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Comparison of genomic alterations between primary breast cancer and local recurrence after surgery

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Comparison of genomic alterations between primary breast cancer and local recurrence after surgery

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Abstract

Comparison of genomic alterations between primary breast cancer and remnant breast or ipsilateral chest wall recurrence

According to domestic and worldwide statistics, breast cancer is the most common cancer in females. With advancement in sequencing technique and targeted therapy based on genomic information, to identify targetable genomic abnormalities is becoming more common. However, it remains challenging to optimize treatment for the patients with second breast malignancies after receiving treatment of primary cancers, without better understanding on the differences between primary and relapsed tumors. Herein, we assessed genomic properties between primary and recurrent tumors of ipsilateral breast or chest wall after curative resection. When we compared the results of targeted next-generation exon sequencing with 121 cancer-related genes on formalin-fixed paraffin-embedded (FFPE) matched samples from 20 patients, genomic alterations showed highly concordant between paired samples, regardless of clinicopathological manifestation known as a factors impacting on recurrent tumors' attributes such as the interval to relapse, change of molecular subtype and therapeutic interventions. The analysis based on targeted exome sequencing results revealed that most of primary tumors and matched local recurrences clustered together and had strong positive linear correlation. We found that 16 out of 20 patients had at least one shared somatic mutation or CNAs between the primary and local recurrence, and the gain or loss of alterations throughout tumor progression developed in 8 patients, 1 case of whom acquired new driver mutations that could be targets for breast cancer.

To our knowledge, this is the first study comparing genomic properties between

primary tumor and matched local recurrence using next-generation sequencing. We found the molecular characteristics consistently retained in majority of local recurrence within the territory of remnant breast or ipsilateral chest wall, showing small number of changes in driver mutations. Based on this findings, genomic profiling on primary cancer may give useful information when considering target therapy in patients with local recurrence.

Keyword: breast cancer, local recurrence, genomic alteration, next-generation sequencing (NGS)

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Contents

11.Abstract (Korean)

List of figures

List of tables

Abbreviations

CNA Copy number alteration

CNV Copy number variation

ER Estrogen receptor

FFPE Formalin fixed paraffin embedded

FISH Fluorescence in situ hybridization

HER2 Human epidermal growth factor receptor 2

HR Hormone receptor

IHC Immunohistochemistry

NGS Next-generation sequencing

NPT New primary tumor

PgR Progesterone receptor (PgR)

SNV single nucleotide variants

TNBC Triple negative breast cancer

TR true recurrence

VUS Variant with unknown significance

Introduction

Breast cancer remains the leading cause of cancer-related mortality in worldwide(1). Although approximately 80% of patients diagnosed breast cancer with early stage and most women with localized disease is amenable to curative therapy, significant proportions of patients suffer from recurrence (2, 3). Breast cancer recurrence may occur in the ipsilateral remnant breast or chest wall where primary cancer was originally diagnosed and resected, regional lymph nodes as well as distant sites. Developing local recurrence does not always herald distant metastases but regarded as the high risk of subsequent distant relapse and poor prognosis (4). Several reports have suggested that local recurrence at the site of ipsilateral breast or chest wall is regarded as true recurrence (TR) consistent with the regrowth of remained malignant cells, which is an independent predictor of distant metastatic disease and poor survival(5, 6). Others have indicated, however, new primary tumor (NPT) described as "de novo malignancies" just gave rise to at the very site previously resected, that it showed quite different features with previously treated primary tumors(7-9). Several studies have attempted to classify these differences by using tumor location at recurrence, histologic subtype or pathologic criteria(8, 10). Some researchers suggested a genetic classification using clonal analysis, genomic expression profiling or quantitative DNA fingerprinting as a potentially valuable tool to improve understanding for the second breast malignancies(11-13).

With advancement in genomic analysis technique and getting genomic data of the tumors more frequently, genomic alteration

s throughout tumor progression may provide an insight helpful for comprehending the features of the relapsing tumors and determining appropriate treatment after recurrence. Genomic evolution have been increasingly studied and have showed that mutational processes are mostly similar in primary and relapsed tumors but continue

to acquire or lose mutations (14). As recent clinical trials enable to select targeted drugs based on genomic information for tailored therapy, genomic profiling has become more important, especially in recurrence or treatment-resistant cancers. The aim of this study is to compare genomic information and targetable genomic changes in both of primary tumors and local recurrence lesions through targeted sequencing of cancer-related genes, it might be useful when determining therapeutic target agents for recurrent tumors.

Patients and Methods

Tumor samples and clinicopathologic information

We reviewed the records of patients with invasive breast cancer who underwent operations for both of primary and relapsed tumor from 2002 to 2015 at the Seoul National University Hospital (Seoul, South Korea). Patients were selected on basis of primary-local recurrent matched tissue availability. Formalin-fixed paraffin blocks in both of primary breast cancer and matched recurrent tumor specimens were obtained. The Formalin fixed paraffin embedded (FFPE) samples were evaluated by a pathologist for selection of tumor areas for microdissection. To exclude germline mutations, we further sequenced the selected patients' blood sample if it had been stocked in our biorepository (Repository of lab of breast cancer biology, Seoul National University Hospital, IRB number: 1405-088-580). DNA was purified and extracted using ReliaPrep™ FFPE gDNA Miniprep System (Promega) and the quality of DNA was assessed through a TapeStation Systems according to the manufacturer's instructions. We confirmed DNA purity as ratio of A260/A280 is between 1.8 and 2.0. Clinicopathologic information including immunohistochemistry and Fluorescence in situ hybridization (FISH) was collected by a retrospective review of patient

medical records. The location of relapsed tumors which were documented in medical records or identified in mammography were obtained whether the tumor recurred at or near the vicinity of the primary tumor site. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB number: 1712-150-911).

Immunohistochemistry (IHC) staining and interpretation

This method was previously used in a published study (15). The samples were immunostained with the following antibodies according to the manufacturers' instructions: Anti-estrogen receptor(ER) (1:100; 1D5; Dako, Glostrup, Denmark), anti-progesterone receptor (PgR) (1:100; 636; Dako), and anti-HER2 (1:200; A0485; Dako). Positive ER and PgR expression were defined as nuclear staining in 1% or more of tumor cells. The HER2 membranous staining was scored on a scale of 0 to 3+ according to the HercepTest protocol. For tissue samples with a human epidermal growth factor receptor-2 (HER2) staining score of 2+, additional HER2 FISH testing was considered. HER2 status was considered positive when the IHC score was 3+ or the gene copy ratio of HER2/CEP17 by FISH was 2.2 or higher.

Genomic profiling

We performed a targeted next-generation sequencing (NGS) assay on FFPE samples by using a multi-gene panel (SNUH BCC (Seoul National University Hospital Breast Care Center) Panel) with average 356X sequencing depth. The SNUH BCC panel consisting of 121 genes was developed based on our previous study in which we had performed whole-exome sequencing and RNA-Seq of 200 pairs of matched clinical breast cancer and normal samples from Korean breast cancer patients. In addition, we had analyzed the mutations, CNAs, and gene expression results of approximately 3000 clinical breast cancer samples in the TCGA and METABRIC databases. As a result, we chose oncogenes, tumor

suppressor genes, or breast cancer-associated genes that showed a highly frequent mutations, genomic copy number variations and expression changes in breast cancer tissues (Table 1). The SNUH BCC panel is unique compared with other cancer panels based on NGS because it includes a certain portion of novel breast cancer-associated genes that have not been included in other recent popular and conventional cancer panels. In this regard, the SNUH BCC panel is not only targeted to worldwide breast cancer patients but is also ethnically directed to Korean breast cancer patients for diagnosis and therapeutic prognostic prediction (16).

Sequence alignment, variant calling and driver landscape for therapeutic target

Raw FASTQ file was filtered and trimmed using Adaptor removal 2.2.2. Burrows-Wheeler aligner (BWA; version 0.7.10) mem with default option was used to align reads to human reference genome sequence GRCh37. Sequence alignment map (SAM) file was converted to BAM format using samtools (version 1.1). Picard tool (version 1.115) was used to sort and remove duplications. GATK (version 4.0.4.0) was used to perform base quality score re-calibration. Samtools mpileup was used to create mpileup file with minimum base-quality of 17 and varscan (version 2.4.0) was used to call variants. Minimum variant frequency was set to 1%, minimum coverage was set to 8, minimum supporting reads was set to 2, and Strand-filter was applied. We excluded germline SNVs as well as technical artifacts observed in the blood samples and known variants from public databases such as the Genome Aggregation Database (gnomAD) and 1000 Genomes. And then variations were filtered by using the dbSNP archive (http://www.ncbi.nlm.nih.gov/projects/ SNP/) and annotated for known somatic mutations by using the COSMIC (17, 18). Somatic copy number alterations (CNAs) were called by calculating the number of mapped reads and then performing normalization using 40 tumor samples and reference samples. The threshold for gains was > 4.0-fold

and for loss <0.25−fold. Each variant is classified according to the Association of Molecular Pathology (AMP) guidelines for somatic cancer variants (19). Any variants classified as benign, likely benign or variants with unknown significance (VUS) were excluded for driver mutation landscape. We considered known oncogenic/likely oncogenic variants and hotspots as reported in the literature so far, and we defined alterations deemed a target of FDA−approved drug or investigational therapeutics on basis of a review of TARGET database v3 in Cancer Genome Analysis (CGA) and oncology knowledge database (OncoKB) database as "actionable" (20, 21). Actionable alterations with clinical or biologic evidence supporting an association with response to targeted drugs were stratified by level of evidence from OncoKB. We used "R version 4.0.1" to compare and visualize the differences and similarities between primary tumors and matched recurrence. The results from paired samples that can be sequenced with high quality were included in calculation to well visualize the patterns of matched samples. MutationMapper was used for visualization of actionable mutations. (22)

Results

Patient and sample characteristics

The clinicopathological characteristics of the 20 patients in our study were summarized at Figure 1 and we described more detail information in Table 2 and 3. The mean age at primary breast cancer diagnosis was 48.6 years (range 37–70) and median follow−up period from the date of primary cancer diagnosis to last visit record to our clinic was 146 months. The mean local recurrence free interval was 32 months (range 6–108). Twelve patients (60%) were diagnosed with hormone receptor (HR) positive primary breast cancer including 3 patients with unknown HER2 status and the number of patients identified with HER2 amplification and

triple negative primary breast cancer (TNBC;ER−, PR−, and HER2−negative) were 4(20%) and 5(25%), respectively. At presentation with primary cancer, 8 patients (40%) had axillary lymph node metastasis and 16 women received neoadjuvant/adjuvant chemotherapy. As the sites of relapse were defined according to surgery type, recurrence at remnant breast (50%) for the patients who underwent breast conserving surgery (BCS) and ipsilateral chest walls (50%) for mastectomy. Eight patients developed subsequent distant metastasis, and two showed synchronous metastatic lesion with local recurrence (Figure 1, Table 2).

When comparing molecular types of the primary and matched recurrent lesions, local recurrence mostly had concordant immunohistochemical characteristics [i.e. hormone receptors (HR) and HER2 status] with that of primary tumor. All triple−negative and HER−2 status retained their characteristics in the matched recurrent lesions, whereas 4 patients appeared to have discordant ER or PgR status. More specifically, three patients lost ER or PgR during progression and 1 recurrent tumor gained PgR. (Table 2, 3).

Somatic mutations and copy number variations of primary tumors and local recurrence

In 40 FFPE samples, we identified a total of 90 driver somatic alterations or copy number variations (CNVs) in 25 genes (Figure 1). There is no germline mutation in 16 blood samples. Genomic sequencing using the 121−gene panel detected at least one known oncogenic or likely oncogenic alteration in 36 of 40 samples (mean 2.25; range, 0–6). In the manner of copy number calculation from NGS data, as ERBB2 gain were observed in the 8 of the 10 tumors that were identified as HER2−positive by IHC or FISH, so the HER2 copy−number amplification results were found to be discordant with that of tested by IHC or FISH in 2 cases (Figure 1). The most frequently mutated gene was *TP53* (23.3%), followed by *PIK3CA* (20.0%) and most common amino acid change was H1047R (11 samples) in

PIK3CA gene. When we excluded the tumors with unknown IHC results, *TP53* alterations were found in 87.5% out of triple-negative samples, 50% in HER2-positive samples, and 43.5% in HR-positive samples. Whereas, *PIK3CA* alterations were observed in 60.9% of HR-positive tumors and only 1 of HR-negative samples. At least one alteration was detected in 6 genes associated with clinical action irrespective of the kind of targeted tumor type, excluding amplifications in *ERBB2* gene (Figure 1 & Table 3).

