

DNA methylation analysis of progression patterns in ductal carcinoma in situ of triple-negative breast cancer

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Abstract

Background:

DNA methylation alterations associated with invasion remain incompletely characterized. In triple-negative breast cancer (TNBC), treatment strategies and indications for systemic therapy differ substantially depending on the presence or absence of invasion. Therefore, predicting the invasive potential of TNBC is of clinical importance. In this study, we compared DNA methylation profiles between matched ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) components of TNBC to identify exploratory methylation candidates potentially associated with invasion.

This single-center exploratory study included five patients: two patients with pure DCIS diagnosed by postoperative histopathological examination and three patients with mixed lesions containing both DCIS (mixDCIS) and invasive ductal carcinoma (mixIDC) components. In all mixed lesions, the invasive component measured less than 1 cm. A total of eight formalin-fixed, paraffin-embedded (FFPE) specimens obtained from these five patients were included in the analysis.

Lesion components were isolated by laser capture microdissection and profiled using the Infinium MethylationEPIC BeadChip v2.0. Differentially methylated positions (DMPs) and regions (DMRs) were evaluated using an absolute mean methylation difference ($|\Delta\beta| \geq 0.25$) and a false discovery rate (FDR) < 0.05 . Exploratory candidates were assessed using unadjusted P values. Stepwise methylation candidates were selected based on effect size and directional concordance across the three groups and all matched pairs.

Results

The mixDCIS–mixIDC comparison identified 977 DMP and 840 DMR candidates with $|\Delta\beta| \geq 0.25$ and unadjusted P < 0.05 ; none remained significant after multiple-testing correction. Of these candidates, 636 DMPs (65.1%) and 507 DMRs (60.4%) were hypermethylated in mixIDC. A *RASSF1*-related region showed the smallest unadjusted P value among DMRs ($\Delta\beta = 0.397$; P = 1.34×10^{-5} ; FDR = 0.424). In the pureDCIS–mixDCIS comparison, 33 DMRs met the FDR threshold, including 32 hypomethylated regions in mixDCIS. Additionally, 419 CpG sites exhibited stepwise methylation patterns, comprising 86 hypermethylation and 333 hypomethylation candidates.

Conclusions

Matched DCIS and IDC components of TNBC showed exploratory methylation differences, including hypermethylation of a *RASSF1*-related region, although none remained significant after multiple-testing correction. These candidates and the stepwise methylation patterns require validation in independent cohorts and do not establish temporal progression or predictive utility.

Introduction

Ductal carcinoma in situ (DCIS), in which neoplastic cells remain confined within the mammary ductal basement membrane, is considered a precursor lesion of invasive ductal carcinoma (IDC) [1, 2]. Although some DCIS lesions progress to invasive carcinoma, many may remain non-invasive throughout a patient's lifetime [1, 3]. Currently available clinicopathological factors cannot reliably distinguish between these lesions, raising concerns regarding the overtreatment of patients with lesions at low risk of invasion [2, 4]. Therefore, identifying molecular alterations associated with the acquisition of invasive potential at the DCIS stage is important for both understanding breast cancer progression and developing individualized treatment strategies.

Breast carcinogenesis involves both genetic alterations and epigenetic dysregulation [5, 6]. DNA methylation is one of the major epigenetic mechanisms and involves the addition of a methyl group to the fifth carbon of cytosine, predominantly at cytosine–guanine dinucleotides known as CpG sites [6]. Hypermethylation of promoter-associated CpG regions can suppress gene transcription, including that of tumor suppressor genes, and such alterations may occur early in breast carcinogenesis [5–7]. Fleischer et al. performed a large-scale analysis of normal breast tissue, pure DCIS, mixed DCIS–invasive breast carcinoma lesions, and invasive breast carcinoma. They reported extensive methylation alterations during the transition from normal breast tissue to DCIS, as well as additional differences involving approximately 1,000 genes between DCIS and invasive carcinoma [8]. Johnson et al. conducted a longitudinal analysis of estrogen receptor-positive DCIS and identified candidate CpG sites associated with the subsequent development of invasive breast cancer, with enrichment of genes involved in developmental regulation and homeobox-related pathways [9]. These findings suggest that DNA methylation may serve as a candidate indicator of DCIS progression [8, 9].

However, DCIS is molecularly heterogeneous, and its progression may differ among breast cancer subtypes [10, 11]. Bediaga et al. demonstrated distinct DNA methylation epigenotypes in basal-like, human epidermal growth factor receptor 2-enriched, luminal A, and luminal B breast cancers [10]. Bergholtz et al. further reported that methylation differences between DCIS and invasive breast carcinoma varied substantially according to molecular subtype and were most pronounced in basal-like lesions [11]. Consequently, comparisons between DCIS and invasive carcinoma without accounting for molecular subtype may be confounded by differences in subtype composition. DNA methylation alterations should therefore be evaluated separately within each breast cancer subtype [10, 11].

