

# PIK3CA-AKT pathway predominantly acts in developing ipsilateral breast tumor recurrence long after breast conserving surgery

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## Research Article

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# Abstract

**Background:** Ipsilateral Breast Tumor Recurrence (IBTR) after breast-conserving therapy is seen after a long interval, but the clinical classification of Residual Tumor Recurrence (RR) or Double Primary (DP) needs to be validated. We used genome profiling to identify the genetic alterations associated with IBTR recurrence after a long period of time.

**Methods:** Among 1,881 breast cancer patients treated with breast-conserving therapy between 1999 and 2018, IBTR occurred in 52 patients (2.8%). Of these, 22 patients who consented for genetic analysis of Primary Breast Cancer (PBC) (T1) and IBTR (T2) were studied. When the same gene mutations in T1 and T2 were identified, it was classified as genomic residual recurrence gRR, and when no shared mutations identified, it was classified as gDP. The differences between clinical and genomic classification were compared. Furthermore, the pathway of the genes which were responsible for recurrence was also examined.

**Results:** Of 13 clinically diagnosed RRs (cRRs), 11 were gRR and 2 were gDPs, while of 9 cDPs, 6 were gDP and 3 gRR, with a match rate of 17/22 (77%). Discrepancy was mostly related to the limitation of clinical judgement, heavily depend on tumor location. We searched for genes involved in IBTR: PIK3CA-AKT pathway mutations were found in 12 of 14 gRRs (86%) in T1, while *PIK3CA* mutations were found in only 2 of 8 gDPs (25%), significant difference was noted ( $p=0.004$ ). When both of PBC and IBTR compared, PIK3CA-AKT pathway abnormalities were 24/28 (86%) in the gRR group and 5/16 (31%) in the gDP group ( $p<0.001$ ).

**Conclusions:** Genome profiling revealed that abnormalities in the PIK3CA-AKT pathway in long-term residential recurrences and are a crucial molecular group in the development of IBTR.

**Trial registration:** not applicable

## Introduction

Breast-conserving therapy (BCT), a combination of breast-conserving surgery (BCS) followed by whole-breast irradiation, is an established standard therapy for early-stage breast cancer. Many clinical trials with subsequent meta-analyses have provided clear evidence that BCT achieves a long-term survival equivalent to mastectomy [1, 2]. Initially, local recurrence (LR) rate after BCS was reported 6-11% in 5 years and 10-13% in 10 years [3]. Improvement of diagnostic modality for the extent of tumor spread and optimal treatment by radiation and adjuvant chemotherapies have obviously contributed to the decrease of LR rate [4]. However, LR still have occurred 1-5% in 10 years [5, 6]. In addition, a question still remains whether this ipsilateral breast tumor recurrence (IBTR) is a true recurrence of the residual primary tumor or clonally different new primaries. Furthermore, the clonal nature of IBTR after radiation and adjuvant therapy has not been fully elucidated by genomic analysis. The aim of this study is to reveal the genomic profiling of IBTR which developed after BCT in long-term interval of  $7.7 \pm 4.6$  years (ranging 0.9 to 19.7).

We believe this analysis may present new insight into the nature of “recurrence” by revealing their genome profiles.

## Methods

### **Selection of patients with IBTR for clinical and genomic analysis**

A total of 2,770 patients with breast cancer underwent surgery from 1999 to 2018 in our hospital; total mastectomy in 889 patients (32%) and breast conserving surgery (BCS) in 1,881 (68 %) (Fig. 1). After BCS, 98% of patients were treated by irradiation of 50 Gy for whole breast and in cases with residual tumor found at margin of resected specimen had additional 10 Gy for the tumor bed. Furthermore, adjuvant systemic therapies were added according to the guidelines [7][8]. Of 1,881 cases with BCS, the ipsilateral breast tumor recurrence (IBTR) had developed in 52 (2.8 %) cases with median follow of  $7.7 \pm 4.6$  years (ranging 0.9 to 19.7 years).

We reviewed clinical features including age at developing PBC, TNM classifications, stage, histology, immunohistochemical subtypes and interval years from PBC to IBTR (Table 1). Among the 52 cases with IBTR, written informed consent for genomic analysis was obtained in 22 cases. We analyzed genomic profile of paired samples of primary breast cancer (PBC or T1) and recurrence (IBTR or T2) (Fig. 2 a, b).

