Targeted next-generation sequencing assays using triplet samples of normal breast tissue, primary breast cancer, and recurrent/metastatic lesions

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Abstract

Background: Next-generation sequencing (NGS) has shown that recurrent/metastatic breast cancer lesions may have additional genetic changes compared with the primary tumor. These additional changes may be related to tumor progression and/or drug resistance. However, breast cancer-targeted NGS is not still widely used in clinical practice to compare the genomic profiles of primary breast cancer and recurrent/metastatic lesions.

Methods: Triplet samples of genomic DNA were extracted from each patient’s normal breast tissue, primary breast cancer, and recurrent/metastatic lesion(s). A DNA library was constructed using the QIAseq Human Breast Cancer Panel (93 genes, Qiagen) and then sequenced using MiSeq (Illumina). The Qiagen web portal was utilized for data analysis.

Results: Successful results for three or four samples (normal breast tissue, primary tumor, and at least one metastatic/recurrent lesion) were obtained for 11 of 35 breast cancer patients with recurrence/metastases (36 samples). We detected shared somatic mutations in all but one patient, who had a germline mutation in TP53. Additional mutations that were detected in recurrent/metastatic lesions compared with primary tumor were in genes including TP53 (three patients) and one case each of ATR, BLM, CBFB, EP300, ERBB2, MUC16, PBRM1, and PIK3CA. Actionable mutations and/or copy number variations (CNVs) were detected in 73% (8/11) of recurrent/metastatic breast cancer lesions.

Conclusions: The QIAseq Human Breast Cancer Panel assay showed that recurrent/metastatic breast cancers sometimes acquired additional mutations and CNV. Such additional genomic changes could provide therapeutic target.

Background

Studies using next-generation sequencing (NGS) have demonstrated remodeling of genes in metastatic cancer compared with the primary tumor from the same individual, possibly as a result of subclonal diversification or mutational evolution [1-10]. Whole-exon or whole-genome analyses can yield valuable information but are costly and time consuming. Whole-exon sequencing analysis of metastatic breast cancers showed drug-targetable mutations in genes such as ERBB4, NOTCH3, and ALK [11]. A targeted NGS assay of metastatic breast cancers and their associated primary tumor using a general cancer panel can also detect drug-targetable mutations. Vasan et al. reported that 84% (43/51) of metastatic breast cancers showed at least one genomic alteration that could be targeted by currently available drugs [12]. In 2016, Muller et al. reported that 45% (10/22) of metastatic breast cancers contained molecular targets for currently available therapies, including for off-label use [13]. In these studies, comprehensive cancer panels that were not breast cancer specific were used. To our knowledge, there has been no study using breast cancer-targeted NGS of paired samples of primary breast cancer and recurrent/metastatic cancer.
The aim of the present study was to perform breast cancer-targeted NGS to compare gene mutations and copy number variations (CNVs) in samples of primary breast cancer and recurrent/metastatic lesions from the same individuals. Because sequencing analyses of matched tumor and normal tissue are essential [14], we performed an NGS study using three or more samples from each patient, consisting of normal breast tissue, primary breast cancer, and recurrent/metastatic lesion(s). This study aimed to clarify the practical possibility of breast cancer-targeted NGS and improve understanding of subclonal diversification or mutational evolution of metastatic breast cancers.

Methods

Patients and samples

One hundred seven cases of distant metastasis or local recurrence of breast cancer were collected from the pathology archives of Kawasaki Medical School Hospital from 2010 to 2017. The microscopic evaluation of these cases found that normal breast tissue, sufficient (20% or more tumor content) primary breast cancer tissue, and tissue from at least one relapse site were available for 35 patients. For 66 patients, the tumor content of samples from primary tumors and/or recurrent/metastatic tumors was less than 20%. For six patients, the primary breast cancers were resected in other institutions, so histologic specimens were not available (Supplementary Fig. 1). The protocol of the present study was approved by the Ethics Committee of Kawasaki Medical School and Hospital (approval number: 2695).

DNA extraction and quality assessment

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were obtained from the Department of Pathology at Kawasaki Medical School Hospital. DNA was extracted from the tumor and normal tissue at the primary site (breast) and from sites of metastasis and/or local recurrence. Samples of pleural or pericardial effusion were collected using the collodion bag method to generate cell blocks. Four 10-mm sections were cut from each paraffin block. Maxwell 16 FFPE Tissue LEV DNA purification kits (#AS1130; Promega, Madison, WI, USA) were employed for DNA extraction. DNA was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit dsDNA BR assay kits (#Q32850; Thermo Fisher Scientific). DNA quality was assessed by calculating the QC score (https://www.qiagen.com/us/resources/download.aspx?id=aae35658-5ef2-44b2-bd02-fbe73fe7737c&lang=en). DNA amplification for library construction was performed by quantitative polymerase chain reaction (qPCR) using QIAseq DNA QuantiMIZE Assay Kits (#DNQC-100Y-R; Qiagen, Hilden, Germany) [15].