DCIS associated with triple-negative breast cancer (TNBC) is relatively uncommon, and few DNA methylation studies have directly compared the DCIS and IDC components of TNBC [11]. In paired within-patient comparisons, both components share patient-specific factors, including genetic background, age, lifestyle, and environmental exposures. This approach can reduce the influence of interindividual variation and facilitate a more direct assessment of methylation alterations associated with the transition from DCIS to IDC. Therefore, we compared the DCIS and IDC components of TNBC within the same patients to identify DNA methylation alterations potentially associated with invasion.

We performed genome-wide DNA methylation profiling using the Infinium MethylationEPIC BeadChip in eight specimens: paired DCIS and IDC components isolated by laser capture microdissection from mixed TNBC lesions in three patients and pure DCIS specimens from two additional patients. The primary objective was to identify differentially methylated positions (DMPs) and differentially methylated regions (DMRs) between the paired DCIS and IDC components. As a secondary objective, we identified CpG sites exhibiting stepwise methylation changes across pure DCIS, DCIS coexisting with IDC, and IDC to explore candidate methylation alterations associated with invasion in TNBC.

Materials and Methods

Study design and tissue samples

This was a single-center exploratory study using formalin-fixed, paraffin-embedded (FFPE) surgical specimens. The study included mixed TNBC lesions containing both DCIS and IDC components from three patients and pure TNBC–DCIS lesions without an invasive component from two patients. The DCIS and IDC components within mixed lesions were designated as mixDCIS and mixIDC, respectively, whereas DCIS obtained from lesions without an IDC component was designated as pureDCIS.

To focus on relatively early invasive lesions and limit the potential influence of secondary molecular alterations accumulated during subsequent tumor growth, only mixed lesions with an IDC component measuring less than 1 cm in maximum diameter were included. The clinicopathological characteristics of the patients are summarized in Table 1.

Table 1. Clinicopathological characteristics of the study samples

Case	Age	Invasive tumor size, (cm)	DCIS size (cm)	ER score	PgR score	HER2 score	Ki-67 %	Nuclear grade	Histological grade	Comedo necrosis
mixDCIS 01 mixIDC 01	57	0.3	2.6	0	0	0	29.5	1	1	Absent
mixDCIS 02 mixIDC 02	70	0.6	2.5	0	0	1	>50	3	3	Absent
mixDCIS 03 mixIDC 03	51	0.8	3.2	1	0	1	>50	3	3	Absent
pureDCIS 01	74	–	3.3	0	0	0	<10	2	–	Present
pureDCIS 02	50	–	4.1	0	0	0	>30	3	–	Present

Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2. ER and PgR were assessed by immunohistochemistry and graded using the J-score of the Japanese Breast Cancer Society; a J-score of 0 or 1 (staining in < 1% of tumor cell nuclei) was regarded as negative. HER2 was scored from 0 to 3+ according to the ASCO/CAP guidelines, and scores of 0 and 1+ were regarded as negative. Triple-negative breast cancer was defined as ER-negative, PgR-negative and HER2-negative by these criteria. Nuclear grade was assessed using the criteria applied in the JCOG1505 trial; histological grade was assigned to the invasive component only and is therefore not applicable to the pureDCIS lesions. Invasive tumor size and DCIS size denote the maximum diameter measured on the resected specimen. Comedo necrosis is recorded as present or absent. "–" indicates not applicable.

Hematoxylin and eosin-stained sections were reviewed by a pathologist to confirm the pathological diagnosis and identify the DCIS and IDC areas. The corresponding areas were selectively isolated from FFPE sections by laser capture microdissection (LCM) using an Arcturus XT™ system. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital (approval no. KEN 2606-040).

DNA extraction and methylation profiling

Genomic DNA was extracted from the laser capture-microdissected tissues using a method optimized for FFPE specimens. DNA quantity and concentration were measured, and the DNA extracts were concentrated, when necessary, because of the limited amount and fragmentation of FFPE-derived DNA. DNA from all eight specimens underwent bisulfite conversion and FFPE DNA restoration. Genome-wide DNA methylation levels were subsequently measured using the Infinium MethylationEPIC BeadChip v2.0 (Illumina).

Calculation of β values and data preprocessing

DNA methylation data were processed using the R package SeSAMe. Signal intensities, detection P values, and β values were calculated for each probe from the IDAT files. Low-quality probes were excluded based on their detection P values, followed by normalization and the exclusion of probes considered unsuitable for between-sample comparisons. For multiple probes targeting the same methylation site, β values were averaged to generate site-level methylation data. Trait associations from the EWAS Atlas and nearby gene annotations were assigned to each probe.

Differential methylation analysis

Differentially methylated positions (DMPs) were analyzed using the DML function in SeSAMe. Untransformed β values were fitted to a linear regression model with group as the sole explanatory variable. Unadjusted P values were calculated using t tests of the group coefficient.

Differentially methylated regions (DMRs) were identified using the DMR function in SeSAMe. Boundaries were defined where the distance between the β values of adjacent probes exceeded a threshold, and consecutive probes between boundaries were grouped into a single region. Regions containing only one probe were also included in the analysis. Region-level P values were calculated by combining constituent probe-level P values using Stouffer's Z-score method.