Comparison of genomic properties in primary tumors and matched local recurrence

The unsupervised hierarchical clustering of the tumors based on genomic mutations revealed that 17 pairs out of 20 matched samples clustered together (Figure 2A). Additional correlation analysis of 18 patients excluding 2 pairs with low sample quality showed the recurrent tumors' genomic characteristics were closely related with that of primary lesions with respect to targeted analysis (Pearson's correlation, Figure 2B).

In a driver perspective, 54 (83.1%) of the 65 somatic mutations and 18 (72%) of 25 CNVs detected were found to be concordant in primary cancer and matched recurrence (Figure 3). Eight patients showed differences in genomic alterations between primary tumors and recurrence, 6 relapsed tumors of whom acquired deleterious alterations compared to primary tumors.

Clinicopathological features and genomic concordance

When we calculated Z score to assess and visualize genomic similarities among samples, the matched samples were mostly concordant regardless of tumor location or molecular subtype (Figure 4, Table 3). Though all pairs were composed of a same histologic subtype with invasive ductal carcinomas in most cases and relapsed tumors mainly developed at near the primary tumor, one recurrent lesion

occurred in remnant breasts was located at a site different from the primary (Patient#6). Of the 12 patients with HR-positive tumors at initial diagnosis, in terms of molecular subtypes, 3 patients revealed changes of HR status but retained either of ER or PR positivity in recurrent tumors and conversion to HR-negative subtype occurred in one patient (Patient #13). Furthermore, local recurrence free interval ranged from 6 to 108 months, showing over 7 years interval in two patients (Patient #5 and #6). Figure 4 showed that genomic features of matched recurrent tumors were highly concordant with those of primary tumors in Patient #5, #6 and #13. The paired samples in these 3 patients had also identical driver mutations and CNVs (Figure 3). Additionally, recurrent tumor showed similarity with the primary tumor in 17 patients who had received adjuvant therapy, 16 women of whom received cytotoxic chemotherapy (Figure 4, Table 3).

Clinical actionability of molecular targets

Overall, 15 of the 20 patients had genomic alterations in approved or potentially "actionable" genes including HER2 amplifications. With exception of HER2 amplifications, genomic profiling detected new potentially actionable alterations that had not been previously identified by standard-of care testing in 14 patients. The targets identified were listed in Table 5. Oncogenic mutations in *PIK3CA* gene were most frequently detected for therapeutic target. Somatic mutations in *CDKN2A* (p. G101L and p. N42Rfs) and *FGFR2* (p. F276V) genes were considered as likely oncogenic changes in well-known functional protein domain that regulate the cell growth or division (Figure 5)(23, 24). We found somatic alterations in six genes with breast cancer-specific or all solid tumor-acceptable target drugs, whereas two (*FLT3* and *SF3B1*) of targetable genes have not yet be approved for breast cancer (Table 4). Six patients subsequently suffered metachronous distant metastasis after receiving treatment for local recurrence. Whereas only one recurrent tumor (patient #14) showed new targetable genomic alteration in *PIK3CA* gene which was not found in primary sample, and the other 5 patients

showed consistent results between primary and recurrent tumors from an actionable point of view.

Discussion

Personalized therapy is based on molecular characterization of the tumor and target aberrations that drive tumor growth(25). As the NGS techniques and target therapy based on genomic information have been advanced, the genetic landscape allows tailored therapy and will overcome tumoral heterogeneity and its resistance to traditional anticancer agents (26, 27).

Breast cancer is a highly heterogeneous disease; the same primary tumor frequently showed different genomic profiles and its recurrent lesion also acquired new molecular aberrations compared to their primary tumors. While several studies have showed the result on genomic evolution between primary breast cancer and its' metastasis (14, 28-30), we studied genomic alterations confined to local recurrence in remnant breast or chest wall where primary tumor had been removed. With respect to ipsilateral breast tumor recurrence, many studies have been tried to classify the new primary and true recurrence on basis of clinical features such as histology, molecular subtype or location between primary and relapsed tumors. To our knowledge, this is the first study comparing between primary tumor and matched local recurrence based on genomic analysis using sequencing data.

We performed target sequencing of 121 cancer-related genes to evaluate and compare the spectrum of genomic alterations between primary breast cancer and matched local recurrence in remnant breast or ipsilateral chest wall after mastectomy. A large proportion of primary and matched recurrent tumors included in our analysis seemed to have similar genomic properties irrespective of clinicopathological characteristics, showing small number of changes in driver

alterations. Previous studies have considered clinical and molecular features like as different histology, tumor occurrence at distant site from primary tumor bed and molecular subtype discordance as an indicator to distinguish between de novo primary tumor and regrowth of remained malignant cell and to impact on post-recurrence survival and potential treatment options(31). We identified, however, secondary cancer developed in remnant breast or chest wall was found as true recurrence rather than new primary tumor, retaining their genomic characteristics of primary cancer even when recurrent tumor occurred at a different site with long periods after primary cancer treatment or with changes in molecular types. Moreover, new primary tumors were known to be developed after a longer interval from their initial treatment than patients with true recurrence, but recurrent lesions developed after long period over than 7 and 9 years in two patients had similar attributes to those of primary tumors.

Previous studies have presented that somatic alterations were more frequently found in recurrent lesions than primary tumors, especially in the analysis focused on distant metastasis (14, 28, 29). However, our result suggested that local relapse within the area of remnant breast or ipsilateral chest wall had similar attributes with primary cancer, and significant evolution throughout local relapse rarely happened (Figure 4). Yates et al revealed genomic evolution exerted by therapeutic interventions response to treatment exposures, as truncating mutations were gained after chemotherapy or cancer genes potentially actionable driver mutations emerged during endocrine therapy(14). When we gave consideration that most of patients included in our study had received systemic adjuvant therapy (chemotherapy and/or hormone therapy) or radiotherapy for local control, however, overall genomic properties of relapsed tumors did not seem to be affected by therapeutic interventions.

In terms of actionable genomic mutations, one patient acquired driver mutation that can be a target for breast cancer-specific drugs (Patient #14, H1047R in

PIK3CA gene) and targetable mutation in *CDKN2A* gene emerged in the other one case(Patient #8). Because the quality of primary FFPE sample in case of Patient#8 was definitely poor and was sequenced with very low depth coverage, there are limitation to interpret the differences. The amplifications in *CNK12* gene and SNVs in *FLT3* and *SF3B1* which were described in Table 5 might be potential candidates related with clinical action with limited evidence, for targeted mutation type or tumor type are different from those of literatures.

Our study has several limitations. The number of cases included in this study is small, for obtaining both of primary tumor and matched FFPE block was challenging. Limited sample size resulted in the failure of the comprehensive statistical analysis on the correlation between genomic alterations and clinical characteristics such as subtypes or disease-free interval. Additionally, as our study was designed as retrospective manner and the samples had not collected with purpose of genomic analysis, the quality of FFPE samples varied depending on the archived periods or status of storage. It might have affected the sequencing result reducing coverage depth in certain region or increasing the rate of variants detected in some of the samples. For these reasons, we consequently applied very strict cut-offs both of depth and allelic frequency for the confirmation of NGS variant calls. Finally, this study limited to perform targeted sequencing only focused on genomic mutations and CNVs and did not analyze other abnormalities in DNA methylation or phosphorylation, gene fusion, RNA or protein expression that can provide novel information. Further comprehensive analysis integrating the genomic profiles, tumor biology and clinical information is essential for better understanding.

In conclusion, we found that genomic characteristics of primary tumor consistently retained in majority of local recurrence within the territory of remnant breast or ipsilateral chest wall, showing small number of changes in driver alterations. So genomic profiling on primary cancer is thought to provide useful information when considering tailored treatment in patients with local recurrence.

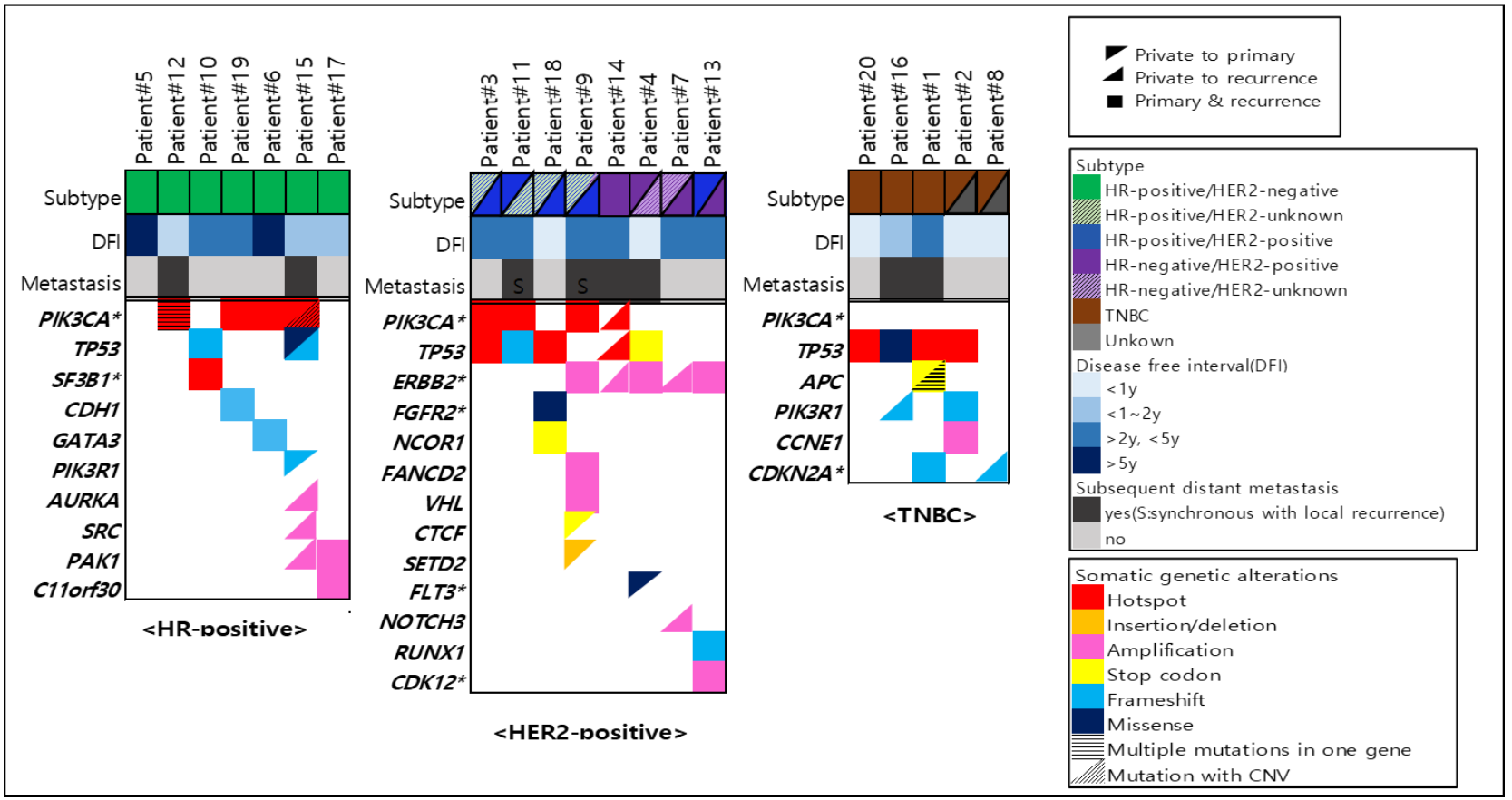

Figure 1 Genomic driver alterations and clinical manifestation of primary and matched recurrent tumors.