### **Clinically defined residual recurrence (RR) and double primaries (DP)**

First, we classified those IBTR whether they are clinical residual recurrence (cRR) of the primary tumors or de novo double primaries (cDP) according to the following gross anatomical and microscopic features. Gross anatomical concordance (AC) was judged, whether the IBTR developed in the same quadrant of the index lesion of PBC [9].

Histological concordance (HC) was judged by the similarity of pathological findings and immunohistochemical subtypes between the PBC and IBTR [10]. We judged IBTR as clinical residual recurrence (cRR) by belonging to both of AC and HC and the others were judged as clinical double primaries (cDP).

### **Genomically defined residual recurrence (gRR) and double primaries (gDP)**

We classified IBTR into two groups by genome profiling, namely, genomic residual recurrence (gRR) and genomic double primary (gDP) by cancer panel analysis [11-14]. We defined gRR by the existence of

identical mutations, especially driver oncogenic mutations, in both tissue of PBC and IBTR, whereas gDP was defined by the absence of shared mutations in both tissues. Correlations of clinically and genomically defined cRR/cDP and gRR/gDP were studied.

## Genome analysis of primary breast cancer and IBTR ( 22 cases)

### Preparation of samples

The tumor samples from PBC and IBTR were obtained from surgical resections or biopsies. A serial section of 10- $\mu$ m-thick, formalin-fixed and paraffin-embedded (FFPE) tissue was stained with haematoxylin-eosin and then microdissected using an Arcturus XT laser-capture microdissection system (Thermo Fisher Scientific, Waltham, MA, USA) [15].

Peripheral blood samples were obtained and buffy coats were isolated following centrifugation of peripheral blood at 820  $\times$  g at 25 °C for 10 min, and subsequently stored at -80 °C until required for DNA extraction. Total DNA was extracted from lymphocytes using the QIAamp DNA blood mini QIAcube kit (Qiagen, Hilden, Germany). The concentration of DNA was determined using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

### Targeted deep sequencing

For targeted deep sequencing analysis, as we previously reported [16], Ion AmpliSeq designer (Thermo Fisher Scientific) was used to design custom primers, which consisted of 2,863 primer pairs in two pools [17]. These primers covered the exons of 53 breast cancer-associated genes (total 287,520 nucleotides) reported by the TCGA project, other studies, and the COSMIC database [18-20] (sTable 1).

Multiplex polymerase chain reaction (PCR) was performed with Ion AmpliSeq Library Kit Plus and two primer pools, as we previously described [21]. PCR amplicons were partially digested with FuPa reagent and subsequently ligated to adapters with Ion Xpress Barcode Adapters. Adaptor-ligated amplicon libraries were purified using Agencourt AMPure XP reagents (Beckman Coulter, Brea, CA, USA). The library concentration was determined by quantitative real-time PCR using an Ion Library Quantitation Kit. Emulsion PCR and chip loading were performed on the Ion Chef with the Ion PI Hi-Q Chef kit. Sequencing was performed on the Ion Proton Sequencer (Thermo Fisher Scientific).

### Data analysis

The sequencing data were processed using standard Ion Torrent Suite software running on a Torrent Server as described previously [16]. Raw signal data were analysed using Torrent Suite. The pipeline

included signalling processing, base calling, quality score assignment, adapter trimming, PCR duplicate removal, read alignment to human genome 19 reference (hg19), quality control of mapping quality, and coverage analysis. Variants calling and annotations were performed using an Ion Reporter Server System, and peripheral blood DNA was used as a control to detect variants in tumours using filtration of “confident somatic variants” in a Tumour-Normal pair pipeline. The minimum count for mutant allele reads was more than 10 and coverage depth was more than 20. Common single nucleotide polymorphisms were excluded from further analysis.

## Annotation of the genome profiling

We compared genome profiling of paired samples of PBC and IBTR.

The significant mutated gene (SMG) which was defined with allelic fraction more than 1 % and the number of driver genes were defined according to the oncoKB (Precision Oncology Knowledge Database, Memorial Sloan Kettering Cancer Center, USA) .