Methylation differences were expressed as $\Delta\beta$. For DMPs, $\Delta\beta$ was calculated as the mean β value in the latter group minus that in the reference group. For the mixDCIS–mixIDC comparison, a positive $\Delta\beta$ indicated higher methylation in mixIDC, whereas for the pureDCIS–mixDCIS comparison, a positive $\Delta\beta$ indicated higher methylation in mixDCIS.

DMPs and DMRs were considered statistically significant when they met both an absolute methylation difference of $|\Delta\beta| \geq 0.25$ and a Benjamini–Hochberg-adjusted P value < 0.05 . When no DMPs or DMRs met the FDR threshold in a given analysis, loci or regions satisfying $|\Delta\beta| \geq 0.25$ and an unadjusted P value < 0.05 were reported as exploratory candidates.

Identification of stepwise methylation candidates

CpG sites showing stepwise methylation changes across pureDCIS, mixDCIS, and mixIDC were identified. Candidates were required to show the same direction of methylation change in both the pureDCIS–mixDCIS and mixDCIS–mixIDC comparisons, with an absolute mean β -value difference of at least 0.10 between pureDCIS and mixDCIS and at least 0.25 between mixDCIS and mixIDC. In addition, all three matched patient pairs were required to show a concordant direction of change from mixDCIS to mixIDC. The identified candidates were ranked according to the absolute total methylation difference between pureDCIS and mixIDC. Statistical significance was not used as a selection criterion; therefore, this analysis was considered a hypothesis-generating exploratory analysis based on effect size and directionality.

Results

Comparison between mixDCIS and mixIDC

In the differentially methylated position (DMP) analysis comparing mixDCIS with mixIDC, 12,403 probes showed an absolute mean β -value difference of at least 0.25. Of these, 977 CpG sites had a nominal P value of < 0.05 , including 636 sites that were hypermethylated and 341 that were hypomethylated in mixIDC. The smallest nominal P value was 2.85×10^{-4} ; however, no DMPs met the false discovery rate (FDR) threshold of < 0.05 after Benjamini–Hochberg correction.

The DMP with the smallest P value was cg18592459, annotated to *GREM2* ($\Delta\beta = -0.308$, $P = 2.85 \times 10^{-4}$), followed by cg19130860, annotated to *MRPL23* ($\Delta\beta = +0.658$, $P = 4.25 \times 10^{-4}$), and cg19352193, annotated to *SIAH3* ($\Delta\beta = -0.269$, $P = 6.60 \times 10^{-4}$). Other candidate CpG sites were annotated to *MAP3K9*, *IRF4*, *RPL9P24*, *BTBD2* and *P3H2-AS1* (Table 2A).

In the differentially methylated region (DMR) analysis comparing mixDCIS with mixIDC, 10,009 regions showed an absolute mean β -value difference of at least 0.25, of which 840 had a nominal P value of <0.05 . Among these regions, 507 (60.4%) were hypermethylated and 333 (39.6%) were hypomethylated in mixIDC. When the analysis was restricted to regions containing at least two CpG sites, 264 regions were identified, including 183 (69.3%) that were hypermethylated and 81 (30.7%) that were hypomethylated in mixIDC.

No DMRs met the FDR threshold of <0.05 . The *RASSF1*-related region showed the smallest nominal P value and was hypermethylated in mixIDC ($\Delta\beta = 0.397$, $P = 1.34 \times 10^{-5}$, FDR = 0.424). Regions associated with *SMG6-IT1*, *LEXM*, *CLCNKA*, and *ZNF154* were also among the leading candidates containing at least two CpG sites (Table 2B).

Table 2. Top differentially methylated positions and regions between mixDCIS and mixIDC

Table 2A. Top DMP candidates between mixDCIS and mixIDC

Rank	CpG ID	Gene	Mean β mixDCIS	Mean β mixIDC	$\Delta\beta$	Direction in mixIDC	P value	FDR
1	cg18592459	<i>GREM2</i>	0.425	0.118	-0.308	Hypomethylated	2.85E-04	9.01E-01
2	cg19130860	<i>MRPL23</i>	0.211	0.869	0.658	Hypermethylated	4.25E-04	9.01E-01
3	cg19352193	<i>SIAH3</i>	0.311	0.042	-0.269	Hypomethylated	6.60E-04	9.01E-01
4	cg02311152	<i>MAP3K9</i>	0.379	0.093	-0.286	Hypomethylated	1.03E-03	9.01E-01
5	cg09033053	<i>IRF4</i>	0.454	0.793	0.339	Hypermethylated	1.10E-03	9.01E-01
6	cg12045560	<i>RPL9P24</i>	0.586	0.860	0.274	Hypermethylated	1.29E-03	9.01E-01
7	cg11894504	<i>BTBD2</i>	0.413	0.045	-0.368	Hypomethylated	1.61E-03	9.01E-01
8	cg00578624	<i>P3H2-AS1</i>	0.596	0.933	0.337	Hypermethylated	1.80E-03	9.01E-01
9	cg15706604	—	0.430	0.109	-0.321	Hypomethylated	1.94E-03	9.01E-01
10	cg21936959	<i>MRGPRF</i>	0.543	0.956	0.413	Hypermethylated	2.61E-03	9.01E-01