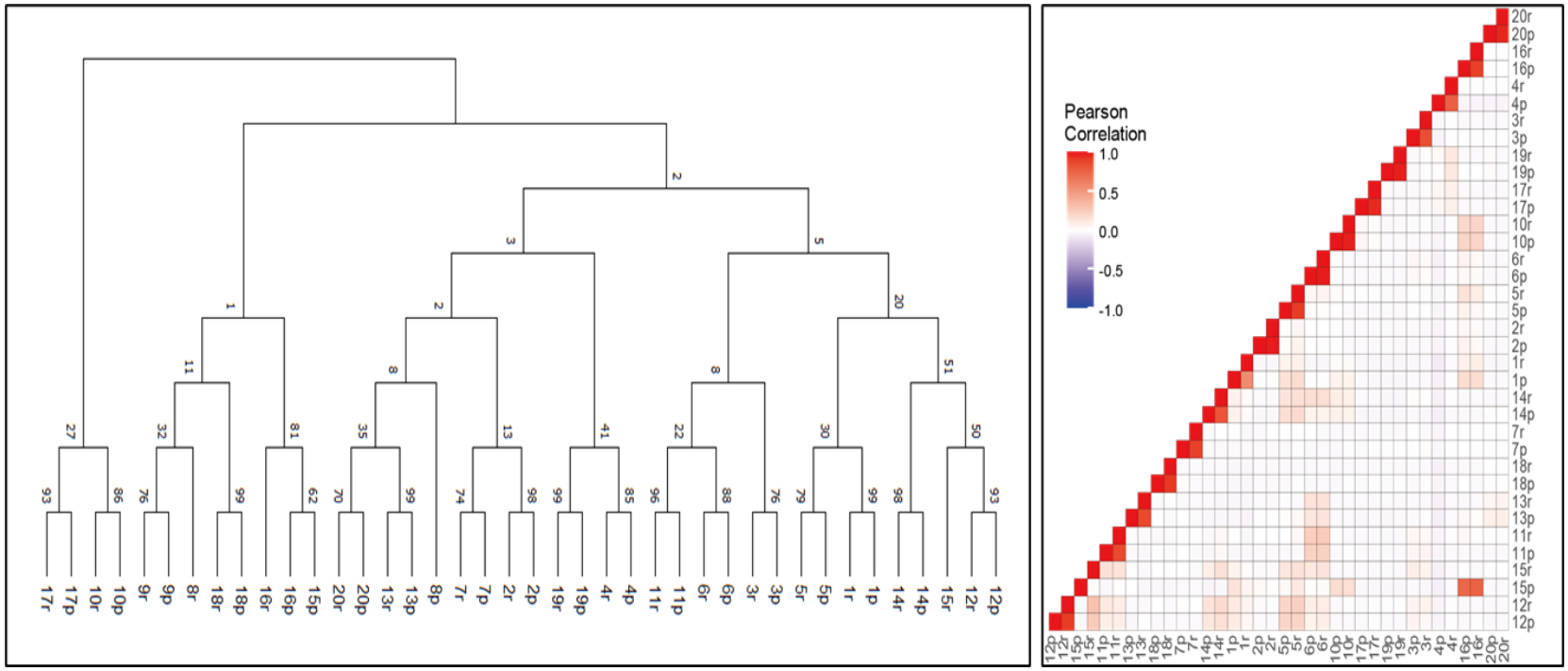

Figure 2 Comparison of genomic features in primary tumors and matched local recurrence. A, Unsupervised hierarchical clustering of samples based on genomic mutations revealed that 17 pairs out of 20 matched samples clustered together. B, Pearson's correlation showed strong positive relation between primary and matched recurrent lesions. The paired samples of Patient #8 and #9 were excluded in this analysis, for primary tumor showed definitely poor quality (Mean depth over target region is below 10 and 150, respectively).

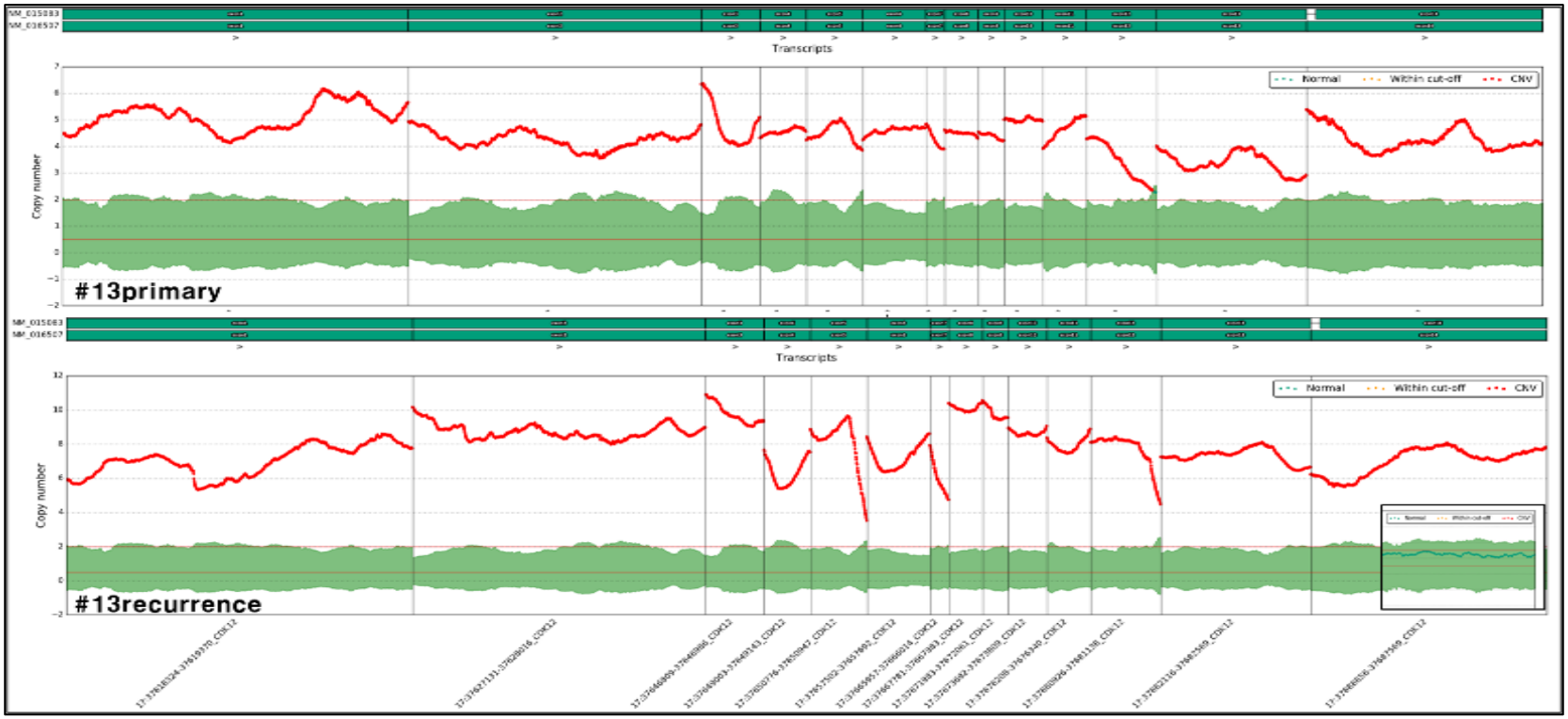

Figure 3 Visualization on an example of concordant copy number variation detected in our series. Gain of copy number in *CDK12* gene was observed in both of primary and recurrent lesion in Patient #13 who had HR status change from ER+/PR- to ER-/PR-.

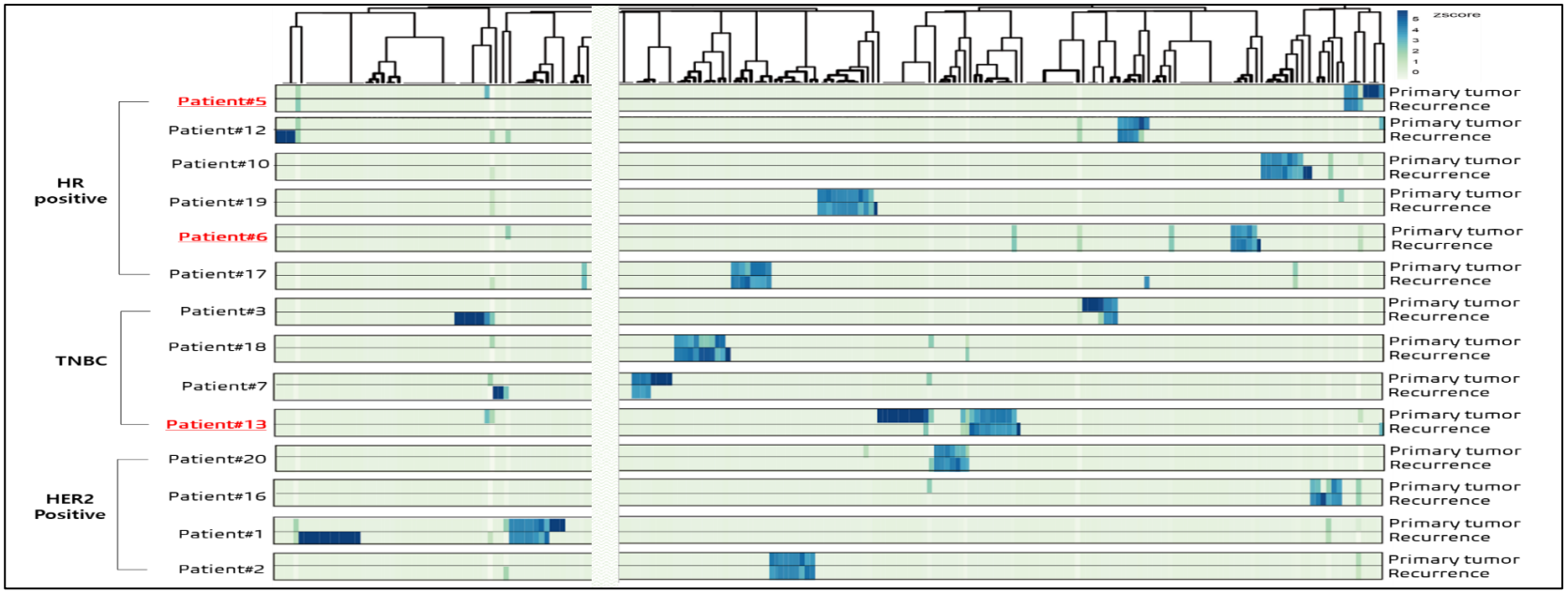

Figure 4 Bar graph representing clusters of primary and matched relapsed tumors in terms of Z scores. Z score plot showed each of matched samples clustered regardless of clinical properties. (Samples with high quality sequencing (;>80% in 100x coverage) included only in this bar graph.) Patient #5 & #6 had long recurrence free interval, 108 and 95 months respectively. Recurrence developed at a different location of remnant breast compared with the site of primary cancer in Patient #6, and Patient #13 revealed HR negative conversion in recurrence (Table 3).

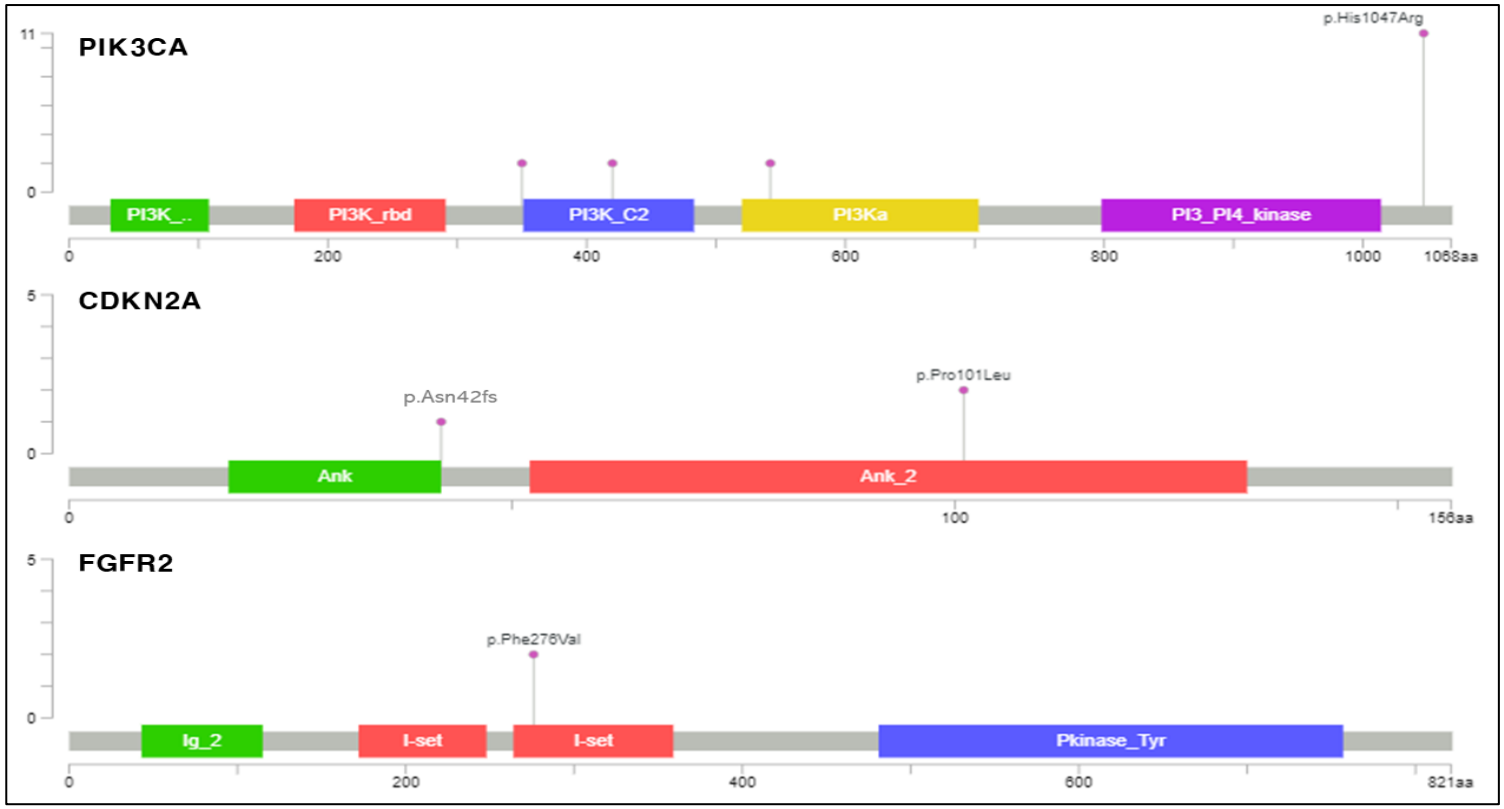

Figure 5 Identification of therapeutic target of functional protein domain or hotspot. The lollipop plot shows mutation in functional domain detected in our series.

