Comparison of the multigene panel test and OncoScan™ for the determination of *HER2* amplification in breast cancer

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Abstract. The diagnostic accuracy of the multigene panel test (MPT) and OncoScan™ in the determination of *HER2* amplification in breast tumors remains controversial. In the present study, *HER2* copy number was analyzed using both MPT and OncoScan™ in 45 breast tumors and was compared with that in fluorescent *in situ* hybridization (FISH) analysis. Tumors with low cellularity were examined using tumor cell enrichment and fluorescence-activated cell sorting. Both MPT and OncoScan™ exhibited significant correlations with FISH with respect to the determination of *HER2* amplification in breast tumors. However, the correlation coefficient was significantly higher for the comparison of MPT and FISH (r=0.770) compared with that between OncoScan™ and FISH (r=0.564). The accuracy of MPT (93.3%) was slightly higher compared with that in OncoScan™ (84.4%) in determining the *HER2* status, which was mostly explained by the higher sensitivity of MPT in tumors with low cellularity (83.3 vs. 33.3%), but not in those with high cellularity (81.8 vs. 72.7%). The specificity was 100% for both tests. The MPT exhibited higher sensitivity in the determination of the amplification of other genes, including *MYC*, fibroblast growth factor receptor 1 and GATA binding protein 3 in tumors with low cellularity compared with that in tumors with high cellularity. OncoScan™ exhibited low sensitivity without tumor cell enrichment. The results suggested that MPT could be a promising method to determine *HER2* status in breast tumors and that it could exhibit improved accuracy compared with that in OncoScan™ in tumors with low cellularity.

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Abbreviations: MPT, multi gene panel test; FISH, fluorescent *in situ* hybridization; FACS, fluorescence-activated cell sorting; CDx, companion diagnostic; FFPE, formalin-fixed, paraffin-embedded; SNP, single nucleotide polymorphism; TCGA, The Cancer Genome Atlas; MAFs, mutant allele frequencies; pan-CK, pancytokeratin; ER, estrogen receptor; PR, progesterone receptor; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma

Key words: copy number aberration, target sequencing, breast cancer, cellularity, multi gene panel test

Introduction

The amplification of a variety of genes, including *HER2*, *MYC*, *FGFR1*, *CCND1*, *GATA3*, *MDM4* and *IGF1R* has been found in a significant proportion of patients with breast cancer (1-5). Among them, *HER2* amplification is routinely assessed using histological assays, such as fluorescent *in situ* hybridization (FISH)/non-fluorescent *in situ* hybridization or immunohistochemistry (IHC) analysis, which are well-accepted companion diagnostic (CDx) for anti-*HER2* therapies (6). In addition, *FGFR1*, *MYC*, *MDM2* or *IGF1R* amplification has potential use as a CDx for antitumor agents targeting each amplified gene (7-11). Furthermore, it has been reported that *HER2*, *FGFR1*, *MYC* or *CCND1* amplification could predict patient prognosis (12-14) or resistance to hormonal therapy and/or chemotherapy (15,16). Therefore, an assay that could simultaneously detect the amplification of these genes would be clinically useful for guiding treatment selection.

However, the assays used for the simultaneous determination of large numbers of genes are considered excessively laborious and time-consuming. Comparative genomic hybridization (17) and single nucleotide polymorphism (SNP) arrays (18,19) have been used as the standard methods for the comprehensive analysis of gene copy number in the whole genome, highlighting their potential applications. However, these assays generally require tumors with high cellularity. For example, in a cross-tumor analysis of The Cancer Genome Atlas (TCGA), tumor cellularity of 60-80% or higher was used as an inclusion criterion for all tumor types (2,20-22). OncoScan™ is the most advanced microarray-based assay for copy number analysis; however, it requires >20% tumor cellularity. A significant proportion of breast cancers, such as lobular carcinoma, scirrhous carcinoma and lymphocyte-predominant carcinoma have shown low cellularity (23-25).

The multigene panel test (MPT), which was designed to analyze the mutation of multiple target genes using next-generation sequencing, is another option for the detection of gene amplification (26,27). Recently, MPT was introduced as a key test for personalized treatment for a variety of tumors, including breast cancer. The main application of MPT was the identification of the gene mutation that dictates the indication of a molecular targeted agent (28,29). In addition, the development of new algorithms, such as PureCN (30) and the copy number variation kit (31) have enabled the analysis of copy numbers using MPT. An MPT assay for *HER2* amplification (FoundationOne™) has been recently approved as a CDx for anti-*HER2* therapy (32). Furthermore, PureCN is expected to be applicable for tumors with low cellularity that cannot be analyzed using OncoScan™. These results suggest the possibility that the simultaneous assessment of multigene amplification is feasible in breast cancer using MPT.