Table 2B. Top DMR candidates containing at least two CpG sites between mixDCIS and mixIDC

Rank	Gene	No. of probes	Mean β mixDCIS	Mean β mixIDC	$\Delta\beta$	Direction in mixIDC	P value	FDR
1	<i>RASSF1</i>	3	0.179	0.576	0.397	Hypermethylated	1.34E-05	0.424
2	<i>SMG6-IT1</i>	4	0.612	0.888	0.275	Hypermethylated	1.91E-04	0.893
3	-	4	0.442	0.713	0.272	Hypermethylated	5.87E-04	0.893
4	<i>LEXM</i>	3	0.613	0.941	0.328	Hypermethylated	6.24E-04	0.893
5	<i>CLCNKA</i>	4	0.349	0.748	0.400	Hypermethylated	6.59E-04	0.893
6	<i>ZNF154</i>	3	0.300	0.774	0.474	Hypermethylated	9.12E-04	0.893
7	<i>TBX5-AS1</i>	3	0.454	0.142	-0.312	Hypomethylated	1.68E-03	0.893
8	<i>GLI2</i>	4	0.478	0.781	0.302	Hypermethylated	1.87E-03	0.893
9	<i>LOC112268198</i>	2	0.237	0.754	0.517	Hypermethylated	2.04E-03	0.893
10	<i>H1-0</i>	3	0.608	0.864	0.255	Hypermethylated	2.08E-03	0.893

The table presents the regions with the smallest unadjusted P values among the 264 differentially methylated regions that contained at least two CpG sites and met the criteria of $|\Delta\beta| \geq 0.25$ and an unadjusted P value of < 0.05 . Regions represented by a single probe were excluded because they are equivalent to the corresponding DMPs shown in Table 2A and cannot be interpreted as region-level events. $\Delta\beta$ was calculated as the mean β value in mixIDC minus that in mixDCIS. Positive and negative values indicate hypermethylation and hypomethylation in mixIDC, respectively. FDR values were calculated using the Benjamini–Hochberg method. None of the candidates met $FDR < 0.05$.

Comparison between pureDCIS and mixDCIS

In the DMP analysis comparing pureDCIS with mixDCIS, 12,031 probes showed an absolute mean β -value difference of at least 0.25, of which 2,771 had a nominal P value of < 0.05 . Among these CpG sites, 471 were hypermethylated and 2,300 were hypomethylated in mixDCIS, indicating a predominance of hypomethylated candidates. The smallest nominal P value was 8.95×10^{-7} ; however, no DMPs met the FDR threshold of < 0.05 , and the smallest FDR was 0.221.

In the DMR analysis comparing pureDCIS with mixDCIS, 9,277 regions showed an absolute mean β -value difference of at least 0.25, of which 2,370 had a nominal P value of < 0.05 . Among these regions, 429 were hypermethylated and 1,941 were hypomethylated in mixDCIS. Of the 33 DMRs that met the FDR threshold of < 0.05 , only one region, annotated to *CARHSP1-DT*, was hypermethylated in mixDCIS (chr16:8,873,116–8,873,117; cg21103526; $\Delta\beta = 0.383$; $P = 8.95 \times 10^{-7}$; $FDR = 0.010$) (Table 3).

Table 3. Top DMR candidates between pureDCIS and mixDCIS

Rank	Gene	No. of probes	Mean β pureDCIS	Mean β mixDCIS	$\Delta\beta$	Direction in mixDCIS	P value	FDR
1	<i>RASSF1</i>	12	0.482	0.180	-0.302	Hypomethylated	4.45E-12	6.60E-07
2	<i>EYA4</i>	10	0.348	0.085	-0.262	Hypomethylated	5.35E-12	6.60E-07
3	<i>RPL5P28</i>	3	0.504	0.099	-0.405	Hypomethylated	1.51E-11	1.24E-06
4	<i>MSC</i>	6	0.483	0.051	-0.432	Hypomethylated	2.51E-11	1.55E-06
5	<i>CPEB1-AS1</i>	5	0.509	0.071	-0.438	Hypomethylated	4.08E-10	1.80E-05
6	<i>ALDH1L1-AS2</i>	7	0.397	0.062	-0.335	Hypomethylated	4.38E-10	1.80E-05
7	<i>ABCC9</i>	6	0.349	0.081	-0.269	Hypomethylated	5.98E-10	2.11E-05
8	<i>NINJ2-AS1</i>	5	0.809	0.437	-0.372	Hypomethylated	7.57E-09	2.34E-04
9	<i>NOL3</i>	8	0.455	0.180	-0.276	Hypomethylated	1.18E-08	3.23E-04
10	<i>CDH13</i>	12	0.425	0.101	-0.324	Hypomethylated	6.91E-08	1.62E-03
Hyper	<i>CARHSP1-DT</i>	1	0.556	0.939	0.383	Hypermethylated	8.95E-07	1.00E-02

The table presents the 10 hypomethylated DMRs with the smallest unadjusted P values among 33 DMRs meeting FDR < 0.05. *CARHSP1-DT* was additionally included because it was the only DMR showing hypermethylation in mixDCIS among the 33 significant DMRs. $\Delta\beta$ was calculated as the mean β value in mixDCIS minus that in pureDCIS. Positive and negative $\Delta\beta$ values indicate hypermethylation and hypomethylation in mixDCIS, respectively. P values were obtained from the unpaired DMR analysis; FDR values were calculated using the Benjamini–Hochberg method.