## Elucidation of driver gene and pathway to promote recurrence

We tried to elucidate whether any genes and pathways which might be significantly altered might play an important role for the development of recurrences after many years.

## Statistical Analysis

Statistical analysis was performed by t test or  $\chi^2$  test as appropriate using Stat Mate (Atoms, Tokyo, Japan).

## Results

### Clinical Features of 1,881 BCS, 52 total IBTR and DNA analyzed 22 IBTR

When comparing the clinical features of all 1,881 patients who underwent breast-conserving surgery and 52 patients who developed IBTR, there were no differences in age, TNM classification, presence of lymph node metastasis, histological type, or immunohistochemical subtypes (Table 1). The clinical features of the 52 patients with IBTR and the 22 patients whose genomic profiles were analyzed showed a difference in age of  $54 \pm 12$  vs.  $47 \pm 9$  ( $p = 0.01$ ), but no difference in TNM classification, histology, or immune subtype (Table 1). The clinical classification of cRR and cDP was 40:12 and 13:9 in all 52 IBTR cases and 22 genomically analyzed cases, respectively ( $p=0.12$ ) (Table 1).

# Comparison of clinical (cRR/cDP) and genomic (gRR/gDP) judgement

The 22 genomically analyzed cases were classified as gRR (genomic residual recurrence) and gDP (genomic double primary) (Fig. 2 a, b). As a result, 14 cases were classified as the former and 8 cases as the latter. Three of 14 gRRs (gRR4, gRR6, and gRR7, Fig.2a) were classified as cDP. The reason for three cases judged as cDP was that the recurrent IBTR was not in the same quadrant. Regarding 8 genomically judged double primaries, two (gDP1 and gDP2) were judged as clinical residual recurrence (cRR) because they were in the same quadrant and histologically consistent (Fig. 2b). In short, the agreement between clinical and genomic profile was 17/22 (77%) and the disagreement was 5/22 (23%) (Fig. 1, 2 a, b). Clinical judgement heavily related on the location of IBTR which might have limited the accuracy of the discription.

## Intervals and pattern of recurrence

Next, we examined the interval to recurrence and the presence of distant metastasis in 14 patients with gRR (Table 2a) and 8 patients with gDP (Table 2b). The time to IBTR for gRR and gDP was  $7.5 \pm 3.7$  (range 2.7-14.2) and  $9.9 \pm 4.3$  (3.3-16.5) years, with no significant difference between the two groups ( $p=0.21$ ) (Table 1). Five of the 14 patients (36%) with gRR were found to have recurrence after more than 10 years (Table 2a). In addition, the presence of distant metastasis was found in 4 of 14 RRs (28%), but not in 8 gDPs (Table 2a, 2b). These results suggest that the recurrence of gRR is unique in that it can occur over a period of more than 10 years with systemic metastasis.

## Immunohistological pattern of recurrence

We examined the immunohistological subtype (HR pos. Her2 pos. Both. neg.) in the gRR and gDP groups (Tables 2 a, b). In all 14 cases of gRR, the same subtype, i.e., 11 HR pos. and 3 Her2 pos. cases was observed (Table 2a). On the other hand, in 3 of 8 gDP cases, the HR pos. at the time of first occurrence (PBC) changed to Both neg. in IBTR (Table 2b).

## Genome Profiling of gRR and gDP

First, we compared the number of oncogenic mutations in the PBC (T1) and in the IBTR (T2) in the two groups (Table 3).

Regarding oncogenic mutations in PBC (T1) they were  $2.2 \pm 1.6$  and  $1.3 \pm 0.7$  in gRR and in gDP, respectively, ( $p=0.11$ ). The mutations in IBTR (T2) were  $1.7 \pm 0.8$  and  $1.0 \pm 0.6$  ( $p=0.06$ ) in gRR and gDP, respectively (Table 3). The difference were not statistically significant. However, when the total number

of mutations of PBC and of IBTR were combined, more oncogenic mutations were found in gRR than in gDP ( $2.0 \pm 1.3$  vs  $1.2 \pm 0.8$ ) ( $p = 0.03$ ) (Table 3).