Table 1 List of 121 cancer related gene panel

ABL1	CDH1	EZH2	INPP4B	MRE11A	PTEN	ZNF703
ABL2	CDK12	FANCD2	INSR	MST1R	PTK2	
AKT1	CDK4	FBXW7	IRS2	MTOR	PTK6	
AKT2	CDK6	FGF3	JAK1	MYC	RB1	
AKT3	CDKN1B	FGF4	JAK2	NAV3	RET	
ALK	CDKN2A	FGFR1	JAK3	NCOR1	ROS1	
APC	CDKN2B	FGFR2	KDM5B	NF1	RPS6KB1	
AR	CTCF	FGFR3	KIT	NOTCH1	RUNX1	
ARID1A	CTNNB1	FGFR4	KMT2C	NOTCH2	SETD2	
ATM	DDR1	FLT3	KMT2D	NOTCH3	SF3B1	
ATR	DDR2	FLT4	KRAS	PAK1	SRC	
AURKA	EGFR	FOXA1	LTK	PARP1	STK11	
AURKB	EIF4EBP1	FOXM1	MALAT1	PDGFRA	SYK	
BRAF	EP300	GATA3	MAP2K4	PDGFRB	TBX3	
BRCA1	EPHA2	GNAS	MAP3K1	PIK3CA	TLR4	
BRCA2	EPHA3	IDH1	MAP4K5	PIK3CG	TOP2A	
C11orf30	ERBB2	IDH2	MCL1	PIK3R1	TP53	
CBFB	ERBB3	IGF1R	MDM2	PIK3R3	TSC2	
CCND1	ERBB4	IGF2R	MEN1	POLQ	TYK2	
CCNE1	ESR1	IKBKE	MET	PRKDC	VHL	

Table 2 Clinicopathologic characteristics of study subjects

Factors	Primary tumor(N=20)	Recurrent tumor(N=20)
Local recurrence site		
Remnant breast		10 (50%)
Chest wall		10 (50%)
Tumor size		
≤ 2cm	12 (60%)	13 (65%)
> 2cm	8 (40%)	6 (30%)
Unknown	0	1 (5%)
Axillary nodal status		
Node negative	12 (60%)	5 (25%)
Node positive	8 (40%)	7 (35%)
Unknown		8 (40%)
AJCC stage (8th edition)		
I	9 (45%)	
II	9 (45%)	
III	2 (10%)	
Histologic type		
ductal carcinoma	19 (95%)	19 (95%)
lobular carcinoma	1 (5%)	1 (5%)
Subtype		
HR+/HER2−	7 (35%)	5 (25%)
HR+/HER2+	2 (10%)	3 (15%)
HR−/HER2+	2 (10%)	3 (15%)
TNBC	5 (25%)	3 (15%)
HR+/HER2unknown	3 (15%)	3 (15%)
HR−/HER2unknown	1(5%)	1(5%)
Unknown	0	2(10%)
Hormone Receptor		
Positive	12 (60%)	11 (55%)
Negative	8 (40%)	7 (35%)

Unknown	0	2 (10%)
Histologic grade		
1	0	1 (5%)
2	4 (20%)	5 (25%)
3	15 (75%)	11 (55%)
unknown	1 (5%)	3 (15%)
Ki-67		
Low (<10%)	9 (45%)	11 (55%)
High (≥10%)	10 (50%)	5 (25%)
Unkonwn	1 (5%)	4 (20%)
Surgery-Breast		
Mastectomy	10 (50%)	7 (35%)
Breast conservation	10 (50%)	0
Tumor excision	0	13 (65%)
Surgery-Axilla		
SLNBx* Only	7 (35%)	1 (5%)
ALND**	14 (65%)	8 (40%)
Others***	0	11 (55%)
Adjuvant treatment		
Chemotherapy	16 (80%)	
Radiotherapy	8 (40%)	
Hormone therapy	10 (50%)	

*The cutoff value of Ki-67 is 10% (32).

* SLNBx; Sentinel lymph node biopsy **ALND; axillary lymph node dissection

*** "Others" in axillary surgery include "not done" and "unknown".

Table 3 Details of clinical information in 20 patients

Patient	Age	Recurrence Type	Primary lesion location	Recurrent lesion location	Hormone Receptor status (Primary & recurrent tumor)	Adjuvant Treatment	Local recurrence free interval (months)
Patient#5	56	chest wall	Rt upper	RUO	ER+ PR− ER+ PR+	CMF, TMF	108
Patient#12	48	chest wall	Lt inner	Lt upper	ER+ PR+ ER− PR+	FAC, TMX	17
Patient#10	46	remnant breast	RUI	RUI	ER+ PR+ ER+ PR+	CMF, Radiotherapy, TMF	47
Patient#19	44	remnant breast	Lt upper~outer	Lt subareola	ER+ PR+ ER+ PR+	Refused by patient	36
Patient#6	33	remnant breast	LLI	LLO (far outer)	ER+ PR+ ER+ PR+	FAC, Radiotherapy, TMX	95
Patient#15	41	chest wall	Lt. subaroelar	Lt chest wall	ER+ PR− ER+ PR−	AT + D, TMX	21
Patient#17	43	remnant breast	LUI	LUI	ER+ PR+ ER+ PR+	Refused by patient	23
Patient#3	38	chest wall	RUO	RUO	ER+ PR+ ER+ PR−	CMF, TMX	32
Patient#11	64	chest wall	Rt outer	RUO	ER+ PR− ER+ PR−	AT + T, Arimidex	34
Patient#18	54	chest wall	LUI	Lt chest wall	ER+ PR− ER+ PR−	Femara	8
Patient#9	70	chest wall	Rt upper	Rt chest wall	ER+ PR− ER+ PR−	CMF, arimidex	31
Patient#14	45	chest wall	LLO	Lt outer	ER− PR−	Refused by patient	26

Patient#4	42	chest wall	Rt upper	Along previous scar	ER− PR− ER− PR−	Adjuvant EC + Doxetaxol	11
Patient#7	35	remnant breast	LLI	LLI	ER− PR− ER− PR−	FAC, Radiotherapy	26
Patient#13	55	remnant breast	Lt upper	Lt upper	ER− PR− ER+ PR−	FAC, Radiotherapy, Arimidex	32
Patient#20	54	remnant breast	RUI	RUI	ER− PR− ER− PR−	FAC, Radiotherapy	9
Patient#16	37	remnant breast	Lt subareolar	Lt subareola	ER− PR− ER− PR−	AT + D, Radiotherapy	14
Patient#1	40	remnant breast	LUO	Lt outer, periareolar	ER− PR− ER− PR−	CMF, Radiotherapy	49
Patient#2	56	remnant breast	Rt outer	Rt outer	ER− PR− ER− PR−	AC + Taxol, Radiotherapy	6
Patient#8	70	chest wall	LUO	LUO	N/A ER− PR− N/A	FAC	10

* N/A not applicable

Table 4 List of actionable gene detected in our series and target drug

Patient ID	Tumor sample	Gene	Amino acid change known to be (likely) oncogenic	Targeted tumor type	Drugs [Evidence level for targeted tumor type]
#3 #6 #15 #12* #11	Primary & recurrence	*PIK3CA*	H1047R *(Most recurrent SNVs in breast cancer)*	Breast Cancer	Alpelisib + Fulvestrant [1] GDC-0077 [3A] Copanlisib+Fulvestrant [3A]
#14**	Recurrence				
#9	Primary & recurrence	*PIK3CA*	E542K		
#12*	Primary & recurrence	*PIK3CA*	D350G		
#19	Primary & recurrence	*PIK3CA*	C420R		
#1	Primary & recurrence	*CDKN2A*	G101L *(G101W is known to be oncogenic.)*	All Solid Tumors	Abemaciclib [4] Palbociclib [4] Ribociclib [4]
#8**	Recurrence	*CDKN2A*	Asn42fs		
#18	Primary & recurrence	*FGFR2*	F276V *(F276C is likely oncogenic.)*	All Solid Tumors	Debio1347 [4] Erdafitinib [4] AZD4547 [4] BGJ398 [4]
#13	Primary & recurrence	*CDK12*	Amplification *(All truncating mutations are likely oncogenic.)*	All Solid Tumors	Cemiplimab [4] Nivolumab [4] Pembrolizumab [4]
#4	Primary	*FLT3*	V643I	Acute Myeloid Leukemia (no evidence in solid tumor)	High Dose Chemotherapy + Midostaurin [1]
#10	Primary & recurrence	*SF3B1*	K700E	Acute Myeloid Leukemia (no evidence in solid tumor)	H3B-8800 [4]

* Multiple mutations in one patient ** Acquired new alteration in recurrent tumor that did not be found in primary sample. # Excluded alterations in ERBB2 gene in this Table.

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국문 초록

유방암 수술 후 국소 재발암과 원발암의 유전적 변이 비교

국내외 통계에 따르면 유방암은 여성에서 발생하는 암 중 가장 흔한 암이다. 유전자 분석 기술과 유방암의 유전 정보에 기초한 표적 치료는 지속적으로 발전하고 있어 치료의 표적이 되는 유전적 이상(genomic alterations)을 확인하는 것이 임상적으로 중요하다. 그러나 원발암의 치료 후 발생하는 재발암의 경우 원발암에 비해 유전적 정보에 대한 연구 결과가 부족하고, 치료에 대한 내성이 발생한 기전 등을 고려해야 하기 때문에 맞춤형 치료를 제시하는 것이 더욱 어렵다. 유방암의 원발 종양과 재발암에서 나타나는 유전적 변화를 확인하기 위해 우리는 유방암의 근치적 치료를 받은 후 동측의 잔존 유방 및 흉벽의 국소 재발을 경험한 20명의 원발암과 재발암 조직에 대해 121개의 암 관련 유전자를 이용하여 차세대 염기 서열 분석(Next-generation sequencing, NGS)을 시행하였다. 원발암과 동측의 유방 혹은 흉벽에 재발한 종양의 유전적 특성을 분석한 결과 동일한 환자에서 발생한 원발암-재발암 조직은 매우 유사한 결과를 보였다. 일부 연구에서 재발암 발생 위치나 분자 아형과 같은 임상학적/분자적 특성의 변화가 발생한 경우나, 무병 기간이 긴 경우 원발암의 특성과는 완전히 다른 특성을 가진 재발암이 발생할 확률이 높은 것이 보고되었으나, 본 연구에서는 무병기간, 아형, 항암 화학 요법, 호르몬 요법 및 방사선 치료 등과 같은 임상적 요소와 관계없이 재발암의 유전적 변이는 원발암의 유전적 변이와 매우 유사한 것으로 나타났다. 또한 대부분의 환자 (80%)에서 원발암과 국소 재발암은 적어도 1개 이상의 동일한 driver alteration(somatic mutation 또는 CNV)을 공유했으며, 재발암이 발생하면서 유전자 변이가 변화(gain or loss)를 보였던 8명의 환자 중 재발암에서 유방암 치료의 타겟이 될 수 있는 새로운 driver mutation을 발견한 것은 1명이었다.

본 연구는 원발암-재발암 조직의 차세대 염기 서열 분석법을 이용하여 원발암과 동측의 잔존 유방 및 흉벽의 국소 재발암의 유전적 특성을 비교한 첫 번째 연구로, 분석 결과 짝지어진 조직의 유전적 특성은 매우 유사한 것으로 확인되었다. 따라서 원발암 조직의 유전자 분석 결과는 국소 재발 환자에서 맞춤형 치료를 고려할 때에도 유용한 정보를 제

공할 것으로 생각된다.