In the present study, the diagnostic accuracy of MPT and OncoScan™ was assessed and compared with that of FISH with respect to the determination of *HER2* amplification. The impact of tumor cell enrichment was assessed using fluorescence-activated cell sorting (FACS). The OncoScan™ results were simultaneously investigated on multiple gene alterations other than *HER2* amplification, which were obtained by MPT.

Materials and methods

Patients and samples. The samples included 45 FFPE tumor samples (fixed in 10% neutral buffered formalin for 6 to 72 h at room temperature) from patients with primary breast cancer (median age, 55 years; range, 34-85 years), who underwent surgery between April 2012 and August 2018 at Osaka University Hospital (Osaka, Japan). The patients did not receive preoperative systemic therapy or radiation delivered to the breast. The clinicopathological characteristics of the patients are shown in Table I. Written informed consent was obtained prior to sampling and the present study was approved by the Ethics Review Board of Osaka University Hospital. All methods were performed in accordance with the relevant guidelines and regulations.

DNA extraction from FFPE tumor samples. A total of 5-10 sections (10-μm thick) were cut and DNA was extracted from the tumor area following macro-dissection. Macroscopic removal of the surrounding non-tumor area was performed and the samples were analyzed using both the OncoScan™ and MPT assays. Genomic DNA for OncoScan™ was extracted from the FFPE tumor slides or FACS-sorted tumor cells using a QIAamp DNA FFPE Tissue kit (Qiagen Corporation) according to the manufacturer's instructions. Genomic DNA for MPT was extracted from FFPE tumor slides using a GeneRead DNA FFPE kit (Qiagen Corporation) according to the manufacturer's instructions.

Copy number and mutation analysis using MPT. A total of 200 ng DNA was extracted from the FFPE tumor samples. The DNA samples were processed by shearing using a Covaris® S220 Focused-Ultrasonicator (Covaris, Inc.) to yield small fragments, which were 150-200 base pairs in size. The enrichment of the sheared DNA was performed using the Agilent SureSelect XT HS Target Enrichment system (Agilent Technologies, Inc.). The probes were designed by SureDesign software (v4.0; https://earray.chem.agilent.com/suredesign) and provided by Agilent (Agilent Technologies, Inc.) to capture 20 target genes, including the top 10 genes with a high frequency of amplification and the top 9 genes with a high frequency of mutation according to TCGA and Molecular Taxonomy of Breast Cancer International Consortium (cBioPortal for cancer genomics; http://www. cbioportal.org) and *ESR1*, whose mutations are important for the ER function of breast cancer (33) (Table SI). The quality of the processed samples was checked with a Bioanalyzer High Sensitivity DNA Assay (Agilent Technologies). The libraries were analyzed using MiSeq and HiSeq 3000 sequencers (Illumina, Inc.) with 2x100 or 2x150 bp paired-end sequencing. The cat. no. and supplier of the sequencing kits are as follows; MiSeq Reagent Nano kit v2 (300 Cycles), MS-103-1001/HiSeq 3000/4000 PE Cluster kit, PE-410-1001 HiSeq 3000/4000 SBS kit (300 Cycles) and FC-410-1003 (all from Illumina, Inc.). The loading concentration of the final library was 2 nM. The average read-depth for the target genes was 2,372. To process the sequencing data, low-quality (<20; where a quality score from the identification of the nucleobases generated by DNA sequencing or Phred score) bases and adaptors were trimmed using Trimgalore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore) and aligned using Burrow-Wheeler Aligner (https://github.com/lh3/bwa/releases/tag/v0.7.17) to the reference human genome (build hg38). The resulting alignment was further processed to fix alignment based on paired read information (https://gatk.broadinstitute.org/hc/en-us/articles/360036713471-FixMateInformation-Picard) using Picard tools (http://broadinstitute.github.io/picard). Subsequently, the Genome Analysis Tool kit (GATK (v4.0.11.0; https://github.com/broadinstitute/gatk/archive/refs/tags/4.0.11.0.tar.gz) was used for base quality score recalibration. SNPs were identified using MuTect (v1.1.7; https://gitlab.bioinfo-diag.fr/Strasbourg/STARK/-/blob/a248629dc0b123d9898c1126b21f78b110c01ffc/sources/tools/mutect/1.1.7/mutect-1.1.7.jar.zip) with default parameters and the copy number was identified using PureCN (http://bioconductor.org/packages/PureCN/). The genes were considered amplified if the segment copy number was ≥6 for focal amplifications (<3 Mb) or ≥7 for non-focal amplifications according to the default standard of PureCN. For the mutational analysis of the panel genes, the SureCall software was used (ver4.0; https://www.agilent.com/en/download-software-surecall), using the default parameters (reference genome; hg19 and minimum allele frequency; 0.001). The following exclusion criteria was used: A depth of <300 reads in the intron or a population frequency of ≥1% reported in dbSNP (https://www.ncbi.nlm.nih.gov/snp/)/1000G (https://www.internationalgenome.org/database).