## Significance of PIK3CA-AKT pathway

We then sorted the individual oncogenic mutations into signaling pathways and compared the two groups. The number of cases with mutations in the PIK3CA-AKT pathway in PBC (T1) was significantly higher in the gRR group; gRR 12/14 (86%) and gDP 2/8 (25%), respectively ( $p=0.004$ ) (Table 3). This PIK3CA-AKT pathway abnormality persisted in IBTR (T2); 12/14 (86%) in gRR group ( $p=0.02$ ). In gDP group, 3 cases (gDP3, gDP4, gDP5) showed PIK3CA-AKT pathways abnormality were seen in IBTR (T2), but none of them preexisted in PBC (T1) (Table 3). Both PBC and IBTR combined, abnormality in PIK3CA-AKT pathway in the gRR group apparent with strong statistical significance ( $p<0.001$ ) (Table 3).

In addition, individual genetic mutations in the PIK3CA-AKT signal pathway were identified. *PIK3CA* mutations were found in 8/14 (57%) of the PBCs in the gRR group, of which *PIK3CA p.His1047Arg* accounted for 4 cases (Fig.2a). In addition, two cases of *AKT* mutations and two cases of *PTEN* mutations were observed, which were mutually exclusive with the occurrence of *PIK3CA* mutations (Table 2a). On the other hand, in PBC (T1) of gDP, *PIK3CA* mutation was found in only one case (13%), and there were no *AKT* and *PTEN* mutations (Table 2b).

TP53/Cell cycle/DNA damage pathway mutations were significantly higher in T1 with gRR 6/14 cases (43%) vs gDP 0/8 (0%) ( $p=0.03$ ), but not in T2 with gRR 5/14 (36%) vs gDP 2/8 (30%) ( $p=0.6$ ). When both combined (T1+T2), gRR showed tendency to have higher abnormality (11/28(39%) vs 2/16(13%)), but the difference did not reach statistical significance ( $p=0.06$ ) (Table 3).

As an additional finding, there was one case of invasive lobular carcinoma (gRR7) among 22 cases. The resected margins of PBC were pathologically confirmed as free, and IBTR occurred in different quadrants after a 6-year interval. Therefore, they were clinically judged as double primaries (Fig. 2a). However, they had four identical mutations (*AKT*, *CDH1*, *KMT2C* and *FOXA 1*) and genomically defined as gRR, (Fig. 2a, Table 2a).

## Discussion

In 20 years from 1999 to 2018, we surgically treated 2,770 patients with breast cancer, of which 1,881 (68%) underwent breast-conserving therapy (BCT). During the same period, we have experienced only 52 cases of recurrence within the conserved breast (2.3%), which is not a high frequency, as the 10-year IBTR has been reported to be 1-5%[5, 6]. However, because of the preservation of breast tissue, it was extremely important to determine whether the recurrence was due to “leftover” or new cancer.

Conventional clinical judgment has been based on whether PBC and IBTR were in the same quadrant and whether they were histologically similar. In this study, we first followed these criteria[9, 10]. It was

previously reported that the percentage of RR cases was higher, while interval was longer and prognosis was better in DP cases<sup>22,23</sup>. In this study, 52 patients with a mean follow-up of 7.7 years (0.9-19.7 years) more cases were judged as cRR (40) than cDP (12 cases), consistent with previous data.

In several different organs, we had previously examined the concordance between primary and metastatic sites in lung and gastric cancers using deep sequencing of tumor tissues[11-14]. We have also reported the usefulness of panel sequencing in differentiating intrahepatic metastases from multiple metastases in liver[16], lung[11], and gastric cancer[12]. In this study, we applied this method to IBTR.

Both PBC and IBTR were analyzed in twenty-two patients who consented to genome analysis. These 22 cases were genomically classified into 14 gRR and 8 gDP. The probability of agreement with clinical classification was 77%, and the probability of disagreement was 23% (Fig. 2 a, b).

There are only a few scattered reports that have confirmed RR and DP by molecular biological methods. Vicini et al. compared clonality by looking at the loss-of-heterogeneity in microsatellite lesions and found that 30% of cases had a discrepancy with clinical classification[22]. This report also showed that DP had a longer interval and better prognosis than RR, as in other reports[10, 23]. In addition, Bollet et al. reported similar results using chromosome copy number alteration to detect clonality[24]. However, there have been no reports of analysis using NGS deep panel sequencing to explore the underlying mechanism of IBTR.