주요어: 유방암, 국소 재발, 유전자 변이, 차세대 염기 서열 분석법

학번: 2016-26831

의학석사 학위논문

유방암 수술 후 국소 재발암과 원발암의 유전적 변이 비교

Comparison of genomic alterations between primary breast cancer and local recurrence after surgery

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Comparison of genomic alterations between primary breast cancer and local recurrence after surgery

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Abstract

Comparison of genomic alterations between primary breast cancer and remnant breast or ipsilateral chest wall recurrence

According to domestic and worldwide statistics, breast cancer is the most common cancer in females. With advancement in sequencing technique and targeted therapy based on genomic information, to identify targetable genomic abnormalities is becoming more common. However, it remains challenging to optimize treatment for the patients with second breast malignancies after receiving treatment of primary cancers, without better understanding on the differences between primary and relapsed tumors. Herein, we assessed genomic properties between primary and recurrent tumors of ipsilateral breast or chest wall after curative resection. When we compared the results of targeted next-generation exon sequencing with 121 cancer-related genes on formalin-fixed paraffin-embedded (FFPE) matched samples from 20 patients, genomic alterations showed highly concordant between paired samples, regardless of clinicopathological manifestation known as a factors impacting on recurrent tumors' attributes such as the interval to relapse, change of molecular subtype and therapeutic interventions. The analysis based on targeted exome sequencing results revealed that most of primary tumors and matched local recurrences clustered together and had strong positive linear correlation. We found that 16 out of 20 patients had at least one shared somatic mutation or CNAs between the primary and local recurrence, and the gain or loss of alterations throughout tumor progression developed in 8 patients, 1 case of whom acquired new driver mutations that could be targets for breast cancer.

To our knowledge, this is the first study comparing genomic properties between

primary tumor and matched local recurrence using next-generation sequencing. We found the molecular characteristics consistently retained in majority of local recurrence within the territory of remnant breast or ipsilateral chest wall, showing small number of changes in driver mutations. Based on this findings, genomic profiling on primary cancer may give useful information when considering target therapy in patients with local recurrence.

Keyword: breast cancer, local recurrence, genomic alteration, next-generation sequencing (NGS)

Student number: 2016-26831

Contents

11.Abstract (Korean)

List of figures

List of tables

Abbreviations

CNA Copy number alteration

CNV Copy number variation

ER Estrogen receptor

FFPE Formalin fixed paraffin embedded

FISH Fluorescence in situ hybridization

HER2 Human epidermal growth factor receptor 2

HR Hormone receptor

IHC Immunohistochemistry

NGS Next-generation sequencing

NPT New primary tumor

PgR Progesterone receptor (PgR)

SNV single nucleotide variants

TNBC Triple negative breast cancer

TR true recurrence

VUS Variant with unknown significance

Introduction

Breast cancer remains the leading cause of cancer-related mortality in worldwide(1). Although approximately 80% of patients diagnosed breast cancer with early stage and most women with localized disease is amenable to curative therapy, significant proportions of patients suffer from recurrence (2, 3). Breast cancer recurrence may occur in the ipsilateral remnant breast or chest wall where primary cancer was originally diagnosed and resected, regional lymph nodes as well as distant sites. Developing local recurrence does not always herald distant metastases but regarded as the high risk of subsequent distant relapse and poor prognosis (4). Several reports have suggested that local recurrence at the site of ipsilateral breast or chest wall is regarded as true recurrence (TR) consistent with the regrowth of remained malignant cells, which is an independent predictor of distant metastatic disease and poor survival(5, 6). Others have indicated, however, new primary tumor (NPT) described as "de novo malignancies" just gave rise to at the very site previously resected, that it showed quite different features with previously treated primary tumors(7-9). Several studies have attempted to classify these differences by using tumor location at recurrence, histologic subtype or pathologic criteria(8, 10). Some researchers suggested a genetic classification using clonal analysis, genomic expression profiling or quantitative DNA fingerprinting as a potentially valuable tool to improve understanding for the second breast malignancies(11-13).

With advancement in genomic analysis technique and getting genomic data of the tumors more frequently, genomic alteration

s throughout tumor progression may provide an insight helpful for comprehending the features of the relapsing tumors and determining appropriate treatment after recurrence. Genomic evolution have been increasingly studied and have showed that mutational processes are mostly similar in primary and relapsed tumors but continue

to acquire or lose mutations (14). As recent clinical trials enable to select targeted drugs based on genomic information for tailored therapy, genomic profiling has become more important, especially in recurrence or treatment-resistant cancers. The aim of this study is to compare genomic information and targetable genomic changes in both of primary tumors and local recurrence lesions through targeted sequencing of cancer-related genes, it might be useful when determining therapeutic target agents for recurrent tumors.

Patients and Methods

Tumor samples and clinicopathologic information

We reviewed the records of patients with invasive breast cancer who underwent operations for both of primary and relapsed tumor from 2002 to 2015 at the Seoul National University Hospital (Seoul, South Korea). Patients were selected on basis of primary-local recurrent matched tissue availability. Formalin-fixed paraffin blocks in both of primary breast cancer and matched recurrent tumor specimens were obtained. The Formalin fixed paraffin embedded (FFPE) samples were evaluated by a pathologist for selection of tumor areas for microdissection. To exclude germline mutations, we further sequenced the selected patients' blood sample if it had been stocked in our biorepository (Repository of lab of breast cancer biology, Seoul National University Hospital, IRB number: 1405-088-580). DNA was purified and extracted using ReliaPrep™ FFPE gDNA Miniprep System (Promega) and the quality of DNA was assessed through a TapeStation Systems according to the manufacturer's instructions. We confirmed DNA purity as ratio of A260/A280 is between 1.8 and 2.0. Clinicopathologic information including immunohistochemistry and Fluorescence in situ hybridization (FISH) was collected by a retrospective review of patient

medical records. The location of relapsed tumors which were documented in medical records or identified in mammography were obtained whether the tumor recurred at or near the vicinity of the primary tumor site. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB number: 1712-150-911).

Immunohistochemistry (IHC) staining and interpretation

This method was previously used in a published study (15). The samples were immunostained with the following antibodies according to the manufacturers' instructions: Anti-estrogen receptor(ER) (1:100; 1D5; Dako, Glostrup, Denmark), anti-progesterone receptor (PgR) (1:100; 636; Dako), and anti-HER2 (1:200; A0485; Dako). Positive ER and PgR expression were defined as nuclear staining in 1% or more of tumor cells. The HER2 membranous staining was scored on a scale of 0 to 3+ according to the HercepTest protocol. For tissue samples with a human epidermal growth factor receptor-2 (HER2) staining score of 2+, additional HER2 FISH testing was considered. HER2 status was considered positive when the IHC score was 3+ or the gene copy ratio of HER2/CEP17 by FISH was 2.2 or higher.

Genomic profiling

We performed a targeted next-generation sequencing (NGS) assay on FFPE samples by using a multi-gene panel (SNUH BCC (Seoul National University Hospital Breast Care Center) Panel) with average 356X sequencing depth. The SNUH BCC panel consisting of 121 genes was developed based on our previous study in which we had performed whole-exome sequencing and RNA-Seq of 200 pairs of matched clinical breast cancer and normal samples from Korean breast cancer patients. In addition, we had analyzed the mutations, CNAs, and gene expression results of approximately 3000 clinical breast cancer samples in the TCGA and METABRIC databases. As a result, we chose oncogenes, tumor

suppressor genes, or breast cancer-associated genes that showed a highly frequent mutations, genomic copy number variations and expression changes in breast cancer tissues (Table 1). The SNUH BCC panel is unique compared with other cancer panels based on NGS because it includes a certain portion of novel breast cancer-associated genes that have not been included in other recent popular and conventional cancer panels. In this regard, the SNUH BCC panel is not only targeted to worldwide breast cancer patients but is also ethnically directed to Korean breast cancer patients for diagnosis and therapeutic prognostic prediction (16).

Sequence alignment, variant calling and driver landscape for therapeutic target

Raw FASTQ file was filtered and trimmed using Adaptor removal 2.2.2. Burrows-Wheeler aligner (BWA; version 0.7.10) mem with default option was used to align reads to human reference genome sequence GRCh37. Sequence alignment map (SAM) file was converted to BAM format using samtools (version 1.1). Picard tool (version 1.115) was used to sort and remove duplications. GATK (version 4.0.4.0) was used to perform base quality score re-calibration. Samtools mpileup was used to create mpileup file with minimum base-quality of 17 and varscan (version 2.4.0) was used to call variants. Minimum variant frequency was set to 1%, minimum coverage was set to 8, minimum supporting reads was set to 2, and Strand-filter was applied. We excluded germline SNVs as well as technical artifacts observed in the blood samples and known variants from public databases such as the Genome Aggregation Database (gnomAD) and 1000 Genomes. And then variations were filtered by using the dbSNP archive (http://www.ncbi.nlm.nih.gov/projects/ SNP/) and annotated for known somatic mutations by using the COSMIC (17, 18). Somatic copy number alterations (CNAs) were called by calculating the number of mapped reads and then performing normalization using 40 tumor samples and reference samples. The threshold for gains was > 4.0-fold

and for loss <0.25−fold. Each variant is classified according to the Association of Molecular Pathology (AMP) guidelines for somatic cancer variants (19). Any variants classified as benign, likely benign or variants with unknown significance (VUS) were excluded for driver mutation landscape. We considered known oncogenic/likely oncogenic variants and hotspots as reported in the literature so far, and we defined alterations deemed a target of FDA−approved drug or investigational therapeutics on basis of a review of TARGET database v3 in Cancer Genome Analysis (CGA) and oncology knowledge database (OncoKB) database as "actionable" (20, 21). Actionable alterations with clinical or biologic evidence supporting an association with response to targeted drugs were stratified by level of evidence from OncoKB. We used "R version 4.0.1" to compare and visualize the differences and similarities between primary tumors and matched recurrence. The results from paired samples that can be sequenced with high quality were included in calculation to well visualize the patterns of matched samples. MutationMapper was used for visualization of actionable mutations. (22)

Results

Patient and sample characteristics

The clinicopathological characteristics of the 20 patients in our study were summarized at Figure 1 and we described more detail information in Table 2 and 3. The mean age at primary breast cancer diagnosis was 48.6 years (range 37–70) and median follow−up period from the date of primary cancer diagnosis to last visit record to our clinic was 146 months. The mean local recurrence free interval was 32 months (range 6–108). Twelve patients (60%) were diagnosed with hormone receptor (HR) positive primary breast cancer including 3 patients with unknown HER2 status and the number of patients identified with HER2 amplification and

triple negative primary breast cancer (TNBC;ER−, PR−, and HER2−negative) were 4(20%) and 5(25%), respectively. At presentation with primary cancer, 8 patients (40%) had axillary lymph node metastasis and 16 women received neoadjuvant/adjuvant chemotherapy. As the sites of relapse were defined according to surgery type, recurrence at remnant breast (50%) for the patients who underwent breast conserving surgery (BCS) and ipsilateral chest walls (50%) for mastectomy. Eight patients developed subsequent distant metastasis, and two showed synchronous metastatic lesion with local recurrence (Figure 1, Table 2).

When comparing molecular types of the primary and matched recurrent lesions, local recurrence mostly had concordant immunohistochemical characteristics [i.e. hormone receptors (HR) and HER2 status] with that of primary tumor. All triple−negative and HER−2 status retained their characteristics in the matched recurrent lesions, whereas 4 patients appeared to have discordant ER or PgR status. More specifically, three patients lost ER or PgR during progression and 1 recurrent tumor gained PgR. (Table 2, 3).

Somatic mutations and copy number variations of primary tumors and local recurrence

In 40 FFPE samples, we identified a total of 90 driver somatic alterations or copy number variations (CNVs) in 25 genes (Figure 1). There is no germline mutation in 16 blood samples. Genomic sequencing using the 121−gene panel detected at least one known oncogenic or likely oncogenic alteration in 36 of 40 samples (mean 2.25; range, 0–6). In the manner of copy number calculation from NGS data, as ERBB2 gain were observed in the 8 of the 10 tumors that were identified as HER2−positive by IHC or FISH, so the HER2 copy−number amplification results were found to be discordant with that of tested by IHC or FISH in 2 cases (Figure 1). The most frequently mutated gene was *TP53* (23.3%), followed by *PIK3CA* (20.0%) and most common amino acid change was H1047R (11 samples) in

PIK3CA gene. When we excluded the tumors with unknown IHC results, *TP53* alterations were found in 87.5% out of triple-negative samples, 50% in HER2-positive samples, and 43.5% in HR-positive samples. Whereas, *PIK3CA* alterations were observed in 60.9% of HR-positive tumors and only 1 of HR-negative samples. At least one alteration was detected in 6 genes associated with clinical action irrespective of the kind of targeted tumor type, excluding amplifications in *ERBB2* gene (Figure 1 & Table 3).