Copy number analysis using OncoScan™. Extracted DNA was processed using copy number analysis with an OncoScan™ CNV FFPE Assay kit (Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Copy number, ploidy and cellularity were calculated using the Allele-Specific Copy number Analysis of Tumors algorithm from the probe data extracted from the OncoScan™

Table I. Clinicopathological characteristics of the patients with breast cancer included in the present study.

Characteristic	Number (%)
Menopausal status	
Pre	21 (47)
Post	24 (53)
pT	
1	25 (56)
2	19 (42)
3	1 (2)
pN	
Positive	16 (36)
Negative	29 (64)
Histopathological type	
IDC	44 (98)
ILC	0 (0)
Other[a]	1 (2)
Histological grade	
1	8 (18)
2	15 (33)
3	22 (49)
Ki67, %	
≥20	31 (69)
<20	14 (31)
ER	
Positive	34 (76)
Negative	11 (24)
PR	
Positive	30 (67)
Negative	15 (33)
HER2 status	
Positive	17 (38)
Negative	28 (62)

[a]Apocrine carcinoma. IDC invasive ductal carcinoma, ILC invasive lobular carcinoma, ER estrogen receptor, PR progesterone receptor.

Console v1.3 software (Affymetrix; Thermo Fisher Scientific, Inc.). The genes were considered amplified according to the default standard of PureCN as aforementioned.

IHC analysis and FISH. The protein expression levels of the ER, PR and *HER2* in the aforementioned FFPE tumor samples were determined using IHC analysis, while FISH was also used for HER2 amplification and was both performed by SRL Inc.. Clinical HER2 status was determined using FISH and/or IHC according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) 2018 guideline and is referred to as 'the clinical test' (6). The cut-offs for the proportion of positive cells were 10% for ER and PR, and 20% for Ki67. Pathological diagnosis and IHC analysis for the protein expression level of Ki67 was performed at the Department of Pathology (Osaka University, Osaka, Japan). Tumor cellularity was microscopically and manually evaluated in increments of 10% based on the proportion of tumor cells per total cells counted in using a light microscope at x400 magnification.

Tumor enrichment by FACS. A total of 40-50 sections (50-μm thick) were cut from the FFPE tumor samples, with low tumor cellularity (<20%), collected in microtubes and dewaxed in xylene, 3 times for 5 min each. Rehydration was performed using two washes (100% ethanol for 5 min each), followed by additional washing in 95, 70, 50 and 30% ethanol. A final wash was performed in distilled water for 1 min. Antigen retrieval was performed using 10 mM Tris-EDTA (pH 9.0; Dako; Agilent Technologies, Inc.) at 80˚C for 60 min in a pre-heated block incubator (AccuBlock; Labnet International, Inc.). The sections were dissociated into single cells using 255 U/ml collagenase type III (Worthington Biochemical Corporation), 300 protease units/ml dispase (Godo Shusei Co., Ltd.), and 0.25 mg/ml RNase (Sigma-Aldrich; Merck KGaA) in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) at 37˚C for 120 min. The cells were filtered using a 40-μm filter, washed in FACS buffer [PBS containing 3% FBS (Thermo Fisher Scientific, Inc.) and 5 mmol/l EDTA] and subsequently stained with anti-pancytokeratin (pan-CK) antibody conjugated with Alexa Fluor 647 (clone C11; Cell Signaling Technology, Inc.) and anti-vimentin antibody conjugated with phycoerythrin (clone RV202; BD Biosciences) overnight at room temperature. DNA staining was performed by incubating the samples with DAPI (Dojindo Molecular Technologies, Inc.) at 4˚C for 30 min. FACSAria II (BD Biosciences) was used for sorting pan-CK-positive/VIM-negative cells from a DAPI-positive single-cell population. A total of $2.1x10^7$-$1.6x10^8$ events were acquired.