Our 22 patients were classified into 14 gRR and 8 gDP. There was no difference in the interval between gRR and gDP ( $7.5 \pm 3.7$  vs.  $9.9 \pm 4.3$ ) ( $p=0.21$ ). In fact, 5 cases (36%) in the gRR group developed IBTR over 10 years, and the longest gRR case (gRR14) was observed in 14 years. All patients received radiation therapy for IBTR prevention and adjuvant therapy according to the guidelines. Since, it is thought that the treatment tolerant persister cells survived and caused IBTR in hormonal therapy, in particular, for long-term (5 to 10 years). However, previous studies had limitations that there were few cases with more than 10 years of follow-up.

The cancer panel deep sequencing revealed not only clonality, but also the genome profile of treatment-resistant tissue that lead to recurrence. In our study, we found that persisters with mutations in the PIK3CA-AKT pathway were more likely to have recurrent disease. In other words, 12/16 (86%) of gRR cases were associated with mutations in the PIK3CA-AKT pathway.

*PIK3CA* mutations are most common in breast cancer, with a frequency of 30-40% in HR pos. and Her2 Pos. subtypes[25]. It is an important mutation involved in oncogenesis, cell proliferation, invasion, and resistance to treatment, and is attracting attention as a therapeutic target[26, 27]. In particular, the mutations are found in the early stages of carcinogenesis and are associated with the development of various types of cancer[28]. The hot spot mutation, *PIK3CA pHis1047Arg*, is reported to be a multipotential genetic change that appears early in the development of breast cancer and progresses to various types of breast cancer[29]. Interestingly, this hot spot mutations were identified in PBC (T1) 5 of 14 cases and all belong to g RR group (Fig.2a). Among these 5 gRRs, 3 (gRR11, gRR13, gRR 14) persisted

over 10 years (Fig.2a). It has been reported that *PIK3CA* mutations were clinically observed not only in invasive carcinoma but also in surrounding intraductal lesions[30]. In addition, *AKT1* mutations have been reported to be associated with breast cancer stem cells[31], although the rate is not high at 3% of breast cancers[25]. These reports suggest that persists with *PIK3CA-AKT* pathway mutations in intraductal breast lesions are resistant to radiation and other therapies, resulting in IBTR, for over 10 years.

Additionally, in one ILC cases (gRR7), a group of cells with mutations in *AKT*, *CDH1*, *FOXA1* and *KMT2C* persisted and developed IBTR after 6 years. This is a group of genes that has been shown in several studies as the genome profile of ILC[32, 33].

The limitation of this study is that the cases we were able to retrieve with consent were mainly HR pos. with only 3 Her2 pos. cases, and we were not able to analyze Both neg. cases. Her2 pos. and Both neg. cases tend to have a shorter time to recurrence clinically, and majority of cases were judged as RR. The majority of patients who were able to obtain IC were those who developed IBTR as late recurrence, resulting in a majority of HR pos. From another point of view, we were able to show the characteristics of late recurrence of breast cancer, that is resistant to treatment, and persisted and hidden in the body in a state of dormancy for a long period and has recurred [34]

## Conclusion

The deep panel sequencing is a useful method to differentiate between RR and DP in IBTR. In addition, the genome profile of treatment-resistant persists after breast-conserving therapy was found to have mutations in the *PIK3CA-AKT* pathway. The application of this interesting biological property to therapy is a future challenge which may including HR therapy over ten years for patients with hot spot mutations such as *PIK3CA H1047Arg*.

## Declarations

### Ethics approval and consent to participant:

This study was approved by the institutional review board in our hospital (No.2018-6). We analyzed 22 cases in whom written informed consent for genomic analysis was obtained.

### Consent for publication:

Consent for publication was included in the written informed consent.

### Availability of data and materials:

The datasets analyzed during current study are available from the corresponding author on reasonable request.

## Competing interests:

The authors declare that they have no competing interest.

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## Authors contribution:

Conceptualization: NH, MO; Data curation: HN, YH, MO; Formal analysis: HN, YH, KA, MO; Funding acquisition: YH, MO; Investigation: HN, YH, KA, MI, KA, HM, YM, Methodology: YH, KA; Project administration: HN, MO; Resources: YH, KA, HM, MO; Software: YH, HM; Validation: MO

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## Tables

Tables 1-4 are available in the Supplementary Files section.

