Genetic heterogeneity during breast cancer progression in young patients

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ABSTRACT

Background: Because a number of years may be required for normal cells to develop into carcinoma, genes involved in tumorigenesis and progression might differ among breast cancers in young women and those in older women. The present study sought to analyze subclonality during breast cancer evolution as well as diversity within each individual in our young patients' cohort.
Methods: A total of 13 women aged <35 years at diagnosis with early breast cancer were recruited. Serial sections of breast samples consisting of synchronous invasive carcinoma, adjacent ductal carcinoma in situ (DCIS), normal breast tissue, and metastatic lymph nodes were collected and prepared for immunohistochemical analysis of estrogen receptor, progesterone receptor, HER2, and Ki67, and for extraction of genomic DNA. Germline and somatic gene alterations of genomic DNA were examined by targeted sequencing.
Results: Genomic DNA from 13 blood samples and 36 breast tissues consisting of 14 invasive carcinomas, nine adjacent DCIS, 11 normal breast tissues, and two metastatic lymph nodes were successfully sequenced. Germline gene alterations including pathogenic variants and gene alterations that were not yet evaluated for their clinical significance were detected in all patients but one. Somatic gene alterations were identified in eight invasive carcinomas, five DCIS, and one metastatic lymph node. Different somatic gene alterations between invasive carcinoma and DCIS were detected in two patients. Somatic gene mutations were present in non-neoplastic tissues in three patients. No two patients had the same gene alterations.
Conclusion: Our results reveal diversity within each individual during breast cancer progression.

1. Introduction

The evolution of cancers involves the gradual accumulation of genetic and epigenetic cancer-promoting changes [1]. The changes accumulate throughout the lifespan, and a number of years may be required for early pre-cancerous lesions to develop into carcinoma in situ and invasive carcinoma. Recent sequencing-based strategies have revealed that mutations could have been acquired prior to the cell becoming a malignant cell, and the amount of exposure to each mutational process could vary from one person to another [2]. Furthermore, each tumor shows individual subclonality in which subclones progress from normal cells to noninvasive and invasive carcinomas, then metastasis [3]. An understanding of cancer evolution might lead to appropriate treatment strategies and improved clinical outcomes [4].

Breast cancer in young women, usually defined as patients aged ≤40 years at diagnosis, is often characterized as aggressive molecular subtypes [5—8], with an increased risk of recurrence and shorter disease-free survival compared to their older counterparts [8—11]. Momozawa and colleagues reported that germline

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pathogenic variants were found in 15% of women diagnosed at < 40 years, and the proportion of patients with a pathogenic variant significantly decreased with advancing age [12]. In addition to genetic factors, it is suggested that genes and/or pathways involved in tumorigenesis and progression differ between breast cancers in young women and those in older women. Although there are numerous reports of genomic features in early and metastatic breast cancers [2,13—15], few studies have reported subclonal evolution in breast cancer in young women. Understanding the key mechanism of progression in each young patient will lead to appropriate prevention and treatment strategies.

We have performed a targeted amplicon exome sequencing for 160 cancer-related genes using genomic DNA from both tumor tissue and blood at our hospital. The clinical utility of our genomic testing for patients with meningioma [16], thyroid carcinoma [17], sinonasal papilloma [18], pancreatic cancer [19], various cancers such as colorectal, stomach, and lung cancers [20], and breast cancer [21] was shown previously.

Here, we analyzed germline and somatic gene alterations of genomic DNA extracted from blood and breast tissues consisting of synchronous invasive carcinoma, adjacent ductal carcinoma in situ (DCIS), normal breast tissue, and metastatic lymph nodes in 13 women aged <35 years at diagnosis by targeted sequencing. We sought to analyze subclonality during breast cancer evolution as well as diversity within each individual.

2. Methods

2.1. Patients

A total of 13 women aged <35 years with early breast cancer who had undergone breast surgery between May 2012 and August 2016 at Hokkaido University Hospital were recruited to this study (Table 1). This study was approved by the Ethics Committee of the Hokkaido University Graduate School of Medicine (No. 16—033), and conformed to the guidelines of the 1996 Declaration of Helsinki. Written informed consent for the use of blood and tumor tissues was provided by each patient. Patients who were positive for axillary lymph nodes or who had human epidermal growth factor receptor 2 (HER2)-positive breast cancer received adjuvant or neoadjuvant chemotherapy including anthracyclines and/or taxanes with or without anti-HER2 drugs. Tumor samples were obtained during surgery. Patients with estrogen receptor (ER)-positive breast cancer received adjuvant endocrine therapy (tamoxifen ± luteinizing hormone-releasing hormone agonist).