Next-generation sequencing

The QIAseq Human Breast Cancer Panel (93 genes, DHS-001Z; Qiagen) and the GeneRead Human Comprehensive Cancer Panel (160 genes, NGHS-501X; Qiagen) were used for library construction according to the manufacturer’s instructions. The libraries were assessed using a QIAseq Library Quant Assay Kit (#QSTF-ILZ-R; Qiagen) and applied to a MiSeq sequencer (Illumina, San Diego, CA, USA).
Qiagen web portal (https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/) was utilized for data analysis [16]. For alignment, GenomeBrowse (http://goldenhelix.com/products/GenomeBrowse/index.html) was used, and GRCH37 was used as the human genome reference. A commercial bioinformatic analysis service (Mitsubishi Space Software Co. Ltd, Tokyo, Japan) was asked to interpret the GeneRead Human Comprehensive Cancer Panel results. CNV was calculated using the cloud analysis pipeline of the Qiagen web portal, and corrected for the percent tumor content. Four or more and one or fewer CNVs were regarded as significant.

**Statistical analyses**

Statistical analyses were performed using IBM SPSS Statistics for Windows (v 25; IBM Corp., Armonk, NY, USA) and $P < 0.05$ was considered significant.

**Results**

**Clinicopathological findings**

For the 11 of the total 107 patients who were successfully analyzed (Supplementary Fig. 1), the median age was 52.5 years at diagnosis of breast cancer. The median time to first relapse was 11 months, and the overall survival was 39 months. All but one patient died from breast cancer. The breast cancer subtypes were six triple-negative, four luminal, and one HER2-enhanced. All patients but one had received chemotherapy and/or hormonal therapy (Table 1, Supplementary Table 1).

**DNA quality and QIAseq Human Breast Cancer Panel**

The data for DNA quality are shown in Supplementary Table 2. The QC score was calculated as the difference between the CT values obtained by qPCR using the 100-bp and 200-bp primers of the QIAseq DNA QuantiMIZE assay kit. A high QC score indicates severe DNA fragmentation. A favorable QC score ($\leq 0.04$) was obtained from 43 of 85 (50.6%) samples. The samples with good DNA quality were collected significantly more recently than those with poor DNA quality ($p < 0.0005$, Fig. 1). The FFPE blocks used in this study were 6 months to 17 years old; 66.7% (38/57) of the blocks that were $\geq 5$ years old had a QC score $>0.04$. We attempted to construct libraries from 14 high-QC-score samples; however, the reading depth for the molecular-tagged sites in eight cases (57.2%) was <10, and failed to identify a mutation. The depth of molecular-tagged sites and all examined sites were both significantly correlated with the storage years ($P < 0.0005$ and $r = -0.586$, $P = 0.004$ and $r = -0.412$, Spearman's correlation).

Successful results were obtained from samples of 11 patients that comprised normal breast tissue, primary tumor, and at least one metastatic/recurrent lesion (36 samples, Table 1), using the QIAseq Human Breast Cancer Panel. We detected somatic driver mutations in all but one case (91%) (Table 2). The mutations shared between the primary tumor and a recurrent/metastatic lesion occurred in $TP53$ (five cases), $PIK3CA$ (three cases), $CDH1$ (one case), $ESR1$ (one case), $GATA3$ (one case), and $PTEN$ (one case). In five cases (45.4%), additional mutations were detected in the recurrent/metastatic lesions.
compared with the primary tumor. These additional mutations occurred in TP53 in three cases and in ATR, BLM, CBFB, EP300, ERBB2, MUC16, PBRM1, and PIK3CA in one case each (Table 2, Fig. 2). No additional mutation was found in one long-surviving patient (K23) at 92 months after partial mastectomy.

The results for CNV are shown in Table 2 and Supplementary Table 3. CNV appears to be higher in distant metastasis than in local recurrence, but this difference was not significant ($P = 0.091$, Fig. 3).