Comparison of genomic properties in primary tumors and matched local recurrence

The unsupervised hierarchical clustering of the tumors based on genomic mutations revealed that 17 pairs out of 20 matched samples clustered together (Figure 2A). Additional correlation analysis of 18 patients excluding 2 pairs with low sample quality showed the recurrent tumors' genomic characteristics were closely related with that of primary lesions with respect to targeted analysis (Pearson's correlation, Figure 2B).

In a driver perspective, 54 (83.1%) of the 65 somatic mutations and 18 (72%) of 25 CNVs detected were found to be concordant in primary cancer and matched recurrence (Figure 3). Eight patients showed differences in genomic alterations between primary tumors and recurrence, 6 relapsed tumors of whom acquired deleterious alterations compared to primary tumors.

Clinicopathological features and genomic concordance

When we calculated Z score to assess and visualize genomic similarities among samples, the matched samples were mostly concordant regardless of tumor location or molecular subtype (Figure 4, Table 3). Though all pairs were composed of a same histologic subtype with invasive ductal carcinomas in most cases and relapsed tumors mainly developed at near the primary tumor, one recurrent lesion

occurred in remnant breasts was located at a site different from the primary (Patient#6). Of the 12 patients with HR-positive tumors at initial diagnosis, in terms of molecular subtypes, 3 patients revealed changes of HR status but retained either of ER or PR positivity in recurrent tumors and conversion to HR-negative subtype occurred in one patient (Patient #13). Furthermore, local recurrence free interval ranged from 6 to 108 months, showing over 7 years interval in two patients (Patient #5 and #6). Figure 4 showed that genomic features of matched recurrent tumors were highly concordant with those of primary tumors in Patient #5, #6 and #13. The paired samples in these 3 patients had also identical driver mutations and CNVs (Figure 3). Additionally, recurrent tumor showed similarity with the primary tumor in 17 patients who had received adjuvant therapy, 16 women of whom received cytotoxic chemotherapy (Figure 4, Table 3).

Clinical actionability of molecular targets

Overall, 15 of the 20 patients had genomic alterations in approved or potentially "actionable" genes including HER2 amplifications. With exception of HER2 amplifications, genomic profiling detected new potentially actionable alterations that had not been previously identified by standard-of care testing in 14 patients. The targets identified were listed in Table 5. Oncogenic mutations in *PIK3CA* gene were most frequently detected for therapeutic target. Somatic mutations in *CDKN2A* (p. G101L and p. N42Rfs) and *FGFR2* (p. F276V) genes were considered as likely oncogenic changes in well-known functional protein domain that regulate the cell growth or division (Figure 5)(23, 24). We found somatic alterations in six genes with breast cancer-specific or all solid tumor-acceptable target drugs, whereas two (*FLT3* and *SF3B1*) of targetable genes have not yet be approved for breast cancer (Table 4). Six patients subsequently suffered metachronous distant metastasis after receiving treatment for local recurrence. Whereas only one recurrent tumor (patient #14) showed new targetable genomic alteration in *PIK3CA* gene which was not found in primary sample, and the other 5 patients

showed consistent results between primary and recurrent tumors from an actionable point of view.

Discussion

Personalized therapy is based on molecular characterization of the tumor and target aberrations that drive tumor growth(25). As the NGS techniques and target therapy based on genomic information have been advanced, the genetic landscape allows tailored therapy and will overcome tumoral heterogeneity and its resistance to traditional anticancer agents (26, 27).

Breast cancer is a highly heterogeneous disease; the same primary tumor frequently showed different genomic profiles and its recurrent lesion also acquired new molecular aberrations compared to their primary tumors. While several studies have showed the result on genomic evolution between primary breast cancer and its' metastasis (14, 28-30), we studied genomic alterations confined to local recurrence in remnant breast or chest wall where primary tumor had been removed. With respect to ipsilateral breast tumor recurrence, many studies have been tried to classify the new primary and true recurrence on basis of clinical features such as histology, molecular subtype or location between primary and relapsed tumors. To our knowledge, this is the first study comparing between primary tumor and matched local recurrence based on genomic analysis using sequencing data.

We performed target sequencing of 121 cancer-related genes to evaluate and compare the spectrum of genomic alterations between primary breast cancer and matched local recurrence in remnant breast or ipsilateral chest wall after mastectomy. A large proportion of primary and matched recurrent tumors included in our analysis seemed to have similar genomic properties irrespective of clinicopathological characteristics, showing small number of changes in driver

alterations. Previous studies have considered clinical and molecular features like as different histology, tumor occurrence at distant site from primary tumor bed and molecular subtype discordance as an indicator to distinguish between de novo primary tumor and regrowth of remained malignant cell and to impact on post-recurrence survival and potential treatment options(31). We identified, however, secondary cancer developed in remnant breast or chest wall was found as true recurrence rather than new primary tumor, retaining their genomic characteristics of primary cancer even when recurrent tumor occurred at a different site with long periods after primary cancer treatment or with changes in molecular types. Moreover, new primary tumors were known to be developed after a longer interval from their initial treatment than patients with true recurrence, but recurrent lesions developed after long period over than 7 and 9 years in two patients had similar attributes to those of primary tumors.

Previous studies have presented that somatic alterations were more frequently found in recurrent lesions than primary tumors, especially in the analysis focused on distant metastasis (14, 28, 29). However, our result suggested that local relapse within the area of remnant breast or ipsilateral chest wall had similar attributes with primary cancer, and significant evolution throughout local relapse rarely happened (Figure 4). Yates et al revealed genomic evolution exerted by therapeutic interventions response to treatment exposures, as truncating mutations were gained after chemotherapy or cancer genes potentially actionable driver mutations emerged during endocrine therapy(14). When we gave consideration that most of patients included in our study had received systemic adjuvant therapy (chemotherapy and/or hormone therapy) or radiotherapy for local control, however, overall genomic properties of relapsed tumors did not seem to be affected by therapeutic interventions.

In terms of actionable genomic mutations, one patient acquired driver mutation that can be a target for breast cancer-specific drugs (Patient #14, H1047R in

PIK3CA gene) and targetable mutation in *CDKN2A* gene emerged in the other one case(Patient #8). Because the quality of primary FFPE sample in case of Patient#8 was definitely poor and was sequenced with very low depth coverage, there are limitation to interpret the differences. The amplifications in *CNK12* gene and SNVs in *FLT3* and *SF3B1* which were described in Table 5 might be potential candidates related with clinical action with limited evidence, for targeted mutation type or tumor type are different from those of literatures.

Our study has several limitations. The number of cases included in this study is small, for obtaining both of primary tumor and matched FFPE block was challenging. Limited sample size resulted in the failure of the comprehensive statistical analysis on the correlation between genomic alterations and clinical characteristics such as subtypes or disease-free interval. Additionally, as our study was designed as retrospective manner and the samples had not collected with purpose of genomic analysis, the quality of FFPE samples varied depending on the archived periods or status of storage. It might have affected the sequencing result reducing coverage depth in certain region or increasing the rate of variants detected in some of the samples. For these reasons, we consequently applied very strict cut-offs both of depth and allelic frequency for the confirmation of NGS variant calls. Finally, this study limited to perform targeted sequencing only focused on genomic mutations and CNVs and did not analyze other abnormalities in DNA methylation or phosphorylation, gene fusion, RNA or protein expression that can provide novel information. Further comprehensive analysis integrating the genomic profiles, tumor biology and clinical information is essential for better understanding.

In conclusion, we found that genomic characteristics of primary tumor consistently retained in majority of local recurrence within the territory of remnant breast or ipsilateral chest wall, showing small number of changes in driver alterations. So genomic profiling on primary cancer is thought to provide useful information when considering tailored treatment in patients with local recurrence.

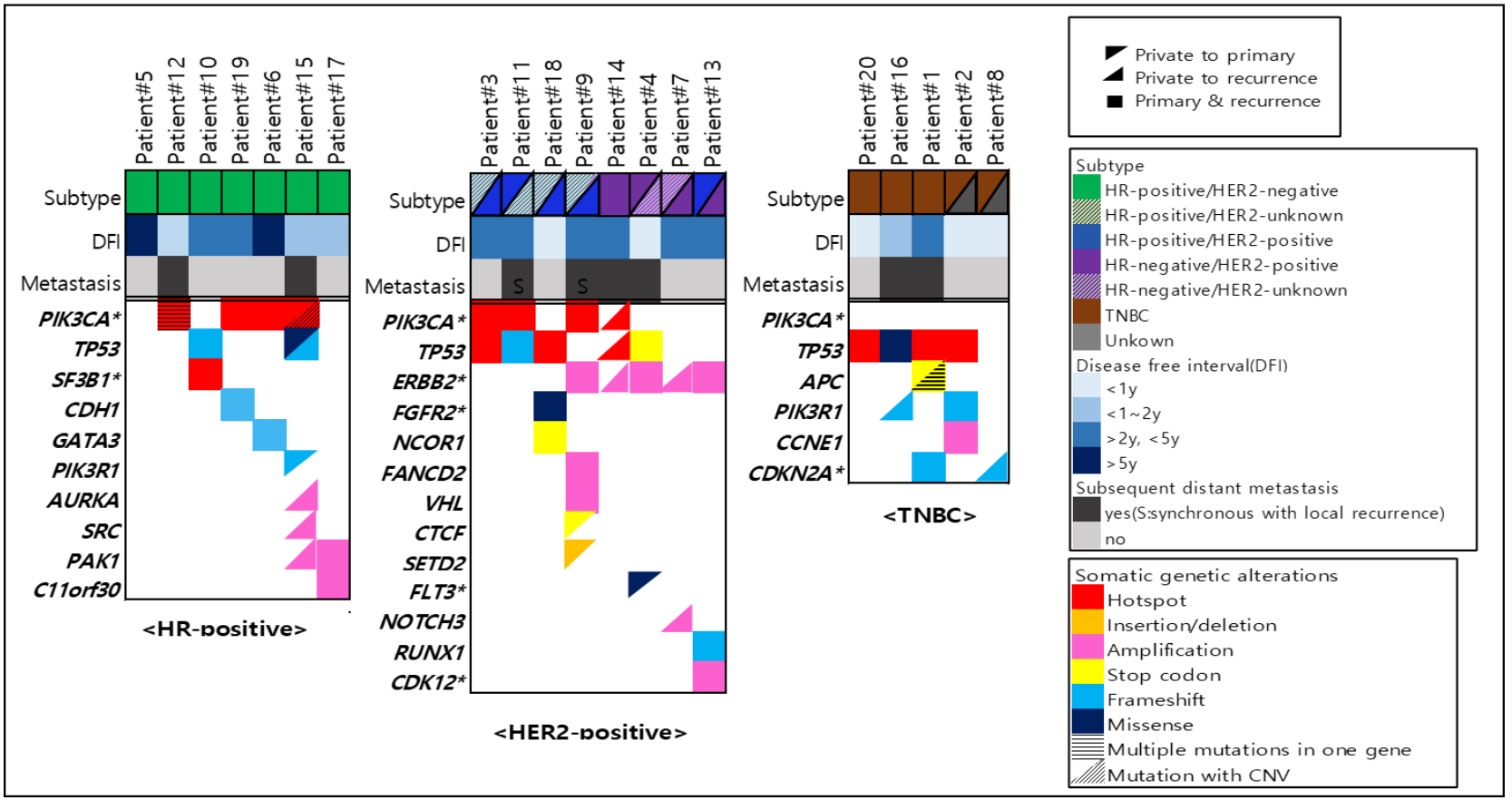

Figure 1 Genomic driver alterations and clinical manifestation of primary and matched recurrent tumors.