Mean β values were calculated from two pureDCIS and three mixDCIS samples across all probes in each region.

Stepwise methylation candidates

CpG sites showing methylation changes in the same direction in both the pureDCIS–mixDCIS and mixDCIS–mixIDC comparisons were identified. Candidates were required to show an absolute mean β -value difference of at least 0.10 between pureDCIS and mixDCIS and at least 0.25 between mixDCIS and mixIDC. In addition, all three matched patient pairs were required to show a concordant direction of methylation change from mixDCIS to mixIDC. A total of 419 CpG sites met these criteria, including 86 stepwise hypermethylation candidates and 333 stepwise hypomethylation candidates. (Details are shown in Additional file 1.)

When the candidates were ranked according to the absolute total methylation difference between pureDCIS and mixIDC, the leading stepwise hypermethylation candidates were cg03044603, annotated to *ZNF835* (total $\Delta\beta$ = 0.600); cg03941417, annotated to *DAND5* (total $\Delta\beta$ = 0.597); and the unannotated cg15691119 (total $\Delta\beta$ = 0.577) (Table 4A). The leading stepwise hypomethylation candidates were cg14582154, annotated to *RPL12P23* (total $\Delta\beta$ = -0.732);

cg16548911, annotated to *MIR6771* (total $\Delta\beta = -0.717$); and the unannotated cg25182165 (total $\Delta\beta = -0.708$) (Table 4B).

Table 4. Top stepwise methylation candidates across pureDCIS, mixDCIS, and mixIDC

Table 4A. Stepwise hypermethylation candidates

Rank	CpG ID	Gene	Mean β pureDCIS	Mean β mixDCIS	Mean β mixIDC	$\Delta\beta 1$ mixDCIS - pureDCIS	$\Delta\beta 2$ mixIDC - mixDCIS	Total $\Delta\beta$ mixIDC - pureDCIS
1	cg03044603	<i>ZNF835</i>	0.087	0.211	0.687	0.124	0.476	0.600
2	cg03941417	<i>DAND5</i>	0.313	0.525	0.910	0.213	0.385	0.597
3	cg15691119	—	0.136	0.273	0.714	0.136	0.441	0.577
4	cg23998167	<i>LOC105372253</i>	0.233	0.395	0.775	0.162	0.380	0.543
5	cg05946697	<i>RASD1</i>	0.314	0.486	0.854	0.172	0.369	0.540
6	cg08358803	<i>RANBP17</i>	0.281	0.415	0.821	0.135	0.406	0.540
7	cg00007845	—	0.413	0.673	0.943	0.260	0.270	0.530
8	cg16063252	<i>RPL36AP37</i>	0.170	0.405	0.695	0.235	0.290	0.525
9	cg00948520	<i>LINC02861</i>	0.254	0.382	0.779	0.127	0.397	0.524
10	cg27267310	<i>RPL36AP37</i>	0.110	0.273	0.632	0.163	0.359	0.522

Table 4B. Stepwise hypomethylation candidates

Rank	CpG ID	Gene	Mean β pureDCIS	Mean β mixDCIS	Mean β mixIDC	$\Delta\beta 1$ mixDCIS - pureDCIS	$\Delta\beta 2$ mixIDC - mixDCIS	Total $\Delta\beta$ mixIDC - pureDCIS
1	cg14582154	<i>RPL12P23</i>	0.821	0.512	0.089	-0.309	-0.422	-0.732
2	cg16548911	<i>MIR6771</i>	0.904	0.711	0.187	-0.193	-0.524	-0.717
3	cg25182165	—	0.910	0.692	0.202	-0.218	-0.490	-0.708
4	cg14001023	<i>PGLYRP1</i>	0.909	0.692	0.230	-0.217	-0.462	-0.680
5	cg26236329	<i>SLC43A3</i>	0.772	0.489	0.113	-0.283	-0.376	-0.659
6	cg13794530	<i>VIPR2</i>	0.812	0.434	0.159	-0.378	-0.275	-0.653
7	cg21928095	<i>HAUS5-DT</i>	0.844	0.725	0.197	-0.119	-0.528	-0.647
8	cg16656411	<i>SPICP3</i>	0.719	0.485	0.075	-0.234	-0.410	-0.644
9	cg07822777	—	0.843	0.506	0.203	-0.338	-0.303	-0.640
10	cg16993923	<i>LOC101929227</i>	0.852	0.565	0.215	-0.287	-0.350	-0.637

Candidates were required to change in the same direction in both comparisons, with $|\Delta\beta1| \geq 0.10$ and $|\Delta\beta2| \geq 0.25$. All three matched mixDCIS–mixIDC pairs were required to show a concordant direction of change.