**GeneRead Human Comprehensive Cancer Panel**

For patient K31, only two mutations were identified by the QIAseq Human Breast Cancer Panel. We suspected the presence of undetected mutations, so performed additional analyses using the GeneRead Human Comprehensive Cancer Panel and a commercial bioinformatic analysis service (Mitsubishi Space Software Co. Ltd). These analyses revealed an additional JAK2 mutation (p.R588Kfs*8) that occurred before metastasis, an ARID1A mutation (p.E672G) that appeared in the first metastasis (pericardial fluid), and additional mutations in XPC (p.R338T) and GATA3 (p.K378R) that were present in the second metastasis (pleural fluid) (Fig. 4). Although GATA3 is included in the QIAseq Human Breast Cancer Panel, we were unable to detect this mutation by web-portal analysis because of its low variant frequency.

For patient K25, only a germ line TP53 mutation was detected by the QIAseq Human Breast Cancer Panel. The GeneRead Human Comprehensive Cancer Panel analysis confirmed this TP53 germ line mutation without identifying other significant mutations.

**Discussion**

We showed that older FFPE materials often had lower DNA quality and could not be analyzed. The depth discrepancy between molecular-tagged sites and all sites is interesting. The Spearman's correlation coefficients with age were $-0.586$ for tagged sites and $-0.412$ for all sites, suggesting that the molecular tag might be sensitive to age-related DNA damage. PAXgene tissue fixation and/or low-temperature paraffin block storing at 4°C or −20°C could improve DNA quality [41].

For 66 of 107 cases (61.7%), the tumor content in samples of primary tumors and/or recurrent/metastatic tumors was less than 20%. The cell blocks of fluid materials frequently included many inflammatory cells and reactive mesothelial cells together with cancer cells; hence, the extraction of tumor DNA from these cell blocks was often inefficient. A primary breast cancer containing abundant stromal lymphoplasmacytic cell infiltration or a severe desmoplastic reaction would also inhibit effective cancer genome recovery. Thus, the development of a more efficient microdissection system is desirable.

We demonstrated that targeted NGS using the QIAseq Human Breast Cancer Panel could detect the driver mutations in all cases of breast cancer examined, except for one case with a germline TP53 mutation that did not meet the classic criteria for Li–Fraumeni syndrome [17] or the Chompret criteria [18].
The most frequently detected mutation shared between primary and metastatic lesions was in \textit{TP53}. Breast cancer patients with a somatic \textit{TP53} mutation have a poor prognosis [19, 20]; unfortunately, \textit{TP53} mutations are not presently targetable. Phosphatidylinositide 3-kinase (PI3K) inhibitor could be a good therapeutic option for cases with \textit{PIK3CA} mutations [21-23]. Alpelisib has been reported to improve the survival patients with \textit{PIK3CA}-altered, ER-positive, HER2-negative breast cancer [24, 25]. Alpelisib is already approved by the US Food and Drug Administration. The Lotus trial showed that ipatasertib, an oral AKT inhibitor, improved the progression-free survival of breast cancer patients with \textit{PIK3CA}/AKT/PTEN mutations [26]. IPATunity130, a pivotal randomized phase III trial evaluating ipatasertib (IPAT) + paclitaxel for \textit{PIK3CA}/AKT1/PTEN-altered advanced triple-negative or hormone receptor-positive HER2-negative breast cancer, is ongoing (http://ascopubs.org/doi/abs/10.1200/JCO.2018.36.15_suppl.TPS1117). Breast cancers with a \textit{PIK3CA} mutation have a good prognosis [20]. \textit{CDH1}-mutated breast cancer cells are sensitive to ROS1 tyrosine kinase inhibitors including foretinib or crizotinib [27]. \textit{ESR1} mutations of breast cancer are often reported after aromatase inhibitor and/or tamoxifen therapy [28]. The SoFEA (Study of Faslodex Versus Exemestane With or Without Arimidex) trial showed that cases with \textit{ESR1} mutations had better survival when treated with fulvestrant compared with exemestane [29].

Of the additional mutations detected, \textit{ERBB2} p.S310F is notable because it results in activation of HER2 without gene amplification or protein overexpression [30]. A tumor with this mutation is likely to be sensitive to neratinib, as are those with G660D, R678Q, E693K, and Q709 mutations [31]. The neratinib HER Mutation Basket Study (SUMMIT) has already started [32]. A \textit{PBRM1} mutation may evoke immunotherapy resistance [33].