2.2. Samples and DNA extraction

Formalin-fixed paraffin-embedded (FFPE) breast samples stained with hematoxylin—eosin (H&E) were reviewed, and adequate tissue blocks consisting of invasive carcinoma, adjacent DCIS, normal breast tissue, and metastatic lymph nodes were selected for each patient (Fig. 1). Macrodissection was performed in order to separate invasive carcinoma from DCIS after pathology review. Serial sections (5 μm) were prepared from the selected blocks for immunohistochemical analysis of ER, progesterone receptor (PgR), HER2, and Ki67, and 5—10 sections were used for extraction of genomic DNA. The Ki67 labeling index was assessed as the percentage of tumor cells showing definite nuclear staining among >5000 invasive tumor cells. Immunohistochemical analysis of PTEN (D4.3, Cell Signaling Technology), p53 (DO-7, Dako), and MDM2 (IF2, Invitrogen) was performed. DNA from peripheral blood cells was also analyzed. Genomic DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), QIAsymphony DSP DNA Mini Kit (Qiagen), or QIAamp DNA Blood Mini Kit (Qiagen).

Table 1
Characteristics of patients and tumors.

Characteristics	Patients (n = 13)
Age, median (range), years	32 (25—34)
Sex	
Female	13 (100%)
Male	0 (0%)
Family history of cancer	
Breast cancer and/or ovarian cancer	6 (46%)
Other cancer	6 (46%)
No family history of cancer	4 (31%)
Stage	
I	4 (31%)
II	8 (62%)
III	1 (8%)
Lymph node status	
Negative	10 (77%)
Positive	3 (23%)
ER and HER2 status	
ER-positive/HER2-negative	11 (85%)
ER-positive/HER2-positive	1 (8%)
ER-negative/HER2-positive	0 (0%)
ER-negative/HER2-negative	1 (8%)
Histopathology	
Invasive ductal carcinoma	9 (69%)
Mucinous carcinoma	3 (23%)
Metaplastic carcinoma	1 (8%)
Neoadjuvant therapy	
Chemotherapy	3 (23%)
Chemotherapy and anti-HER2 therapy	1 (8%)
None	9 (69%)
Postoperative adjuvant therapy	
Endocrine therapy alone	7 (54%)
Chemotherapy alone	2 (15%)
Endocrine therapy and chemotherapy	3 (23%)
Endocrine therapy and anti-HER2 therapy	1 (8%)
Recurrence (distant metastasis)	
No	10 (77%)
Yes	3 (23%)
Follow-up, median (range), months	53.1 (8.6—82.3)

The concentration of the genomic DNA was measured by Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, USA) and Qubit 3.0 Fluorometer (Thermo Fisher Scientific). After checking the quality of the DNA based on the DNA integrity number (DIN) score calculated using the Agilent 2000 TapeStation (Agilent Technologies, Waldbronn, Germany), the minimum amount of DNA was 50 ng and the minimum quality of DNA had a DIN score over 3.1.