Discussion

Overview and clinical context

This exploratory study compared DNA methylation profiles among three groups by separating the ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) components coexisting within the same surgical specimens of triple-negative breast cancer (TNBC) using laser capture microdissection and including pureDCIS as an additional comparison group. Hypermethylation candidates predominated in the mixDCIS–mixIDC comparison, whereas hypomethylation candidates predominated in the pureDCIS–mixDCIS comparison. Although no differentially methylated positions (DMPs) or differentially methylated regions (DMRs) in the mixDCIS–mixIDC comparison remained significant after correction for multiple testing, several exploratory candidates, including a *RASSF1*-related region, were identified. In addition, 419 CpG sites showing stepwise methylation changes across the three groups were identified.

The clinical impetus for identifying molecular alterations associated with invasion arises from ongoing efforts to omit or defer surgery in ductal carcinoma in situ (DCIS) judged to be at low risk of progression. Prospective trials of active surveillance and non-surgical management—LORIS, LORD, COMET and LORETTA—have tested whether selected patients can be managed without immediate surgery [12–15]. In the COMET randomized noninferiority trial, 957 women aged 40 years or older with screen-detected, nuclear grade 1–2, hormone receptor-positive, human epidermal growth factor receptor 2 (HER2)-negative DCIS and no palpable or imaging-detected mass were assigned to guideline-concordant care ($n = 473$) or active monitoring ($n = 484$) [14]. At a median follow-up of 36.9 months, ipsilateral invasive breast cancer was diagnosed in 27 and 19 women, respectively; the 2-year cumulative incidence was 5.9% (95% confidence interval [CI], 3.71–8.04) with guideline-concordant care and 4.2% (95% CI, 2.31–6.00) with active monitoring, meeting the prespecified criterion for noninferiority [14]. Two features of these data bear directly on the question addressed here. First, in the per-protocol analysis, the 2-year cumulative incidence of ipsilateral invasive breast cancer was 8.7% (95% CI, 5.06–12.21) in the guideline-concordant care group and 3.1% in the active monitoring group [14]. These estimates reflect invasive cancer diagnoses over a 2-year period and should not be interpreted as the prevalence of occult invasion at baseline. Second, the invasive cancers that were identified were not uniformly indolent: axillary nodal involvement was present in 18.5% of the invasive cancers in the guideline-concordant care group and in 10.5% of those in the active monitoring group, and 15.8% of the invasive cancers arising during active monitoring were of high histological grade [14].

The Japanese experience points in the same direction. In LORETTA (JCOG1505), a single-arm confirmatory trial in which 341 women with estrogen receptor-positive, HER2-negative, nuclear grade 1–2 DCIS without comedo necrosis received tamoxifen alone without surgery, the 5-year cumulative incidence of ipsilateral invasive breast cancer was 9.8% (95% CI, 5.2–16.1) at a median follow-up of 36 months, exceeding the prespecified safety threshold of 7%; 9.7% of patients ultimately underwent surgery, and overall survival at 5 years was 98.8% [15]. The result was not interpreted as excluding non-surgical management in carefully selected patients, but it demonstrates, as COMET does, that a non-negligible proportion of lesions satisfying stringent low-risk criteria either harbor occult invasion or acquire invasive potential during observation [14,15]. Consistent with this, Semba et al. recently reviewed neoadjuvant endocrine therapy and nonoperative management of estrogen receptor-positive DCIS and concluded that the biomarkers currently used to gauge response, principally Ki-67 suppression and radiological volume reduction, remain experimental and have not been validated against long-term oncological outcomes; across the neoadjuvant endocrine therapy series they summarized, upstaging to invasive carcinoma ranged from 10.2% to 30% [16].

Two further lines of evidence indicate that the selection of DCIS for de-escalated management cannot rest on conventional clinicopathological assessment alone. First, the histopathological criteria that define low-risk disease are themselves imperfectly reproducible. In the interobserver agreement study conducted to support the eligibility assessment for JCOG1505, agreement among pathologists was only moderate for nuclear grade ($\kappa = 0.595$ and 0.519 across two sessions), although substantial for comedo necrosis ($\kappa = 0.753$) [17]. Second, a preoperative diagnosis of DCIS is frequently incomplete. In a Japanese multi-institutional cohort of 2,293 patients preoperatively diagnosed with DCIS, 27.5% were upstaged to invasive carcinoma at surgery; even among patients lacking all four independent predictors of upstaging (palpable mass, mammographic category ≥ 4 , a mass on ultrasonography, and tumor size >20 mm on magnetic resonance imaging), the upstaging rate remained 10.3% [18]. Clinical, radiological and morphological variables therefore capture only part of the biological variation that determines whether a given DCIS lesion has acquired, or will acquire, the capacity to invade [16–18].