Cases with PTEN loss could be treated with \textit{PI3K/AKT} inhibitor [34]. Case K18 and K28 (triple-negative cancers) with \textit{CCND1} gene amplification might be sensitive to CDK4/6 inhibitors [35]. Meanwhile, erdafitinib and cetuximab might be effective for cases K30 and K32 in which the metastatic lesions contained \textit{FGFR} and \textit{EGFR} amplifications [36, 37]. Case K30 and K32, which had decreased \textit{ATM} might be sensitive to topotecan or the poly-(ADP-ribose) polymerase inhibitor olaparib [38]. \textit{CDKN2A} or \textit{RB1} downregulation could be target of palbociclib [39].

The present study with only 93 genes analyzed showed actionable mutations or CNVs in 73\% (8/11) of recurrent/metastatic breast cancer lesions. This is comparable to the findings of previous studies including MSK-IMPACT (61\%), the study by Vasan et al. (84\%), and the study by Muller et al. (45\%) [12, 13, 40].

The major limitations of the present study are its small scale and subtype bias, for which problems with DNA availability are responsible. Patients with luminal breast cancer often show late recurrence/metastasis, such as up to 18 years after diagnosis in our series. The primary tumor blocks of such cases are too old and rarely maintain sufficient DNA quality. In contrast, triple-negative breast cancer usually recurs shortly after surgery. Another limitation is the determination of the cutoff level for CNV. The relationship between drug sensitivity and CNV remains to be elucidated. Moreover, additional
immunohistochemistry might be helpful for cases with altered CNVs of \textit{EGFR} or \textit{FGFR}. We could detect the somatic driver mutations or germline mutations in five triple-negative cancers; however, the genes covered by the QIAseq Human Breast Cancer Panel might be inadequate for analysis of triple-negative breast cancers because these cancers are known to have highly variable mutations [41].

**Conclusions**

Our targeted NGS assay using the QIAseq Human Breast Cancer Panel showed that recurrent/metastatic breast cancer lesions sometimes gained additional mutation and CNV. This method assists the identification of drug-targetable mutations and changes in CNV levels. The performance of an expanded study including an analysis of drug sensitivity is to be encouraged.

**Declarations**

**Ethics approval and consent to participate**

Written informed consent was obtained for use of retrospective tissue samples from the patients within this study. Ethnical approval was obtained from Institutional Review Board of Kawasaki Medical School ethics committee (approval number: 2695).

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets generated and/or analysed during the current study are available in the SYNAPSE repository, https://www.synapse.org/#!Synapse:syn22364283/files/.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

TA and AT were responsible for NGS, NK was responsible for histology and drafted the manuscript; OH and NK contributed statistical analyses; TY and JK contributed clinical information. TM contributed data analysis and interpretation. TM also supervised NK. All authors read and approved the manuscript.
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Abbreviations

**CNV**: Copy number variation

**CPA**: Cyclophosphamide

**DM**: Distant metastasis

**DOD**: Dead of disease

**DTX**: Docetaxel

**EPI**: Epirubicin

**EXE**: Exemestane

**FFPE**: Formalin-fixed paraffin-embedded

**GEM**: Gemcitabine

**ICNOS**: Invasive carcinoma, NOS

**ILC**: Invasive lobular carcinoma

**LND**: Live without disease

**LR**: Local recurrence

**na**: Not available

**NGS**: Next-generation sequencing

**PB**: Primary breast tumor

**PTX**: Paclitaxel

**TAM**: Tamoxifen

**TRA**: Trastuzumab

**X**: Capecitabine,
DFUR: Doxifluridine

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**Tables**

Due to technical limitations, tables 1-2 are only available as downloads in the supplemental files section.

**Figures**
The samples with good QC scores (≤0.04) were collected more recently than those with poor QC scores (>0.04) (P < 0.0005, Mann–Whitney U test). A QC score threshold of 0.04 is recommended by Qiagen.
Figure 2

Case K22. (A) Normal breast tissue, (B) primary breast invasive lobular carcinoma showing loose trabecular growth with CDH1 p.Q264* mutation, and (C) pleural effusion containing metastatic cancer cells with CDH1 p.Q264*, ERBB2 p.S310F, CBFB p.X27_splice and TP53 p.R248Q mutations.
Figure 3

CNV seems to be higher in distant metastasis than in local recurrence, but the difference was not significant (P = 0.091, Mann–Whitney U test).

Figure 4

GeneRead Human Comprehensive Cancer Panel analysis for patient K31. The primary and metastatic cancer cells have similar morphology, but the pericardial and pleural disseminations contained different mutations.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SuppleTable1.xlsx
- SuppleTable2.xlsx