2.3. Targeted amplicon sequencing and data analysis

Using the obtained genomic DNA, we then amplified the target genes by a polymerase chain reaction (PCR) method with Gene Read DNA seq Panel PCR Regent V2 (Qiagen) and Human Comprehensive Cancer Panel (Qiagen). We performed targeted amplicon exome sequencing for 160 cancer-related genes based on the Illumina MiSeq sequencing platform (Illumina, San Diego, USA) with an average depth of coverage of approximately 500 × with at least 80% of bases covered >50 × [16—21]. Sequencing (paired-end, 150 bp) of samples and demultiplexing of libraries was performed by Illumina MiSeq Reporter. The list of 160 genes is shown in Supplementary Table 1 [19,20]. Details of data analysis are described in Supplementary Methods. Germline variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines [22], and retained as pathogenic based on the recommendations of ACMG and ClinVar [23]. Germline secondary findings were disclosed only to those patients who agreed to receive this information.
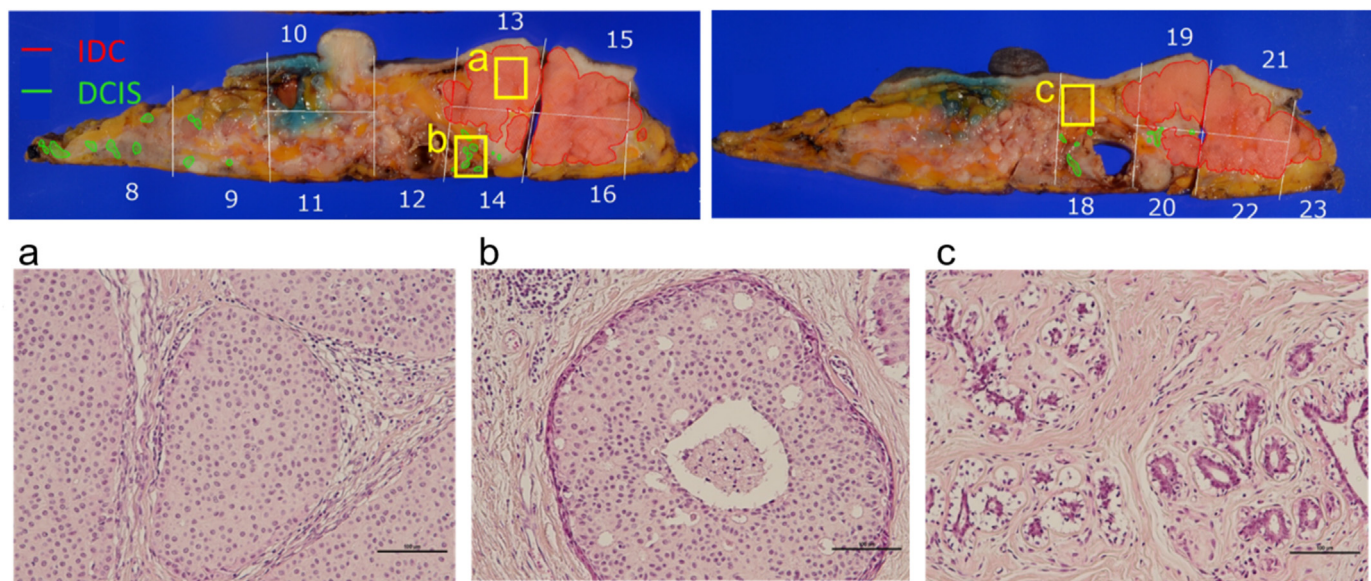
Fig. 1. Selection of adequate tissue blocks for immunohistochemical staining and genomic DNA extraction consisting of invasive carcinoma, adjacent DCIS, and normal breast tissue. Surgically resected breast samples of a representative case (No. 2) are shown. Micrographs (200 ×) of H&E-stained tissue sections of invasive ductal carcinoma (**a**, tumor content 70%), DCIS (**b**, tumor content 60%), and normal breast tissue (**c**, epithelial cell content 40%) in the lower panels are details from the areas delineated by yellow squares in the upper panels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Patient characteristics

Characteristics of the 13 patients recruited in this study are summarized in Table 1, and detailed information of past and family histories of each patient is shown in Table 2. Case 2 had been diagnosed with Cowden syndrome, and case 5 had been diagnosed with neurofibromatosis 1. Six patients had a family history of breast and/or ovarian cancers, and six patients had a family history of other cancers including gastric cancer and thyroid cancer. Eleven were ER-positive/HER2-negative, and case 1 (Table 4) was triple-negative. Case 10 (Table 6) had ER-positive/HER2-positive breast cancer and was treated with neoadjuvant chemotherapy and anti-HER2 therapy. Three patients (cases 11, 12, and 13) received neo-adjuvant chemotherapy. All patients received adequate postoperative adjuvant therapy including endocrine therapy, chemotherapy, and anti-HER2 therapy. Three patients (cases 2, 5, and 13) experienced recurrence. First sites of metastatic disease were bone in cases 2 and 5, and lung and brain in case 13. Biopsy at the recurrent sites was not performed in all three cases. The median follow-up time was 53.1 months (range 8.6–82.3). Genomic DNA from 13 blood samples and 36 breast tissue samples consisting of 14 invasive carcinomas, nine adjacent DCIS, 11 normal breast tissues, and two metastatic lymph nodes were successfully sequenced.

3.2. Germline gene alterations

Detailed information regarding germline gene alterations including pathogenic, uncertain significance (VUS), and benign variants is shown in Table 3. Germline pathogenic variants were

Table 2
Past and family histories of 13 patients.

No.	Age	Past history	Family history of cancer	Other cancer
			Breast cancer and/or ovarian cancer	
1	32	Breast cancer	Breast cancer (great grandmother)	
2	33	Cowden syndrome (breast cancer, endometrial cancer, thyroid adenoma)	Breast cancer (aunt)	Bladder cancer (grandfather)
3	32	Thyroid cancer		Gastric cancer (grandmother), Lung cancer (grandfather), Thyroid cancer (grandmother)
4	33	Benign ovarian tumor		
5	30	Neurofibromatosis 1	Breast cancer (grandmother, grandaunt)	Thyroid cancer (grandmother), Multiple myeloma (uncle)
6	33		Ovarian cancer (grandmother)	
7	31	Endometriosis		Gastric cancer (mother, cousin)
8	33	Systemic lupus erythematosus		
9	32			
10	33	Infertility		Gastric cancer (grandmother), Pancreatic cancer (grandmother)
11	25	Mature cystic teratoma	Breast cancer (aunt, cousin)	
12	34		Breast cancer (cousin)	Colon cancer (uncle)
13	32	Partial anomalous pulmonary venous connection		

identified in two patients: *PTEN* p.E242* in case 2 and *BRCA2* p.R2336H in case 3. VUS were detected in three patients: *TERT* p.R951Q in case 1, *RET* p.E459K in case 6, and *CHEK2* p.H414Y in case 11. A benign *BRCA2* p.I1929V variant was identified in case 5. This patient was diagnosed with type 1 neurofibromatosis when she was a teenager. However, *NF1* and *NF2* gene alterations were not detected. Variants with conflicting interpretations between ACMG values and any non-ACMG value were detected in three patients, and variants without an interpretation of clinical significance were detected in eight patients. Germline gene alterations were detected in all the tissue samples including normal breast tissue, DCIS, invasive carcinoma, and metastatic lymph nodes as well as the blood samples. The median variant allele frequency among breast tissues was 50% (range 19–87%).