A further constraint concerns hereditary predisposition: DCIS forms part of the phenotype of pathogenic germline variants in *BRCA1* and *BRCA2*, with an estimated lifetime risk of approximately 6.2% in carriers compared with about 1.0% in the general population [19], and such variants are detected at appreciable frequency in DCIS diagnosed before 50 years of age, with *BRCA1* variants found predominantly in estrogen receptor-negative lesions [20]. Carriers are nevertheless excluded from the non-surgical strategies described above, not because the in situ-to-invasive transition has been shown to be faster, but because the competing risk of a new ipsilateral or contralateral primary cancer means that risk-reducing surgery or intensified surveillance is already indicated on independent grounds [13, 21, 22]. This is directly relevant to the present work, because the *BRCA1*-deficient, basal-like phenotype can arise epigenetically as well as genetically – *BRCA1* promoter hypermethylation has been reported in 24.1% of early-stage triple-negative breast cancers and in 46.2% of women aged under 50 years, and such tumors are homologous recombination-deficient and otherwise molecularly indistinguishable from *BRCA1*-mutated tumors [23, 24] – yet germline testing results were not available for the five patients in this series and no directed analysis of *BRCA1* promoter methylation was undertaken, both of which are straightforward extensions of the present dataset and should be incorporated into any larger series.

Positioning of the present study

Against this background, molecular characterization of the DCIS component itself represents a potentially useful complementary approach for identifying lesions with invasive potential. In the present study, we compared the DCIS and IDC components within the same lesion to minimize the effects of interindividual differences, including genetic background, age, lifestyle, and environmental exposures. This matched approach may therefore provide a more direct assessment of DNA methylation alterations associated with the transition from DCIS to IDC.

Several considerations, however, are important when interpreting our findings. First, the present study focused on TNBC, whereas the clinical trials investigating non-surgical management have primarily enrolled estrogen receptor-positive, low-grade DCIS [14,15]. TNBC is not currently included in these non-surgical management strategies. Therefore, our findings cannot be directly applied to patient selection for active surveillance or other non-surgical approaches.

Nevertheless, restricting the analysis to TNBC was intentional. DNA methylation differences between DCIS and invasive carcinoma have been reported to vary substantially according to molecular subtype and to be particularly pronounced in basal-like lesions [11]. Subtype-specific analysis is therefore important when investigating molecular alterations associated with invasion. In addition, DNA methylation studies directly comparing DCIS and IDC components in TNBC remain limited [11]. The present study therefore provides an exploratory framework for investigating invasion-associated methylation alterations within a specific breast cancer subtype.

Major DNA methylation changes

In the mixDCIS–mixIDC comparison, 65.1% of the DMP candidates and 69.3% of the DMR candidates containing at least two CpG sites were hypermethylated in mixIDC. Fleischer et al. reported that, among 2,000 CpG sites differing between DCIS and invasive carcinoma, 1,745 were hypermethylated and 255 were hypomethylated in invasive carcinoma [8]. The predominance of hypermethylation candidates in mixIDC in the present study is therefore directionally consistent with previous findings.

However, the numbers and proportions of candidates cannot be directly compared between the two studies because of differences in the breast cancer subtypes examined, tissue sampling methods, DNA isolation procedures, analytical platforms, and candidate-selection criteria. Furthermore, given the very small sample size of the present study, whether the observed predominance of hypermethylation represents a reproducible feature of TNBC invasion remains uncertain.

In contrast, in the pureDCIS–mixDCIS comparison, 83.0% of the DMP candidates and 81.9% of the DMR candidates meeting the criteria of nominal $P < 0.05$ and $|\Delta\beta| \geq 0.25$ were hypomethylated in mixDCIS. Although no DMPs remained significant after correction for multiple testing, 33 DMRs met the FDR threshold of < 0.05 , of which 32 were hypomethylated in mixDCIS.

These findings raise the possibility that DCIS coexisting with invasive carcinoma may have epigenetic characteristics distinct from those of pureDCIS. However, this comparison should be interpreted cautiously because pureDCIS and mixDCIS were obtained from different patients. Consequently, interindividual differences in genetic background, age, environmental exposures, and other clinicopathological characteristics could not be excluded. In addition, the pureDCIS and mixed lesions differed morphologically: both pureDCIS lesions showed comedo necrosis, whereas none of the mixed lesions did, and the pureDCIS lesions were relatively large, measuring 3.3 and 4.1 cm. Thus, the pureDCIS–mixDCIS comparison confounds the presence or absence of an invasive component with differences in histological features and tumor size.

Accordingly, the observed regional hypomethylation in mixDCIS may reflect either the coexistence of an invasive component or differences in the underlying clinicopathological characteristics of the lesions. Therefore, these findings should not be interpreted as direct evidence of temporal progression from pureDCIS to mixDCIS and subsequently to IDC. In particular, the term "stepwise" in the present study describes concordant directional changes across the three groups rather than a demonstrated temporal sequence.

RASSF1-related region

Among the individual candidates, a *RASSF1*-related region showed the smallest nominal P value in the DMR analysis and was hypermethylated in mixIDC compared with mixDCIS ($\Delta\beta = 0.397$, $P = 1.34 \times 10^{-5}$). *RASSF1A*, a major isoform of *RASSF1*, is a tumor suppressor involved in cell-cycle regulation, apoptosis, microtubule stability, and Hippo signaling [25, 26]. Transcriptional silencing of *RASSF1A* through promoter hypermethylation has been widely reported in breast cancer [27–29].