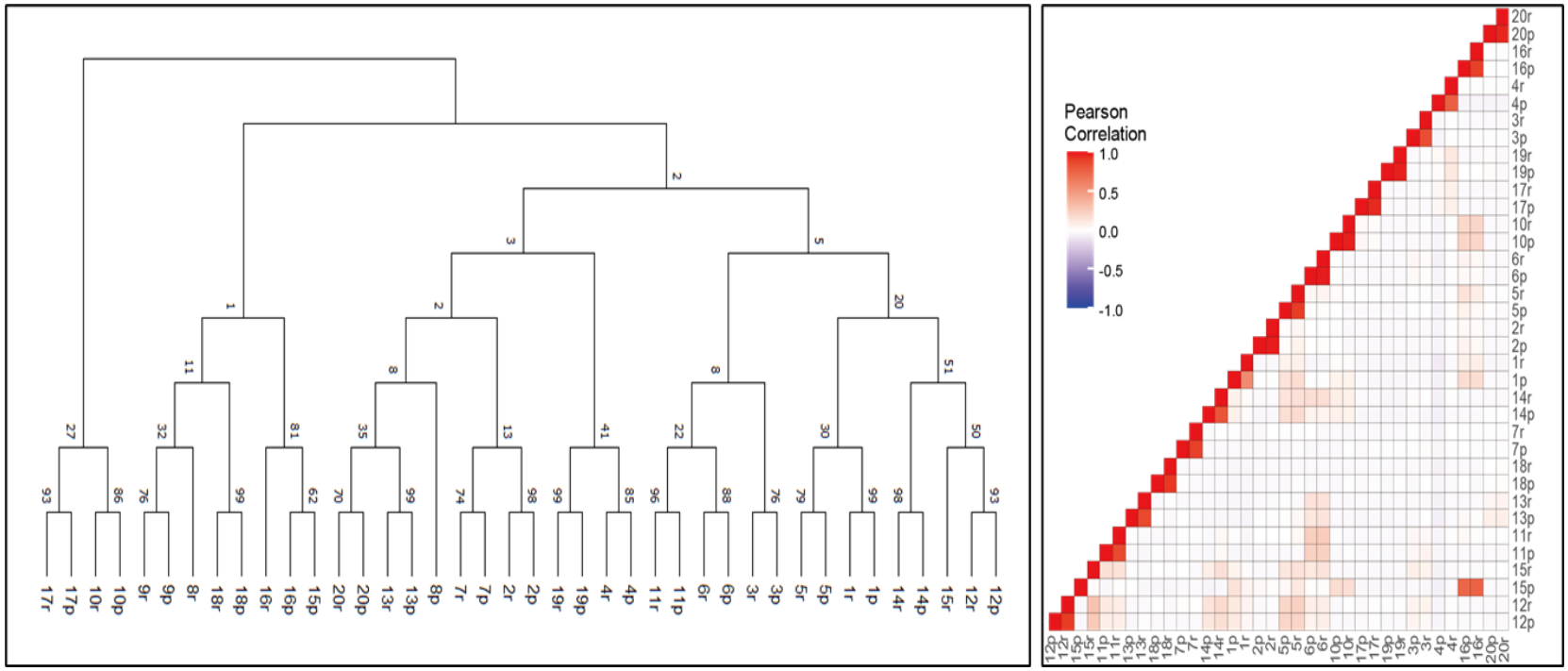

Figure 2 Comparison of genomic features in primary tumors and matched local recurrence. A, Unsupervised hierarchical clustering of samples based on genomic mutations revealed that 17 pairs out of 20 matched samples clustered together. B, Pearson's correlation showed strong positive relation between primary and matched recurrent lesions. The paired samples of Patient #8 and #9 were excluded in this analysis, for primary tumor showed definitely poor quality (Mean depth over target region is below 10 and 150, respectively).

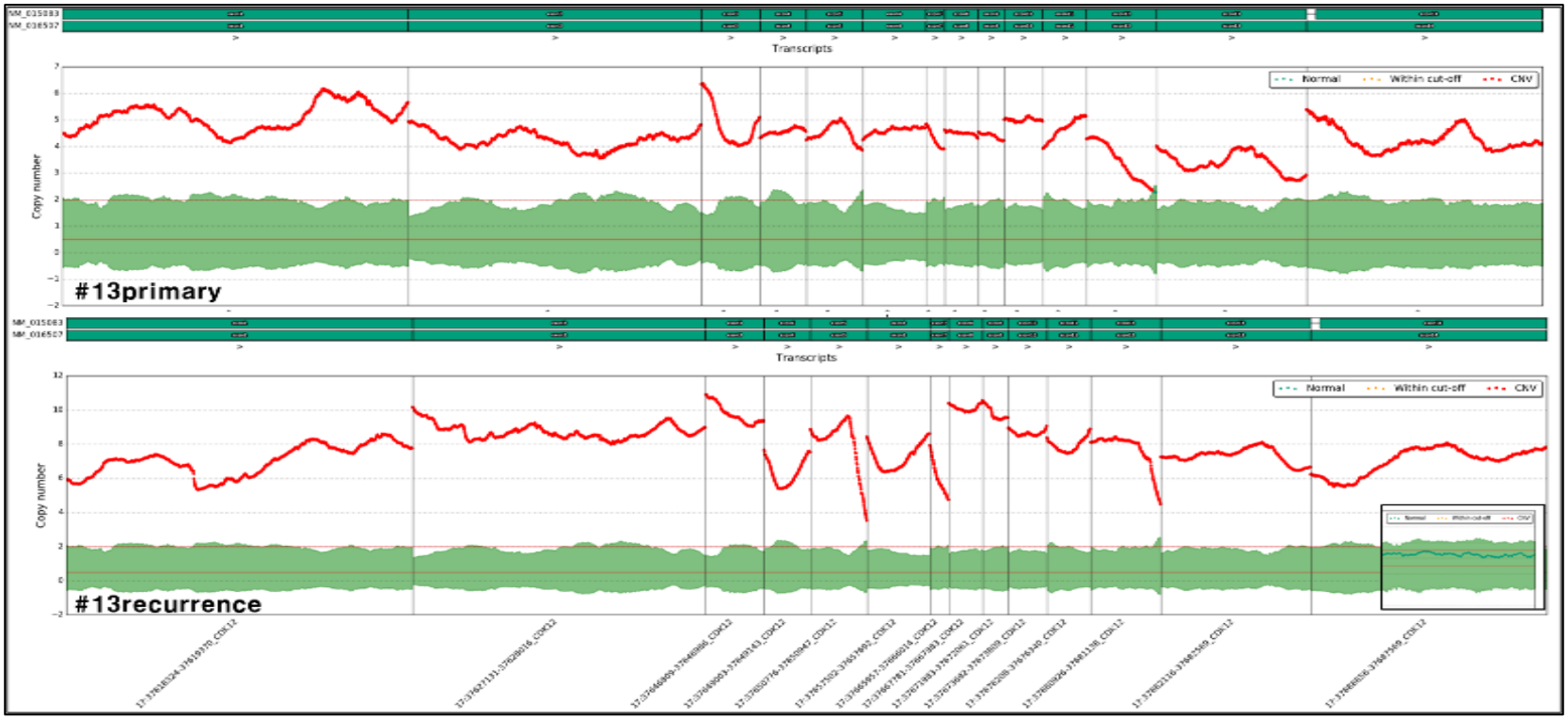

Figure 3 Visualization on an example of concordant copy number variation detected in our series. Gain of copy number in *CDK12* gene was observed in both of primary and recurrent lesion in Patient #13 who had HR status change from ER+/PR- to ER-/PR-.

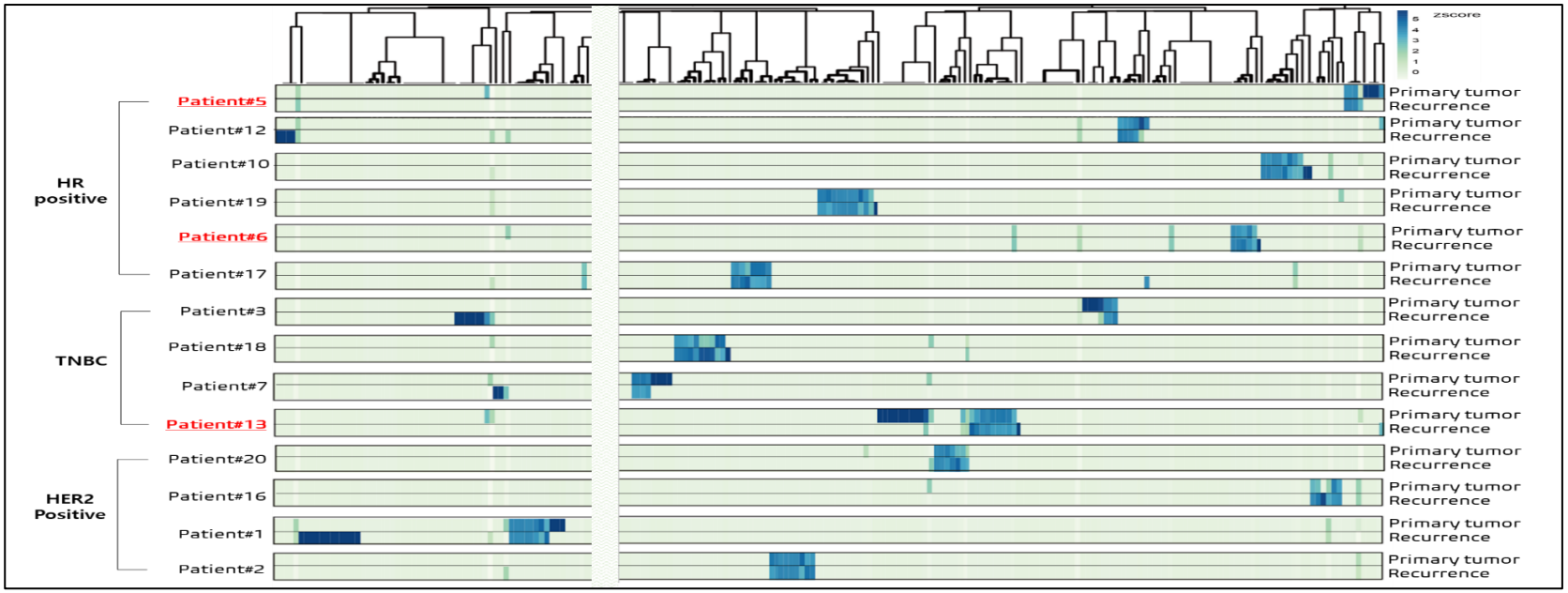

Figure 4 Bar graph representing clusters of primary and matched relapsed tumors in terms of Z scores. Z score plot showed each of matched samples clustered regardless of clinical properties. (Samples with high quality sequencing (;>80% in 100x coverage) included only in this bar graph.) Patient #5 & #6 had long recurrence free interval, 108 and 95 months respectively. Recurrence developed at a different location of remnant breast compared with the site of primary cancer in Patient #6, and Patient #13 revealed HR negative conversion in recurrence (Table 3).

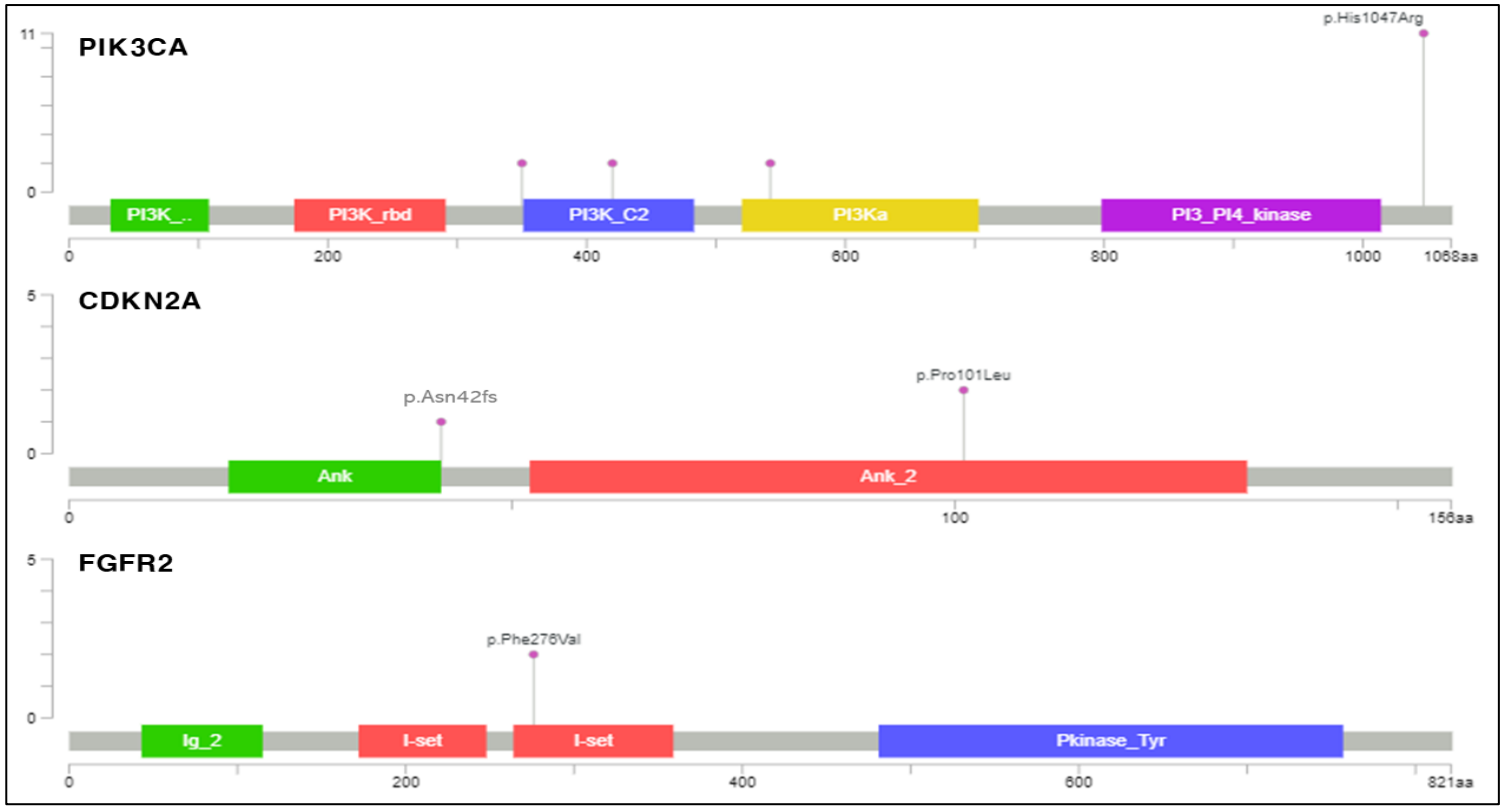

Figure 5 Identification of therapeutic target of functional protein domain or hotspot. The lollipop plot shows mutation in functional domain detected in our series.