3.3. Pathological characteristics and somatic gene alterations in patients treated with primary surgery

Eight synchronous breast samples consisting of invasive carcinoma, adjacent DCIS, and normal breast tissue, and one synchronous breast sample consisting of invasive carcinoma and normal breast tissue were evaluated in nine patients who received primary

surgery (Tables 4 and 5). Synchronous metastatic lymph nodes were also examined in two patients (cases 3 and 4). One lymph node with micrometastasis (0.3 mm, case 2) could not be evaluated. Somatic gene alterations were identified in two normal breast tissues (cases 1 and 4), four DCIS (cases 2, 4, 5, and 6), six invasive carcinomas (cases 1, 2, 4, 5, 6, and 7), and one metastatic lymph node (case 4).

In case 1 with triple-negative breast cancer, high expression of ER and PgR and mutations of *TP53* p.P72S and *PPP2R1A* p.S256F were detected in normal breast tissue, but not in DCIS or invasive carcinoma. Instead, a somatic *BRCA2* p.E2198* mutation was identified in invasive carcinoma. In case 2 with ER-positive/PgR-positive/HER2-negative breast cancer who had a germline *PTEN* p.E242* pathogenic variant, somatic *PTEN* p.E18* and *GATA3* gene mutations were detected in both DCIS and invasive carcinoma. PTEN protein expression was observed in normal epithelial cells, but not in cancer cells in DCIS or invasive carcinoma (Supplementary Fig. 1). Because this somatic *PTEN* p.E18* mutation most likely represents a second hit, it is possible that the two-hit mechanism of PTEN loss in carcinoma occurred in case 2. In case 4 with ER-positive/PgR-positive/HER2-negative breast cancer, a *TP53* p.R213L mutation was identified in DCIS, invasive carcinoma,

Table 3
Germline gene alterations and variant frequency in 13 patients.

No.	Germline gene alteration				Variant allele frequency (%)			
	Pathogenic/likely pathogenic	Uncertain significance (VUS)	Other[a]	Not evaluated[b]	Benign/likely benign	Normal breast tissue	Ductal carcinoma *in situ*	Invasive carcinoma	Metastatic lymph node
1		*TERT* p.R951Q				47	51	50	N/A
				CDK12 p.L1383I		50	41	58	N/A
2	*PTEN* p.E242*					51	53	54	–
3	*BRCA2* p.R2336H					51	67	61	54
4				*KMT2D* p.P2938L		61	58	60	63
				FLT3 p.T382 M		50	48	51	50
5					*BRCA2* p.I1929V	50	34	19	N/A
6		*RET* p.E459K				48	52	47	N/A
			CDH1 p.E880K			47	48	51	N/A
7			*PALB2* p.Q460R			51	50	50	N/A
				SETD2 p.E639K		51	42	40	N/A
8			*FUBP1* p.T601A			50	55	51	N/A
			TP53 p.E11Q			49	50	51	N/A
				FLT3 p.T820 N		49	48	47	N/A
9						–	–	–	N/A
10				*ASXL1* p.E1033V[c]		–	49[c]	47[c]	N/A
				ASXL1 p.E1033V[d]		51[d]	–	48[d]	N/A
11		*CHEK2* p.H414Y				45	–	53	N/A
				ERBB4 p.G319R		53	–	47	N/A
12				*BRCA2* p.L1908V		–	–	50	N/A
				ATRX splice site		–	–	68	N/A
13				*SLC7A8* p.R181Q		–	–	49	N/A
				EP300 p.M556V		–	–	45	N/A
				SETD2 p.M761I		–	–	87	N/A

[a] Conflicting interpretations between ACMG values and any non-ACMG value.
[b] Submissions without an interpretation of clinical significance.
[c] Samples before neoadjuvant chemotherapy.
[d] Samples after neoadjuvant chemotherapy; N/A, not applicable; –, not evaluated.

Table 4
Samples for clinical sequencing in nine patients treated with primary surgery.