Statistical analysis. The R software was used for statistical processing (v3.5.1; http://www.r-project.org). The Williams' test was used to compare the Pearson's correlation coefficient between MPT and FISH, and OncoScan™ and FISH. The McNemar's test was used to compare the concordance of HER2 amplification status between MPT or OncoScan™ and FISH. The frequency of amplification of 4 genes (*HER2*, *MYC*, *FGFR* and *GATA3*), obtained by MPT and OncoScan™, were compared between the 2 methods using the McNemar's test. Table SII represent the numbers of amplification for each of the 4 genes in the samples. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of HER2 copy number detection using MPT or OncoScan™ and FISH. Initially, MPT and OncoScan™ were quantitatively compared with FISH to determine the copy number of *HER2* in 45 FFPE tumor samples. Both MPT and OncoScan™ displayed significant correlations with FISH; however, the correlation coefficient was significantly higher between MPT and FISH compared with that for OncoScan™ and FISH (r=0.770 vs. 0.564; Williams' test P=0.022; Fig. 1). Secondly, MPT and OncoScan™ were qualitatively compared with the clinical test (determined using FISH and/or IHC according to ASCO/CAP guideline) with respect to the determination of the *HER2* status.

Table II. Concordance of *HER2* amplification status between MPT and OncoScan™, and fluorescence *in situ* hybridization/ immunohistochemistry (the clinical test) in 45 FFPE tumors.

HER2 status		MPT			OncoScan™		
		Positive n (%)	Negative n (%)	P-value[g]	Positive n (%)	Negative n (%)	P-value[g]
The clinical test for all tumors	Positive[a]	14 (82)	3 (18)	0.248	10 (59)	7 (41)	0.023
	Negative[b]	0 (0)	28 (100)		0 (0)	28 (100)	
The clinical test for high tumor cellularity (≥40%)	Positive[c]	9 (82)	2 (18)	0.480	8 (73)	3 (27)	0.248
	Negative[d]	0 (0)	16 (100)		0 (0)	16 (100)	
The clinical test for low tumor cellularity (<40%)	Positive[e]	5 (83)	1 (17)	>0.999	2 (33)	4 (67)	0.134
	Negative[f]	0 (0)	12 (100)		0 (0)	12 (100)	

[a]n=17; [b]n=28; [c]n=11; [d]n=16; [e]n=6; [f]n=12; [g]McNemar's test. MPT, multigene panel test.

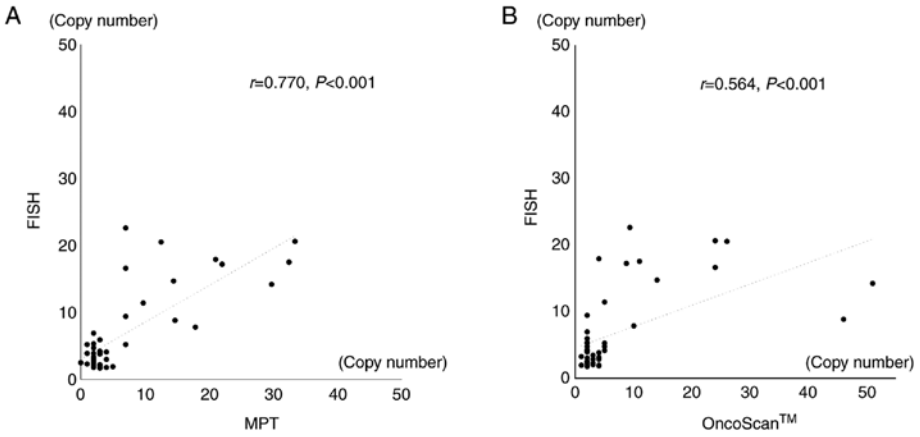

Figure 1. Correlation of *HER2* copy number between the MPT and OncoScan™, and FISH. *HER2* copy number analysis was performed in 45 breast tumors using (A) MPT and (B) OncoScan™, and the results were compared with those of FISH using a Williams' test. MPT, multigene panel test; FISH, fluorescence *in situ* hybridization.

MPT displayed a high concordance with the clinical test, for total HER2 status; however, OncoScan™ showed a significant difference with the clinical test (P=0.248 and P=0.023 for MPT and OncoScan™, respectively) (Table II). The specificity was 100% for both tests, and the false negative rate was 18% for MPT and 41% for OncoScan™.

