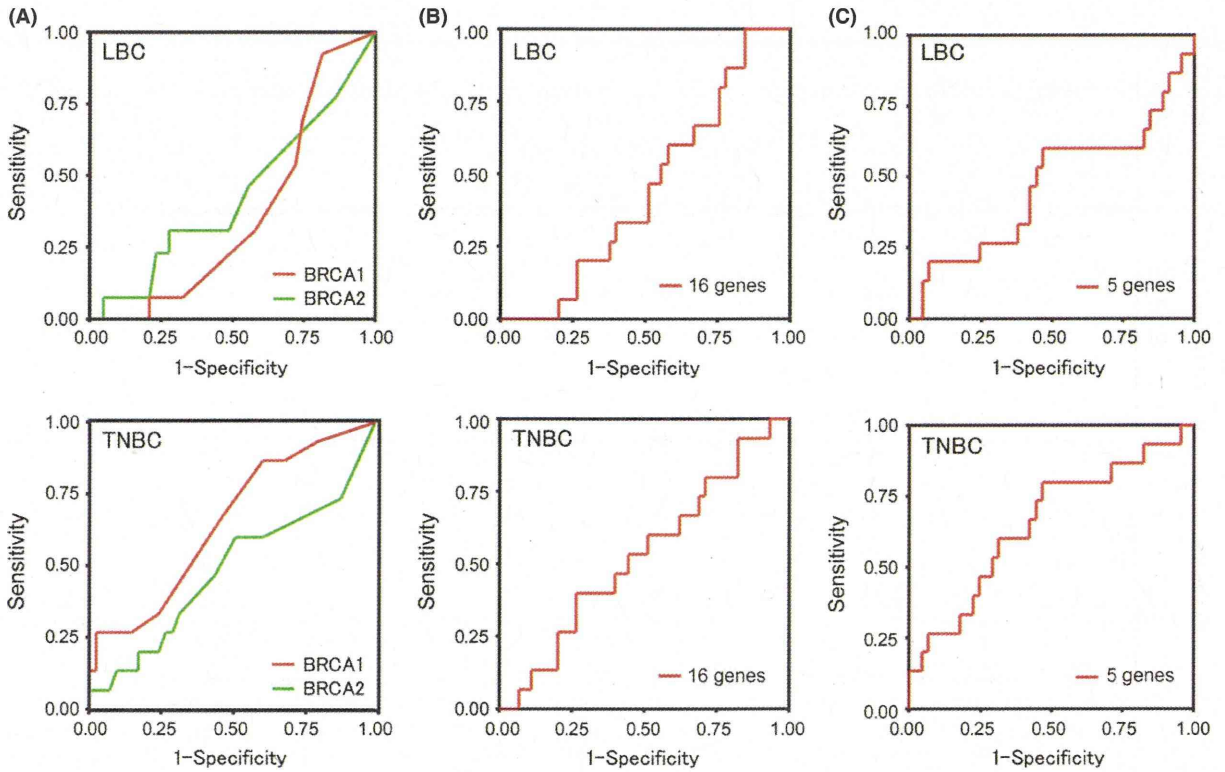

Figure 3 Receiver operating characteristic (ROC) curve of multiple gene panels in luminal breast cancer (LBC) and triple-negative breast cancer (TNBC) samples. The methylation status of BRCA1 and BRCA2 (a), 16 HR gene combination (b) and 5 HR gene (BRCA1 + BRCA2 + MDC1 + ABRA1 + PALB2) combination (c) in their association with tumor response to neoadjuvant chemotherapy in LBC (upper panels) and TNBC (lower panels) samples is shown. *Z*-score s are indicated as red or green polygonal lines.

methylation was reported to be inversely correlated with ERα status (Catteau *et al.* 1999; Niwa *et al.* 2000). Matros *et al.* (Matros *et al.* 2005) investigated the methylation status using MSP and reported that methylation was significantly associated with ERα -positive tumors and was absent in the basal-like subtypes, contradicting the previous reports. However, later studies with MSP were not consistent with the report and demonstrated a relatively higher incidence of BRCA1 methylation in ERα -negative or triple-negative breast cancers (Wei *et al.* 2005, 2008; Chen *et al.* 2009; Hsu *et al.* 2013; Xu *et al.* 2013). The incidence was reported to be remarkably high in special histological types of breast cancer, including medullary, mucinous (Esteller *et al.* 2000) or metaplastic (Turner *et al.* 2007) in TNBC. On the other hand, a recent large cohort of ERα -positive breast cancer examined with pyrosequencing indicated that the BRCA1 methylation level was low with a median value of 2.53% (Cerne *et al.* 2012). In the current study, we also analyzed the methylation status by pyrosequencing for both LBC and TNBC. The results suggest a higher incidence of BRCA1 methylation in TNBC compared with LBC, consistent with the general assumption that BRCA1 deficiency is associated with TNBC and basal-like breast cancer, as is the case with BRCA1 germline mutations or micro-RNA inhibition of BRCA1 (Sorlie *et al.* 2003; Moskwa *et al.* 2011). Furthermore, it is noteworthy that the TNBC-pCR group included 4 cases of highly methylated BRCA1 (30, 44, 91 and 94%) of 15 cases. Interestingly, one case was metaplastic breast cancer, which is generally chemoresistant. It should be noted that the TNBC-pCR group was biased with respect to tumor size; the group contained six T1 cases, whereas the other groups contained no T1 cases (Table 1). This bias is most likely attributable to the fact that small tumors favorably achieve pCR and that TNBC was preferentially treated with neoadjuvant chemotherapy. Interestingly, the T1 cases in TNBC-pCR