No.	Histo-pathology	pT	pN	Grade	Normal breast tissue				Ductal carcinoma in situ				Invasive carcinoma				Metastatic lymph node			
					ER	PgR	HER2	Ki67	ER	PgR	HER2	Ki67	ER	PgR	HER2	Ki67	ER	PgR	HER2	Ki67
1	IDC	16	0	3	95	95	0	20	0	0	1+	60	<1	0	1+	99	N/A			
2	IDC	57	1	3	10	60	0	10	90	90	1+	15	90	90	1+	10	−	−	−	−
3	IDC	28	7	2	30	30	0	2	80	50	2+	20	80	<1	1+	40	95	10	0	30
4	IDC	25	1	2	10	10	0	5	90	90	0	5	90	90	0	25	95	50	1+	30
5	IDC	28	0	3	20	5	0	10	90	20	1+	30	70	20	1+	60	N/A			
6	IDC	13	0	1	40	40	0	10	100	70	1+	20	100	80	2+*	18	N/A			
7	IDC	21	0	2	10	<5	0	5	90	90	0	5	90	80	1+	29	N/A			
8	Mucinous	26	0	1	40	40	0	10	95	90	0	10	95	90	1+	5	N/A			
9	Mucinous	60	0	1	30	30	0	5	N/A				95	95	0	10	N/A			

pT, pathological tumor size (mm); pN, number of positive lymph nodes; Grade, tumor grade; ER, estrogen receptor (% of positive cells); PgR, progesterone receptor (% of positive cells); HER2, score; Ki67, labeling index (%); IDC, invasive ductal carcinoma; Mucinous, mucinous carcinoma; N/A, not applicable; −, not evaluated; *equivocal by HER2 in situ hybridization.

Table 5
Somatic gene alterations in nine patients treated with primary surgery.

No.	Somatic gene alterations								
	Normal breast tissue		Ductal carcinoma in situ		Invasive carcinoma		Metastatic lymph node		
	Gene (VAF)	ECC	Gene (VAF)	TC	Gene (VAF)	TC	Gene (VAF)	TC	
1	TP53 p.P72S[c] (4%) PPP2R1A p.S256F (16%)	30%	none	10%	BRCA2 p.E2198*[a] (14%)	20%	N/A		
2	none	40%	PTEN p.E18* (22%) GATA3 splice site (7%)	60%	PTEN p.E18* (28%) GATA3 splice site (28%)	70%	−		
3	none	20%	none	50%	none	70%	none	50%	
4	ERBB2 p.W169* (5%)	20%	TP53 p.R213L[b] (17%)	50%	TP53 p.R213L[b] (31%)	40%	TP53 p.R213L[b] (6%)	40%	
5	none	40%	PIK3CA p.E81K[b] (19%)	50%	PTEN p.R130G[a] (40%)	80%	N/A		
6	none	40%	MAP2K4 del	60%	MYC amp, CDK12 amp	50%	N/A		
7	none	30%	none	30%	TP53 p.T230S (34%) DNMT3A p.R320Q (4%)	40%	N/A		
8	none	20%	none	30%	none	65%	N/A		
9	none	20%	N/A		none	70%	N/A		

VAF, variant allele frequency; ECC, epithelial cell content; TC, tumor content; del, deletion; amp, amplification; N/A, not applicable; −, not evaluated.
[a] pathogenic, [b] likely pathogenic, [c] uncertain significance, [d] conflicting interpretations of pathogenicity.

Table 6
Samples for clinical sequencing in four patients treated with neoadjuvant therapy.

No.	Tissue samples	Histo-pathology	ypT	ypN	Normal breast tissue				Ductal carcinoma in situ				Invasive carcinoma			
					ER	PgR	HER2	Ki67	ER	PgR	HER2	Ki67	ER	PgR	HER2	Ki67
10	before NAC	IDC	N/A						95	0	3+	30	100	0	3+	30
	after NAC		16	0	20	10	0	5	−				>90	2	2+	1.5
11	after NAC	IDC	19	0	80	10	0	1−2	−				100	90	0	10
12	after NAC	Mucinous	34	0	−				−				90	70	0	15
13	after NAC	Metaplastic	47	0	−				−				10	0	0	70

NAC, neoadjuvant chemotherapy; ypT, pathological tumor size after NAC (mm); ypN, number of positive lymph nodes after NAC; ER, estrogen receptor (% of positive cells); PgR, progesterone receptor (% of positive cells); HER2, score; Ki67, labeling index (%); IDC, invasive ductal carcinoma; Mucinous, mucinous carcinoma; Metaplastic, metaplastic carcinoma; before NAC, samples from primary tumors before neoadjuvant chemotherapy; after NAC, samples from primary tumors after neoadjuvant chemotherapy; N/A, not applicable; −, not evaluated.