Table 1 List of 121 cancer related gene panel

ABL1	CDH1	EZH2	INPP4B	MRE11A	PTEN	ZNF703
ABL2	CDK12	FANCD2	INSR	MST1R	PTK2	
AKT1	CDK4	FBXW7	IRS2	MTOR	PTK6	
AKT2	CDK6	FGF3	JAK1	MYC	RB1	
AKT3	CDKN1B	FGF4	JAK2	NAV3	RET	
ALK	CDKN2A	FGFR1	JAK3	NCOR1	ROS1	
APC	CDKN2B	FGFR2	KDM5B	NF1	RPS6KB1	
AR	CTCF	FGFR3	KIT	NOTCH1	RUNX1	
ARID1A	CTNNB1	FGFR4	KMT2C	NOTCH2	SETD2	
ATM	DDR1	FLT3	KMT2D	NOTCH3	SF3B1	
ATR	DDR2	FLT4	KRAS	PAK1	SRC	
AURKA	EGFR	FOXA1	LTK	PARP1	STK11	
AURKB	EIF4EBP1	FOXM1	MALAT1	PDGFRA	SYK	
BRAF	EP300	GATA3	MAP2K4	PDGFRB	TBX3	
BRCA1	EPHA2	GNAS	MAP3K1	PIK3CA	TLR4	
BRCA2	EPHA3	IDH1	MAP4K5	PIK3CG	TOP2A	
C11orf30	ERBB2	IDH2	MCL1	PIK3R1	TP53	
CBFB	ERBB3	IGF1R	MDM2	PIK3R3	TSC2	
CCND1	ERBB4	IGF2R	MEN1	POLQ	TYK2	
CCNE1	ESR1	IKBKE	MET	PRKDC	VHL	

Table 2 Clinicopathologic characteristics of study subjects

Factors	Primary tumor(N=20)	Recurrent tumor(N=20)
Local recurrence site		
Remnant breast		10 (50%)
Chest wall		10 (50%)
Tumor size		
≤ 2cm	12 (60%)	13 (65%)
> 2cm	8 (40%)	6 (30%)
Unknown	0	1 (5%)
Axillary nodal status		
Node negative	12 (60%)	5 (25%)
Node positive	8 (40%)	7 (35%)
Unknown		8 (40%)
AJCC stage (8th edition)		
I	9 (45%)	
II	9 (45%)	
III	2 (10%)	
Histologic type		
ductal carcinoma	19 (95%)	19 (95%)
lobular carcinoma	1 (5%)	1 (5%)
Subtype		
HR+/HER2−	7 (35%)	5 (25%)
HR+/HER2+	2 (10%)	3 (15%)
HR−/HER2+	2 (10%)	3 (15%)
TNBC	5 (25%)	3 (15%)
HR+/HER2unknown	3 (15%)	3 (15%)
HR−/HER2unknown	1(5%)	1(5%)
Unknown	0	2(10%)
Hormone Receptor		
Positive	12 (60%)	11 (55%)
Negative	8 (40%)	7 (35%)

Unknown	0	2 (10%)
Histologic grade		
1	0	1 (5%)
2	4 (20%)	5 (25%)
3	15 (75%)	11 (55%)
unknown	1 (5%)	3 (15%)
Ki-67		
Low (<10%)	9 (45%)	11 (55%)
High (≥10%)	10 (50%)	5 (25%)
Unkonwn	1 (5%)	4 (20%)
Surgery-Breast		
Mastectomy	10 (50%)	7 (35%)
Breast conservation	10 (50%)	0
Tumor excision	0	13 (65%)
Surgery-Axilla		
SLNBx* Only	7 (35%)	1 (5%)
ALND**	14 (65%)	8 (40%)
Others***	0	11 (55%)
Adjuvant treatment		
Chemotherapy	16 (80%)	
Radiotherapy	8 (40%)	
Hormone therapy	10 (50%)	

*The cutoff value of Ki-67 is 10% (32).

* SLNBx; Sentinel lymph node biopsy **ALND; axillary lymph node dissection

*** "Others" in axillary surgery include "not done" and "unknown".

Table 3 Details of clinical information in 20 patients

Patient	Age	Recurrence Type	Primary lesion location	Recurrent lesion location	Hormone Receptor status (Primary & recurrent tumor)	Adjuvant Treatment	Local recurrence free interval (months)
Patient#5	56	chest wall	Rt upper	RUO	ER+ PR− ER+ PR+	CMF, TMF	108
Patient#12	48	chest wall	Lt inner	Lt upper	ER+ PR+ ER− PR+	FAC, TMX	17
Patient#10	46	remnant breast	RUI	RUI	ER+ PR+ ER+ PR+	CMF, Radiotherapy, TMF	47
Patient#19	44	remnant breast	Lt upper~outer	Lt subareola	ER+ PR+ ER+ PR+	Refused by patient	36
Patient#6	33	remnant breast	LLI	LLO (far outer)	ER+ PR+ ER+ PR+	FAC, Radiotherapy, TMX	95
Patient#15	41	chest wall	Lt. subaroelar	Lt chest wall	ER+ PR− ER+ PR−	AT + D, TMX	21
Patient#17	43	remnant breast	LUI	LUI	ER+ PR+ ER+ PR+	Refused by patient	23
Patient#3	38	chest wall	RUO	RUO	ER+ PR+ ER+ PR−	CMF, TMX	32
Patient#11	64	chest wall	Rt outer	RUO	ER+ PR− ER+ PR−	AT + T, Arimidex	34
Patient#18	54	chest wall	LUI	Lt chest wall	ER+ PR− ER+ PR−	Femara	8
Patient#9	70	chest wall	Rt upper	Rt chest wall	ER+ PR− ER+ PR−	CMF, arimidex	31
Patient#14	45	chest wall	LLO	Lt outer	ER− PR−	Refused by patient	26

Patient#4	42	chest wall	Rt upper	Along previous scar	ER− PR− ER− PR−	Adjuvant EC + Doxetaxol	11
Patient#7	35	remnant breast	LLI	LLI	ER− PR− ER− PR−	FAC, Radiotherapy	26
Patient#13	55	remnant breast	Lt upper	Lt upper	ER− PR− ER+ PR−	FAC, Radiotherapy, Arimidex	32
Patient#20	54	remnant breast	RUI	RUI	ER− PR− ER− PR−	FAC, Radiotherapy	9
Patient#16	37	remnant breast	Lt subareolar	Lt subareola	ER− PR− ER− PR−	AT + D, Radiotherapy	14
Patient#1	40	remnant breast	LUO	Lt outer, periareolar	ER− PR− ER− PR−	CMF, Radiotherapy	49
Patient#2	56	remnant breast	Rt outer	Rt outer	ER− PR− ER− PR−	AC + Taxol, Radiotherapy	6
Patient#8	70	chest wall	LUO	LUO	N/A ER− PR− N/A	FAC	10

* N/A not applicable

Table 4 List of actionable gene detected in our series and target drug

Patient ID	Tumor sample	Gene	Amino acid change known to be (likely) oncogenic	Targeted tumor type	Drugs [Evidence level for targeted tumor type]
#3 #6 #15 #12* #11	Primary & recurrence	*PIK3CA*	H1047R *(Most recurrent SNVs in breast cancer)*	Breast Cancer	Alpelisib + Fulvestrant [1] GDC-0077 [3A] Copanlisib+Fulvestrant [3A]
#14**	Recurrence				
#9	Primary & recurrence	*PIK3CA*	E542K		
#12*	Primary & recurrence	*PIK3CA*	D350G		
#19	Primary & recurrence	*PIK3CA*	C420R		
#1	Primary & recurrence	*CDKN2A*	G101L *(G101W is known to be oncogenic.)*	All Solid Tumors	Abemaciclib [4] Palbociclib [4] Ribociclib [4]
#8**	Recurrence	*CDKN2A*	Asn42fs		
#18	Primary & recurrence	*FGFR2*	F276V *(F276C is likely oncogenic.)*	All Solid Tumors	Debio1347 [4] Erdafitinib [4] AZD4547 [4] BGJ398 [4]
#13	Primary & recurrence	*CDK12*	Amplification *(All truncating mutations are likely oncogenic.)*	All Solid Tumors	Cemiplimab [4] Nivolumab [4] Pembrolizumab [4]
#4	Primary	*FLT3*	V643I	Acute Myeloid Leukemia (no evidence in solid tumor)	High Dose Chemotherapy + Midostaurin [1]
#10	Primary & recurrence	*SF3B1*	K700E	Acute Myeloid Leukemia (no evidence in solid tumor)	H3B-8800 [4]

* Multiple mutations in one patient ** Acquired new alteration in recurrent tumor that did not be found in primary sample. # Excluded alterations in ERBB2 gene in this Table.

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국문 초록

유방암 수술 후 국소 재발암과 원발암의 유전적 변이 비교

국내외 통계에 따르면 유방암은 여성에서 발생하는 암 중 가장 흔한 암이다. 유전자 분석 기술과 유방암의 유전 정보에 기초한 표적 치료는 지속적으로 발전하고 있어 치료의 표적이 되는 유전적 이상(genomic alterations)을 확인하는 것이 임상적으로 중요하다. 그러나 원발암의 치료 후 발생하는 재발암의 경우 원발암에 비해 유전적 정보에 대한 연구 결과가 부족하고, 치료에 대한 내성이 발생한 기전 등을 고려해야 하기 때문에 맞춤형 치료를 제시하는 것이 더욱 어렵다. 유방암의 원발 종양과 재발암에서 나타나는 유전적 변화를 확인하기 위해 우리는 유방암의 근치적 치료를 받은 후 동측의 잔존 유방 및 흉벽의 국소 재발을 경험한 20명의 원발암과 재발암 조직에 대해 121개의 암 관련 유전자를 이용하여 차세대 염기 서열 분석(Next-generation sequencing, NGS)을 시행하였다. 원발암과 동측의 유방 혹은 흉벽에 재발한 종양의 유전적 특성을 분석한 결과 동일한 환자에서 발생한 원발암-재발암 조직은 매우 유사한 결과를 보였다. 일부 연구에서 재발암 발생 위치나 분자 아형과 같은 임상학적/분자적 특성의 변화가 발생한 경우나, 무병 기간이 긴 경우 원발암의 특성과는 완전히 다른 특성을 가진 재발암이 발생할 확률이 높은 것이 보고되었으나, 본 연구에서는 무병기간, 아형, 항암 화학 요법, 호르몬 요법 및 방사선 치료 등과 같은 임상적 요소와 관계없이 재발암의 유전적 변이는 원발암의 유전적 변이와 매우 유사한 것으로 나타났다. 또한 대부분의 환자 (80%)에서 원발암과 국소 재발암은 적어도 1개 이상의 동일한 driver alteration(somatic mutation 또는 CNV)을 공유했으며, 재발암이 발생하면서 유전자 변이가 변화(gain or loss)를 보였던 8명의 환자 중 재발암에서 유방암 치료의 타겟이 될 수 있는 새로운 driver mutation을 발견한 것은 1명이었다.

본 연구는 원발암-재발암 조직의 차세대 염기 서열 분석법을 이용하여 원발암과 동측의 잔존 유방 및 흉벽의 국소 재발암의 유전적 특성을 비교한 첫 번째 연구로, 분석 결과 짝지어진 조직의 유전적 특성은 매우 유사한 것으로 확인되었다. 따라서 원발암 조직의 유전자 분석 결과는 국소 재발 환자에서 맞춤형 치료를 고려할 때에도 유용한 정보를 제

공할 것으로 생각된다.

주요어: 유방암, 국소 재발, 유전자 변이, 차세대 염기 서열 분석법

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