## Figures

Fig.1

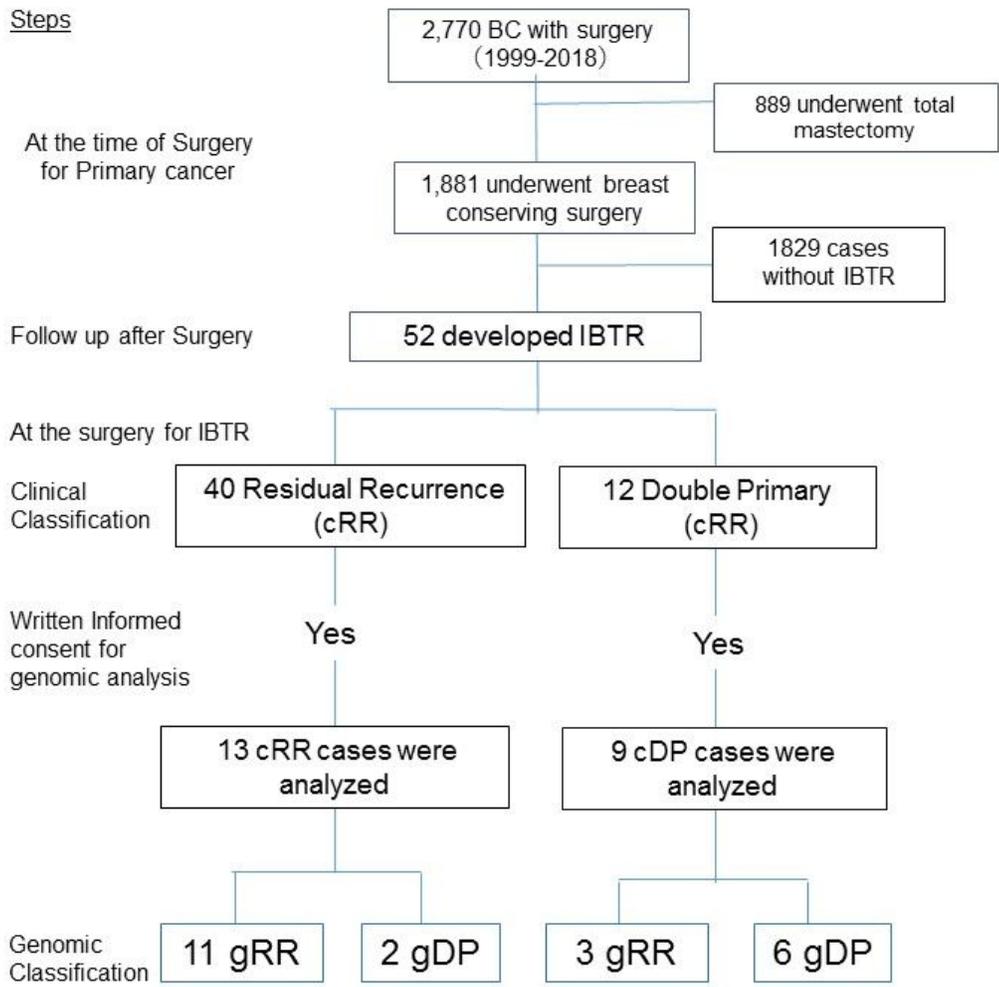


Figure 1

Flow chart of 2,770 patients with breast cancer to study clinical and genomic features for Ipsilateral Breast Tumor Recurrence