The cellularity of the 45 tumors ranged from 10 to 80% (median; 40%), and the tumors were divided into high (n=27; >40% cellularity) and low cellularity groups (n=18; <40% cellularity) using the median as the cut-off. The representative figures of tumors with high and low cellularity are presented in Fig. S1. In the high tumor cellularity group, MPT and OncoScan™ showed a similar concordance with the clinical test (McNemar's test P=0.480 and P=0.248 for MPT and OncoScan™, respectively; Table II). However, in the low tumor cellularity group, it was revealed that MPT showed an improved concordance with the clinical test compared with that for OncoScan™ (McNemar's test P>0.999 and P=0.134 for MPT and OncoScan™, respectively; Table II); however, both were non-significant (P>0.05).

Impact of tumor cellularity on copy number analysis using MPT and OncoScan™. The frequencies of *HER2*, *MYC*, *FGFR1* and *GATA3* amplification, which were the four most frequently amplified genes in the present cohort, were compared between MPT and OncoScan™ according to tumor cellularity (Fig. 2). In all the tumors (n=45), *HER2*, *MYC*, *FGFR1* and *GATA3* amplification was found in 31.1, 15.6, 11.1 and 11.1% of the samples by MPT respectively, and in 22.2, 13.3, 6.7 and 2.2% by OncoScan™ respectively. The MPT resulted in higher frequency of amplification in all four genes compared with that in OncoScan™ (Fig. 2A), although the difference was not significant (Table SII). When analyzed by tumor cellularity, the MPT and OncoScan™ detected the gene amplifications in high cellularity tumors (n=27) with approximately the same frequency (Fig. 2B). By contrast, in low cellularity tumors (n=18), MPT showed even higher frequency of the amplification for all four genes compared with that in OncoScan™ (Fig. 2C). Although, no statistically significant difference was seen in the small cohort, MPT was more sensitive at detecting gene amplification than OncoScan™, particularly in samples with low tumor cellularity.

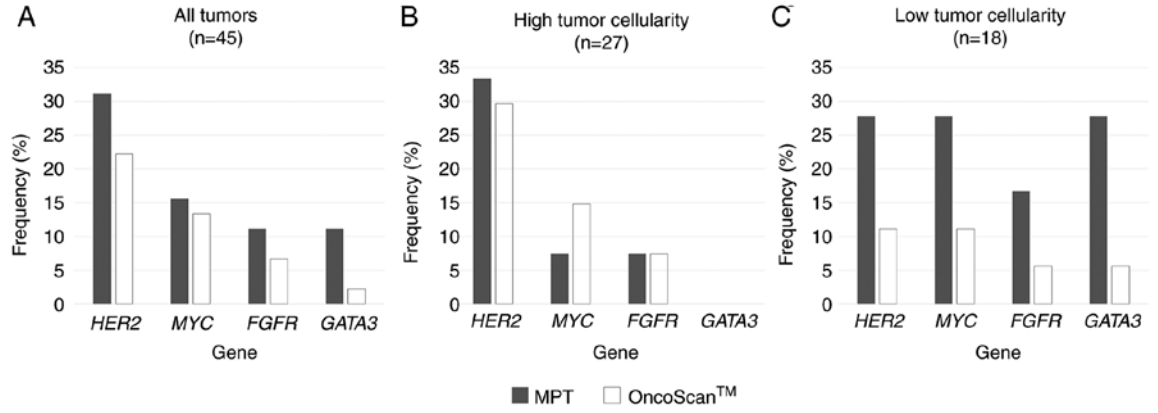

Figure 2. Comparison of the frequency of *HER2*, *MYC*, *FGFR1*, and *GATA3* amplification between the MPT and OncoScan™. Frequency of *HER2*, *MYC*, *FGFR1* and *GATA3* amplification was compared between MPT and OncoScan™ in (A) all breast tumors, (B) tumors with high cellularity and (C) tumors with low cellularity. The McNemar's test to analyze the concordance between the two methods for each gene amplification was performed (Table SII). MPT, multigene panel test.

Copy number analysis using OncoScan™ following tumor cell enrichment by FACS in tumors with low cellularity. The aforementioned results suggested that the inferiority of OncoScan™ in the detection of gene amplification in tumors with low cellularity was attributable to the contamination with normal cells. Therefore, the effect of tumor cell enrichment was investigated using FACS on the detection of gene amplification by OncoScan™. Tumor cell enrichment was performed prior to OncoScan™ testing, but not prior to MPT. The enrichment was performed on tumors with low cellularity. A total of 4 tumor samples with low cellularity and absence of *HER2*, *MYC*, *FGFR1* and *GATA3* amplification, according to OncoScan™ analysis, were subjected to tumor cell enrichment using FACS (Fig. 3A). A comparison of mutant allele frequencies (MAFs), prior to and following FACS analysis, indicated that tumor cells were enriched by 2.7-fold (range 2.1-3.3) (Fig. 3B). In cases #1 and #2, the MAFs were increased to 47.9 and 35.0%, respectively.