included fewer cases with aberrant DNA methylation in HR genes. In contrast, six cases of the remaining 9 cases (66.7%) of TNBC-pCR exhibited remarkable hypermethylation in HR genes, including three BRCA1 cases (44, 91, and 94%), one PALB2 case (78%), one MDC1 case (48%) and one case with moderate methylation of multiple HR genes (MDC1:12%, PALB2:33%, MRE11:15% and ATM:17%) (Table S2 in Supporting Information). Therefore, the contribution of the hypermethylation of HR genes to pCR establishment in TNBC could be underestimated in the current study and may be of greater impact.

For the aberrant DNA methylation status of BRCA2 in breast cancer, the absence of methylation in 18 breast and ovarian cancer cell lines was first reported (Collins *et al.* 1997). This is consistent with our observation that there was no detectable BRCA2 methylation and only a few other methylated HR genes in all of the 24 cancer cell lines analyzed. We speculate that this is presumably due to negative selection of the cells with HR deficiency during cell line establishment. For clinical breast cancer samples, the incidence was reported to range from 15 to 64% using MSP or methyl-specific multiplex-ligation probe amplification assay (MS-MLPA) (Cucer *et al.* 2008; Moelans *et al.* 2011; Branham *et al.* 2012). In our study, 9 of 57 cases (15.8%) exhibited more than 10% methylation in the region analyzed, an incidence consistent with the data reported by Branham *et al.* using MS-MLPA(Branham *et al.* 2012). Seven of the 9 cases were TNBC, although there was no statistically significant difference between LBC and TNBC.

Because the phenotype of BRCA2 germ-line mutation exhibits among all of the subtypes, including LBC and TNBC (Sorlie *et al.* 2003; Jönsson *et al.* 2010), we expected that the aberrant DNA methylation of BRCA2 would also be observed in LBC and that LBC-pCR may exhibit higher incidence of BRCA2 methylation. However, the incidence of methylation was rather low. Interestingly, one case with an extremely high level (100%) of methylation was observed for PALB2 in LBC-pCR group. In this case, PALB2 dysfunction may contribute to the hypersensitivity of the cancer cells to chemotherapy because PALB2 is required for BRCA2 recruitment to damaged DNA sites (Zhang *et al.* 2009). BRCA2 and PALB2 are Fanconi anemia genes FANCD1 and FANCN, respectively, and biallelic loss of these genes causes Fanconi anemia, a genome instability syndrome (Rahman *et al.* 2007), suggesting an indispensable role of PALB2 in DNA damage response.

Germline mutations of a number of HR genes other than BRCA1 and BRCA2 are also implicated in breast cancer susceptibility, indicating that the defect of HR function is associated with breast cancer development. These genes include PALB2, ATM, NBS1, RAD50 and RAD51C. Among them, the aberrant DNA methylation status of PALB2 and ATM in breast cancer tissue has previously been investigated using MSP (Vo *et al.* 2004; Treilleux *et al.* 2007; Potapova *et al.* 2008; Moelans *et al.* 2011). Aberrant DNA methylation of PALB2 was detected in 6 of 68 (8.8%) cases of inherited and sporadic primary breast cancers, including 2 cases of ERα -positive and 4 cases of ERα -negative cancer (Potapova *et al.* 2008). In our study, 8 of 48 (16.7%) cases exhibited more than 10% methylation in the CpG island region analyzed. The discrepancy could be ascribed not only to the different technique used to analyze the methylation, but also to the bias that the current study includes approximately half pCR cases, which exhibit an increased incidence of PALB2 hypermethylation (6/25 cases: 24%). For ATM, the absence (Treilleux *et al.* 2007) or high incidence (18/23 cases: 78%) (Vo *et al.* 2004) of aberrant DNA methylation detected by MSP using the same primers has been reported. Another study with MS-MLPA demonstrated a 12% incidence of hypermethylation in invasive breast cancer (Moelans *et al.* 2011). Our results demonstrated that although the level of methylation was modest (18% at highest), 8 out of 57 cases (14.0%) exhibited methylation in more than 10% of the CpG island region examined.

The aberrant DNA methylation status in breast cancer tissues of other genes, including BARD1, MDC1, RNF8, RNF168, UBC13, ABRA1, RAD 50, RAD51, RAD51C, MRE11, NBS1 and CtIP, has not been previously investigated. Our data indicate that there are some incidences of methylation for these genes. Among them, the incidence of methylation of RNF8 was significantly higher in TNBC than that in LBC. RNF8, an E3 ubiquitin ligase, is one of the upstream proteins in both HR and nonhomologous end-joining pathways and is required for retention of BRCA1-ABRA1-RAP80 complex and 53BP1 at sites of DNA damage (Ohta *et al.* 2011). Down regulation of the two major pathways to repair DNA double-strand breaks caused by RNF8 methylation could be involved in cancer development of TNBC. Although the level of methylation was relatively low, the highest incidence (11/45 cases: 24%)