Fig. 2a

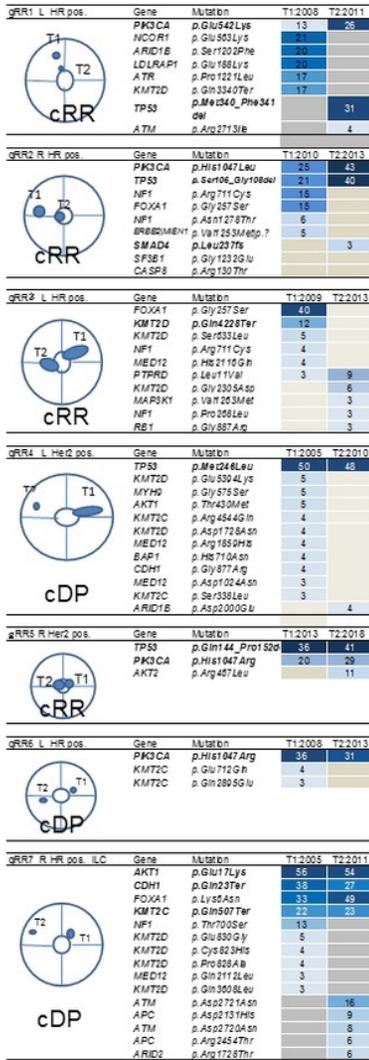


Fig. 2b

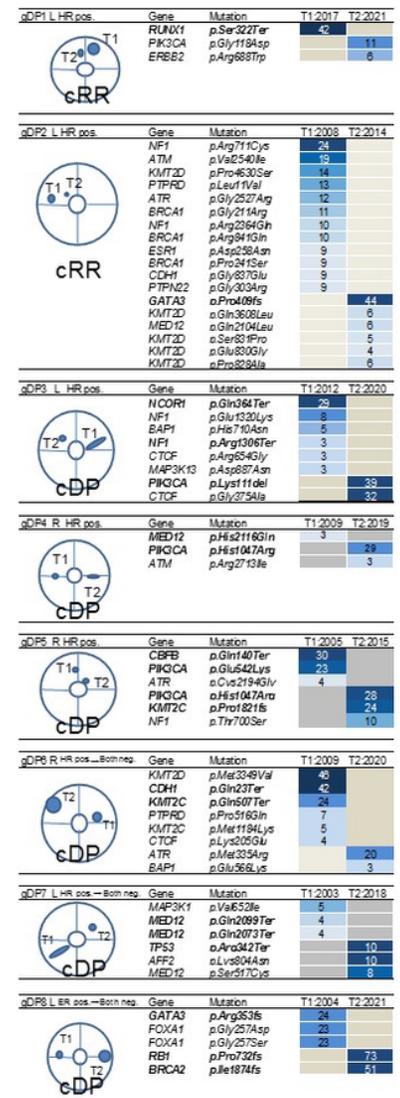
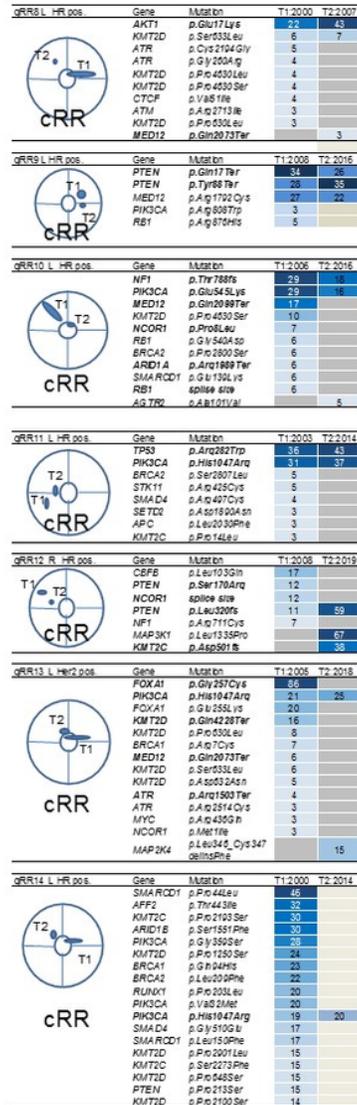


Figure 2

a Genome profiling of primary breast cancer (PBC) and Ipsilateral breast tumor recurrence (IBTR) a ; 14 cases of gRR.

14 cases were judged as genomic residual recurrence (gRR) because of shared mutations were identified between PBC (T1) and IBTR (T2). Oncogenic mutations are indicated by bold italic. In gRR3, no common oncogenic mutation was found in T1 and T2, but a common PTPRD mutation was found and included in gRR.

b; 8 cases of gRR

b 8 cases were judged as gDP because of absence of shared mutations between T1 and T2.

## Supplementary Files

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