The copy number analysis of the FACS-sorted tumor cells was performed using OncoScan™. The data demonstrated *MYC* amplification in case #1, and *FGFR1* and *GATA3* amplifications in case #2 (Fig. 3C). The results obtained following FACS analysis were consistent with those obtained for MPT, in the absence of tumor cell enrichment. Conversely, the MAFs remained low following FACS analysis for the other two tumors and were calculated to be 11.4% in case #3 and 5.5% in case #4. OncoScan™ could not detect *GATA3* amplification following enrichment by FACS, despite this amplification being detected in both tumors using the MPT assay. In addition, the results of copy number analysis obtained by OncoScan™, which covered all chromosomes, revealed amplifications in additional genes, other than for *FGFR1*, *MYC* and *GATA3* following enrichment by FACS, but not prior to this process, as shown by blue dots in Fig. 4. This was notably observed in cases #1 and #2 and not in cases #3 and #4. These data demonstrated that tumor cell enrichment by FACS improved the detection of copy number aberration in all the chromosomes.

Discussion

In the present study, the data revealed the accuracy of MPT in determining the copy number of *HER2* and the results were compared with the OncoScan™ method. The detection of *HER2* amplification using FISH and IHC analysis was used as a reference. The results indicated that the superiority of MPT over OncoScan™ was possibly due to its higher sensitivity in tumors with low cellularity. Therefore, the effect of tumor cellularity was investigated on the detection of *MYC*, *FGFR1* and *GATA3* amplifications. The analysis confirmed that MPT improved the results, possibly due to its higher efficiency to detect gene amplifications in tumors with low cellularity. OncoScan™ is not widely used in routine clinical practice, but has been well-accepted as a standard method for the comprehensive analysis of the gene copy number (6). It is therefore widely used in clinical research (34). OncoScan™ has the potential to be used as a clinical test in future studies; therefore, the detection sensitivity of MPT was compared with that of OncoScan™. The latter is a current standard method; however, it is limited by its application in tumors with low cellularity.

The effect of tumor cell enrichment was assessed on the results obtained using OncoScan™ analysis. Tumor cell enrichment could improve the accuracy of this method if low cellularity is the main cause of the inconsistent results. The findings illustrated that the detection of gene amplification using OncoScan™ could be improved by tumor cell enrichment, provided a sufficient MAF could be achieved. However, gene amplification was not accurately detected using OncoScan™ in samples with low MAFs, even though it was readily detected using MPT. These results reinforced the higher sensitivity of the MPT method compared with that in OncoScan™ in tumors with low cellularity. This finding could be due to a higher accuracy of MPT in quantifying the SNP alleles compared with that in OncoScan™. The precise reason is unknown; however, it may involve the read-depth of the MPT analysis. It is hypothesized that the detection sensitivity of copy number aberrations could be improved by increasing the read-depth using the MPT method, whereas for OncoScan™ analysis, it is restricted by the resolution of the microarray chip (18). This has been confirmed in the present study by deep sequencing (average read-depth, 2,372).

Molecular tests, including the MPT assay, are not based on visual inspection and cannot be used to assess tumor heterogeneity, such as that noted in ductal carcinoma *in situ* (DCIS), which occurs within invasive ductal carcinoma (IDC) tumors. However, adjacent DCIS is found in ~45% of IDC tumors of