and metastatic lymph nodes, suggesting that this TP53 mutation was a driver for the progression through DCIS and invasive carcinoma to metastasis. Interestingly, a somatic ERBB2 p.W169* mutation was detected in normal breast tissue, but not in DCIS, invasive carcinoma, or metastatic lymph nodes in case 4. In case 5 with ER-positive/PgR-positive/HER2-negative breast cancer, ER-positive cell rates decreased and Ki67-positive cell rates increased during the progression from DCIS to invasive carcinoma. A PIK3CA mutation, but not a PTEN mutation, was identified in DCIS, whereas a PTEN mutation but not a PIK3CA mutation was detected in invasive carcinoma in case 5. Previous studies mentioned the discordance between in situ and invasive areas of the same tumor regarding PIK3CA status, and suggested that PIK3CA gene mutation is more likely to play a role in tumor initiation than in invasive progression [24,25]. Different gene mutations detected in DCIS and invasive carcinoma were also identified in case 6 with ER-positive/PgR-positive/HER2-negative breast cancer: MAP2K4 deletion in DCIS and amplifications of MYC and CDK12 genes in invasive carcinoma. In case 7 with ER-positive/PgR-positive/HER2-negative breast cancer, mutations of TP53 p.T230S and DNMT3A p.R320Q were detected in invasive carcinoma, but no mutations were identified in DCIS. Somatic gene alterations in invasive carcinoma were not detected in cases 3, 8, and 9, although case 3 had a germline pathogenic BRCA2 variant and case 8 had germline gene alterations that were not yet evaluated of clinical significance. In case 9 with ER-positive/PgR-positive/HER2-negative mucinous carcinoma, neither germline nor somatic gene alterations were detected in either normal breast tissue or in invasive carcinoma.

3.4. Pathological characteristics and somatic gene alterations in patients treated with neoadjuvant therapy

Samples taken both before and after neoadjuvant treatment were evaluated in case 10 with ER-positive/HER2-positive breast cancer (Tables 6 and 7). *RET* p.V906M mutation and amplifications of *MDM2*, *CDK12*, *ERBB2*, *SPOP*, *BRIP1*, and *CD79B* genes were identified in both DCIS and invasive carcinoma before neoadjuvant therapy. However, *RET* p.V906M mutation and amplifications of *SPOP*, *BRIP1*, and *CD79B* genes were not detected, and *APC* p.S2766* mutation was detected in invasive carcinoma after neoadjuvant treatment. This nonsense mutation of the *APC* gene might be linked to chemotherapy resistance [26,27]. A somatic *BRCA2* p.R1512H mutation was also identified in normal breast tissue after neoadjuvant chemotherapy in case 10, whereas this mutation was not detected in DCIS or in invasive carcinoma after the treatment. Samples before neoadjuvant therapy were not available in cases 11, 12, and 13. *PBRM1* mutation and deletions of *PIK3R1*, *CDKN2A*, *CRLF2*, and *ZRSR2* genes were detected in invasive carcinoma after neoadjuvant chemotherapy in case 13, a patient with metaplastic breast cancer who died 8 months after operation. Somatic gene alterations were not detected in invasive carcinoma after neoadjuvant chemotherapy in cases 11 and 12. Sequencing analysis was not performed for normal tissue after neoadjuvant therapy in cases 12 and 13 because of low amounts of DNA.

3.5. Gene alterations during breast cancer progression

Overall, somatic gene alterations were detected in at least two regions of normal breast tissue, DCIS, invasive carcinoma, or metastatic lymph nodes in six cases (Fig. 2). Somatic gene mutations of *TP53* and *PPP2R1A* (case 1), *ERBB2* (case 4), or *BRCA2* (case 10) were present in normal breast tissue. However, neither of these mutations were detected in DCIS nor invasive carcinoma in the same patients. Different somatic gene mutations identified in DCIS and invasive carcinoma were detected in two patients (cases 5 and 6). On the other hand, somatic gene mutations of *PTEN* and *GATA3* (case 2), *TP53* (case 4), and *RET, SPOP* etc. (case 10) present in DCIS were also detected in invasive carcinoma. Immunohistochemical examination of PTEN (case 2), p53 (cases 4 and 7), and MDM2 (case 10) is shown in Supplementary Figures.

4. Discussion

In this study, we demonstrated germline and somatic gene alterations in blood and breast tissues simultaneously obtained from

invasive carcinoma, adjacent DCIS, normal breast tissue, and/or metastatic lymph nodes in each patient aged <35 years at diagnosis. Interestingly, somatic gene mutations were also present in non-neoplastic tissue samples in three patients. However, neither of these mutations were detected in DCIS nor invasive carcinoma in the same patients, suggesting that these mutations were not drivers for progression of their breast cancer, and that different subclones carrying other mutated genes might progress to carcinoma. Another possibility is that the mutations present in the non-neoplastic epithelial cells might be present in other subclones of DCIS and/or invasive carcinoma that were not revealed in this study. Danforth indicated that normal breast tissues contain evidence of early breast carcinogenesis including loss of heterozygosity, DNA methylation of tumor suppressor and other genes, and telomere shortening [28]. The presence in normal breast tissues of multiple, genetically distinct abnormal clones that could progress independently and simultaneously, provides a possible explanation for the genetic heterogeneity noted in many breast tumors [28].

Different somatic gene mutations identified in DCIS and invasive carcinoma were detected in two patients. It is suggested that the invasive-specific mutations may have occurred at low frequencies in DCIS prior to invasion or, alternatively, after invasion during the expansion of the invasive tumor mass. Another possibility is that the mutations existing in DCIS might be present in other subclones of invasive carcinoma that were not examined in this study, although this is unlikely because both DCIS and invasive carcinoma were collected from adjacent regions. Synchronous normal breast tissue, DCIS, and invasive carcinomas differ in their repertoire of somatic gene alterations even in the absence of systemic therapy, suggesting the contribution of spatial intratumor genetic heterogeneity [2]. On the other hand, somatic gene mutations present in DCIS were also detected in invasive carcinoma in three cases. Casasent and colleagues used single cell sequencing to demonstrate a multiclonal invasion model, in which one or more clones escape the ducts and migrate into the adjacent tissues to establish invasive carcinomas [29]. Their results showed a direct genomic lineage between the *in situ* and invasive tumor cell subpopulations, and revealed that most mutations and copy number aberrations evolved within the ducts prior to invasion [29].

Somatic gene alterations of *TP53*, *PIK3CA*, *GATA3*, *PTEN*, *ERBB2*, *MAP2K4*, *MDM2*, *PIK3R1*, and *CDKN2A* that were reported as the significantly mutated genes in primary breast cancer in the study of the Cancer Genome Atlas Network [13] were also identified in cancer tissues analyzed in this study. On the other hand, germline variants including pathogenic variants and gene alterations that were not yet evaluated of clinical significance were also detected in

Table 7
Somatic gene alterations in four patients treated with neoadjuvant therapy.

No	Tissue samples	Normal breast tissue		Ductal carcinoma *in situ*		Invasive carcinoma	
		Gene (VAF)	ECC	Gene (VAF)	TC	Gene (VAF)	TC
10	before NAC	N/A		*RET* p.V906M[c] (13%) *MDM2* amp, *CDK12* amp, *ERBB2* amp, *SPOP* amp, *BRIP1* amp, *CD79B* amp	50%	*RET* p.V906M[c] (13%) *MDM2* amp, *CDK12* amp, *ERBB2* amp, *SPOP* amp, *BRIP1* amp, *CD79B* amp	40%
	after NAC	*BRCA2* p.R1512H[d] (7%)	30%	–		*APC* p.S2766* (6%) *MDM2* amp, *CDK12* amp, *ERBB2* amp	40%
11	after NAC	none	40%	–		none	50%
12	after NAC	–		–		none	80%
13	after NAC	–		–		*PBRM1* splice site (8%) *PIK3R1* del, *CDKN2A* del, *CRLF2* del, *ZRSR2* del	80%