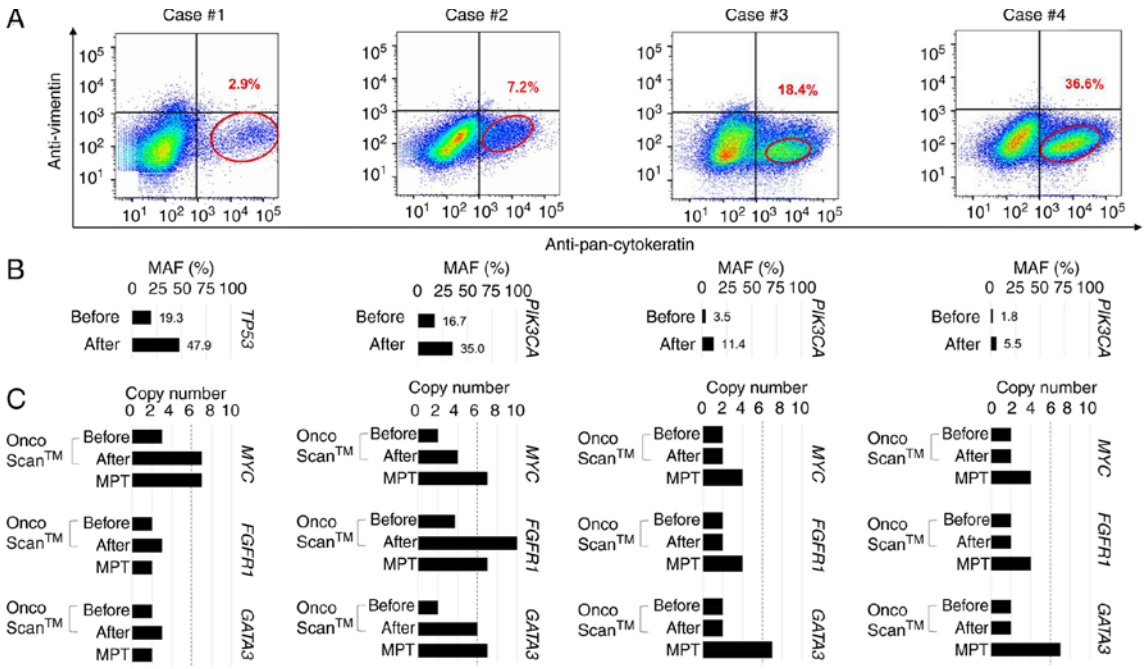

Figure 3. Effect of tumor cell enrichment using FACS on the accuracy of gene copy number analysis using OncoScan™. (A) Breast tumors (n=4) with low tumor cellularity (<20%) were subjected to FACS to separate the pan-cytokeratin-positive and vimentin-negative tumor cells for enrichment. (B) MAFs of *TP53* in case #1 and *PIK3CA* in cases #2, #3 and #4 before and after tumor cell enrichment using FACS are presented to show the effect of enrichment. (C) The copy numbers of *MYC*, *FGFR1* and *GATA3* were analyzed using OncoScan™ before and after tumor cell enrichment using FACS. The copy numbers of these genes were also determined using the MPT before tumor cell enrichment. FACS, fluorescence-activated cell sorting; MPT, multigene panel test.