VAF, variant allele frequency; ECC, ductal epithelial cell content; TC, tumor content; NAC, neoadjuvant chemotherapy; del, deletion; amp, amplification; before NAC, samples from primary tumors before neoadjuvant chemotherapy; after NAC, samples from primary tumors after neoadjuvant chemotherapy; N/A, not applicable; –, not evaluated.
[a] pathogenic, [b] likely pathogenic, [c] uncertain significance, [d] conflicting interpretations of pathogenicity.
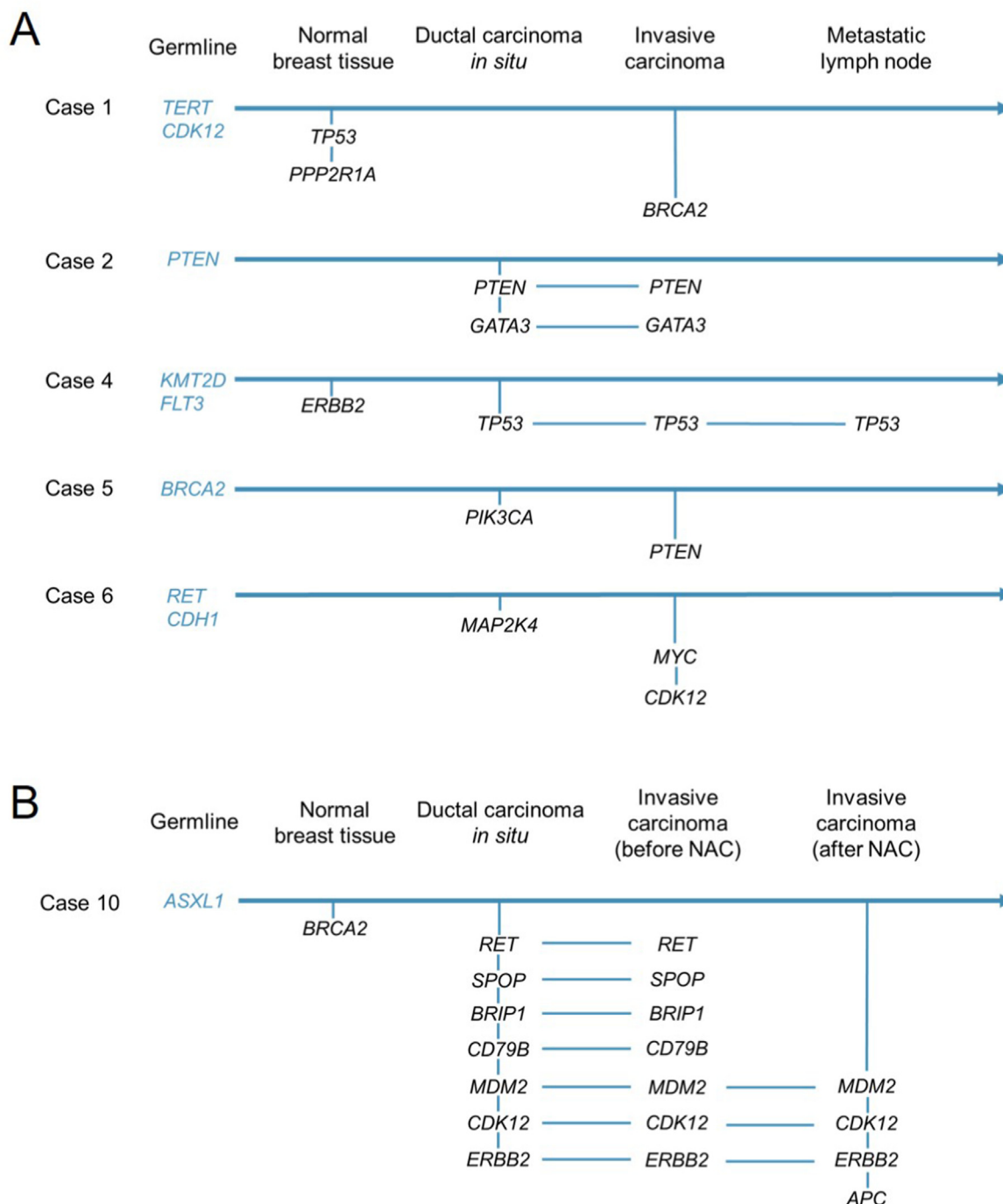
Fig. 2. Gene alterations during breast cancer progression. (A) Changes of gene alterations in five cases in which somatic gene alterations were detected in at least two areas of normal breast tissue, DCIS, invasive carcinoma, or metastatic lymph nodes. (B) Changes of gene alterations in case 10 who was treated with neoadjuvant anti-HER2 therapy and chemotherapy.

all patients but one in our cohort. Interestingly, all patients except case 9 had past and/or family histories. It is considered that hereditary factors affect tumorigenesis and progression of breast cancer disproportionately in young women compared to tumors occurring in older adults. Recognition of yet unexplained variants as a heritable cause might be important to provide not only a better understanding of the biological characteristics of breast cancer but also optimal care to individual young patients.

The present study has several limitations. First, the size of our cohort was small. However, we do show details of the

characteristics, immunohistochemical findings, and gene alterations of each patient. Furthermore, gene alterations in breast tissues simultaneously obtained from normal breast tissue, DCIS, invasive carcinoma, and/or metastatic lymph nodes were examined in each patient. Second, the cancer panel for a targeted amplicon exome sequencing used in this study consists of 160 cancer-related genes, and these 160 genes were selected based on previous reports of general cancers in adults [16–21]. Thus, some genes essential for breast cancer progression especially in young women, both germline, such as BARD1, RAD51C, and RAD51D [30], and somatic

alterations, might not be covered. In fact, the number of genes detected as germline and somatic gene alterations in each patient were low, and in one patient none were detected. Whole-exome sequencing is required for more appropriate analysis especially for young patients. Fourth, DNA was extracted from only one region of each DCIS, invasive carcinoma, and/or normal breast tissue. Furthermore, because of lack of appropriate quality and/or quantity of extracted DNA, not all samples were evaluated for sequencing. Examining more than one region of normal breast tissue and carcinomas in each patient is necessary for the detailed analysis for cancer evolution.

5. Conclusions

We report germline and somatic gene alterations in blood and breast tissues obtained from synchronous invasive carcinoma, adjacent DCIS, normal breast tissue, and/or metastatic lymph nodes in patients aged <35 years. Our results show diversity within each individual during breast cancer progression. Heritable causes might be important to provide not only understanding of the biological characteristics of breast cancer but also optimal care to individual young patients.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2021.10.011.

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