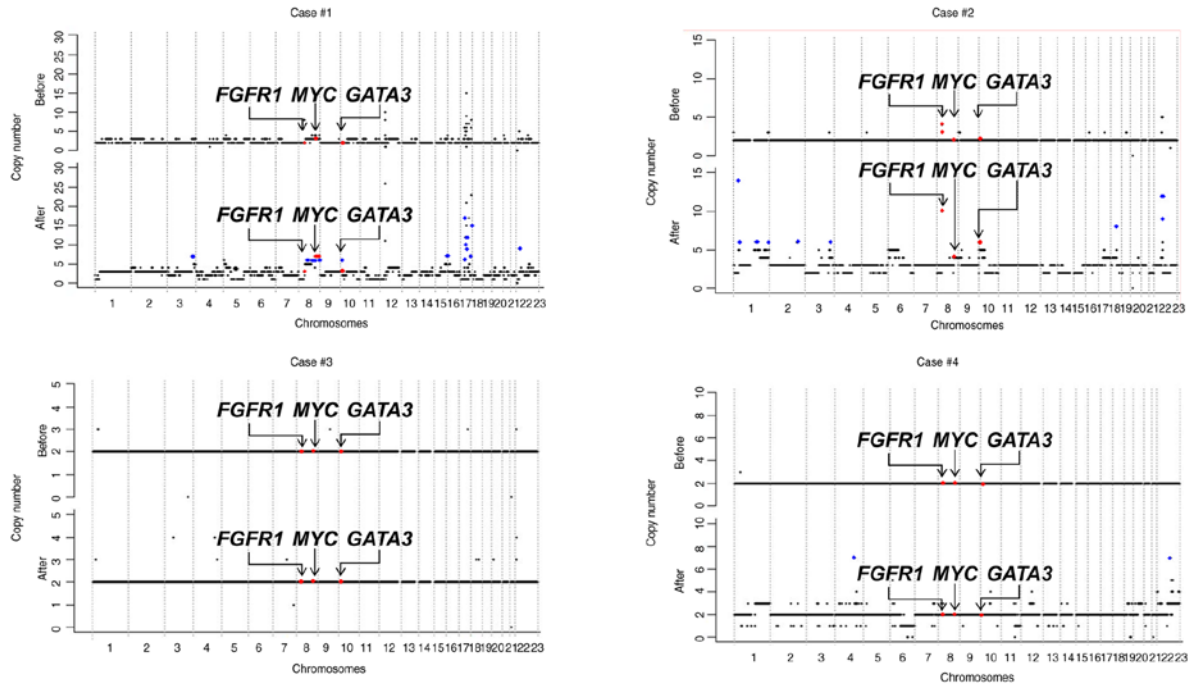

Figure 4. Copy number analysis of all the chromosomes using OncoScan™ before and after tumor cell enrichment by FACS. OncoScan™ copy number analysis was performed before and after tumor cell enrichment by FACS for cases #1-4 and the results were plotted against the entire chromosome. The red dots indicate the FGFR1, MYC, and GATA3. The blue dots indicate the additional genes which were detected following cell sorting. FACS, fluorescence-activated cell sorting.

the breast and their *HER2* status was associated with tumor status (35). Therefore, the MPT assay is unlikely to be affected by such heterogeneity. Genetic heterogeneity of *HER2* gene amplification has also been reported in 13-18% of breast cancer cases (36,37). The false positive rate of the MPT assay in comparison with the clinical test may be due to such genetic heterogeneity since different parts of the tumor were used in each assay. The HER2 FISH assay typically analyzes a small number of cells, while the MPT assay examines DNA from a large number of tumor cells in the whole FFPE section. Therefore, we hypothesized that the MPT assay is less likely to be affected by tumor heterogeneity and more likely to

represent the average HER2 copy number in the whole tumor. These characteristics were not noted for FISH.

HER2 amplification is currently routinely analyzed in practice; however, it has been hypothesized that assays for the amplification of other multiple genes could be required in the near future, due to the development of antitumor agents which target gene amplifications. Furthermore, this information would also be useful for the prediction of patient prognosis (14,16,28). Recently, several multiple gene tests, such as FoundationOne™ CDx and National Cancer Center OncoPanel have been used for an increasing number of patients with numerous types of cancer (38,39). These tests are essential for the implementation of personalized medicine. FISH is widely used and less expensive than MPT, which can analyze only a limited number of genes per assay. It is time consuming and involves significant cost and labor in case of multigene analysis for the development of multiple molecular-targeting agents. In contrast to these observations, OncoScan™ and MPT can immediately analyze the multigene set, without previous sample processing (40-42). The present study revealed that MPT could be superior to OncoScan™ since it is applicable to tumors with low cellularity, in the absence of enrichment by FACS, unlike OncoScan™. Therefore, we hypothesized that the use of MPT could increase as the numbers of analyzed genes and low-cellularity tumors increase, due to its time, cost and labor effectiveness.

A limitation to the present study is that tumor cell enrichment was performed only prior to OncoScan™, but not prior to MPT. This was due to the amount of tumor cells collected, after sorting, from a limited number of FFPE slides, and it was too small to perform OncoScan™ analysis. Another limitation is that analyzing a limited part of the tumor could not completely rule out the effect of intratumoral heterogeneity on the results. Therefore, the benefits of MPT require further investigation from future research, which would analyze the entire tumor tissue.

In conclusion, the present study found that MPT exhibited higher accuracy in determining *HER2* amplification compared with that for OncoScan™, notably for tumors with low cellularity. The data further supported the use of MPT as a CDx, to comprehensively determine the amplification of various genes in addition to *HER2*. The preliminary results of the current study require confirmation from future research, including a larger number of patients.

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Availability of data and materials

The raw sequencing and microarray data are not publicly available due to information that could compromise the privacy of the research participants. The other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KA participated in data analysis and interpretation, and wrote the manuscript. NK was involved in designing the experiments, acquired the funding and drafted the manuscript. YS, DM, SNa, TM, TT, YN, MS and SJK were responsible for providing the resources, analyzing and interpretating the data, and revising the discussion. SNo and KS conceptualized and supervised the study. All authors read and approved the final manuscript. KA and NK confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study involving human samples was approved by the Ethics Review Board of the Osaka University Hospital and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent prior to surgery.

Patient consent for publication

Not applicable.

Competing interests

NK received a research grant from Novartis. YN received a research grant from AstraZeneca and Sysmex and honoraria from AstraZeneca, Sysmex and Chugai pharmaceutical. SN is an advisor for AstraZeneca, Sysmex and Nittobo and has received honoraria from AstraZeneca, Chugai, Eli Lilly and Sysmex. His research funding from Sysmex and the joint patents with Sysmex are not related to the present study. The other authors declare that they have no competing interests.

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