Hypermethylation of the *RASSF1A* promoter has been detected not only in invasive breast carcinoma but also at the DCIS stage. Honorio et al. compared normal breast tissue, DCIS, and IDC and detected *RASSF1A* promoter hypermethylation in 65% of IDC specimens and 42% of DCIS components from the same breasts, whereas no methylation was detected in normal breast tissue. Hypermethylation was also detected in 3 of 10 pure DCIS lesions without an invasive component, suggesting that *RASSF1A* inactivation may arise relatively early during breast carcinogenesis[29].

Pang et al. further reported that *RASSF1A* was the most frequently methylated gene in microdissected DCIS specimens, with methylation detected in 72 of 80 cases (90%). *RASSF1A* methylation was also associated with comedo necrosis, suggesting a possible relationship with aggressive pathological features already present at the DCIS stage [30].

However, previous studies have not consistently demonstrated a uniform increase in *RASSF1A* methylation during the transition from DCIS to IDC. Van Hoesel et al. analyzed normal breast epithelium, usual ductal hyperplasia, atypical ductal hyperplasia, DCIS, and early IDC isolated by laser capture microdissection. Although *RASSF1A* methylation increased markedly between normal breast epithelium and ductal hyperplasia, the methylation indices were similar in DCIS and IDC, at 0.79 and 0.76, respectively. These findings suggest that *RASSF1A* methylation may be established early in breast carcinogenesis rather than being newly acquired at the time of invasion [28].

An apparent discordance within our own data merits comment. A *RASSF1*-related region also ranked first among the DMRs distinguishing pureDCIS from mixDCIS, but in the opposite direction: mean methylation was lower in mixDCIS ($\beta = 0.180$) than in pureDCIS ($\beta = 0.482$; $\Delta\beta = -0.302$; $\text{FDR} = 6.60 \times 10^{-7}$), whereas it was higher in mixIDC ($\beta = 0.576$) than in mixDCIS. The pattern across the three groups is therefore non-monotonic rather than stepwise, and this region was accordingly not retained among the stepwise candidates. Non-monotonic methylation trajectories have been described in breast cancer progression; Fleischer et al. identified regions in which methylation decreased from normal breast tissue to DCIS and increased again in invasive carcinoma [8]. In the present data, however, at least three alternative explanations cannot be distinguished: interindividual variation between the unrelated pureDCIS and mixDCIS patients; the histological differences between these groups noted above, given the reported association of *RASSF1A* methylation with comedo necrosis, which was present in both pureDCIS lesions [30]; and differences in probe coverage between the two comparisons (12 probes versus 3 probes). This observation should therefore be regarded as a reason for targeted analysis rather than as evidence of a biphasic trajectory.

The potential significance of *RASSF1* methylation has also been investigated in TNBC. In a study comparing 40 patients with TNBC and 50 with non-TNBC, *RASSF1* promoter methylation was more frequent in TNBC and was associated with shorter disease-free and overall survival within the TNBC group [31]. However, *RASSF1* methylation did not remain an independent prognostic factor in multivariable analysis, and an association with disease-free survival was also observed in non-TNBC [31]. Furthermore, previous studies investigating associations between *RASSF1A* methylation and hormone receptor status or molecular subtype have yielded inconsistent findings [32–34]. Thus, although *RASSF1A* methylation represents an important molecular alteration observed in TNBC, it cannot currently be regarded as specific to this subtype.

The hypermethylation of the *RASSF1*-related region observed in mixIDC in the present study warrants further investigation in light of previous findings concerning *RASSF1A* methylation in breast cancer [32–34]. Nevertheless, methylation frequency in previous studies and the mean β -value difference evaluated in the present study represent different measures and cannot be directly compared. Moreover, the FDR for this region was 0.424, indicating that the difference was not statistically significant after correction for multiple testing. This region should therefore be regarded as an exploratory candidate. After confirming its positional relationship with the *RASSF1A* promoter, targeted bisulfite analysis and gene expression analysis will be required to evaluate the reproducibility and functional relevance of this finding.

Stepwise methylation candidates

We identified 419 CpG sites that changed in the same direction in both the pureDCIS–mixDCIS and mixDCIS–mixIDC comparisons and met the prespecified thresholds for mean β -value differences and directional concordance across all

three matched patient pairs. Stepwise methylation alterations accompanying breast cancer progression have also been reported previously. Fleischer et al. demonstrated that methylation of *CPA1*, *CUL7*, *LRRTM2*, and *POU2AF1* increased sequentially from normal breast tissue to DCIS and invasive carcinoma [8]. They also identified regions in which methylation decreased from normal breast tissue to DCIS and subsequently increased in invasive carcinoma, indicating that breast cancer progression may involve both monotonic and non-monotonic methylation patterns [8].

Johnson et al. identified 641 CpG sites associated with the subsequent development of invasive breast cancer in a study of 40 patients with estrogen receptor-positive DCIS [9]. At 276 of these sites, methylation changes observed between normal tissue and DCIS were further accentuated in DCIS lesions from patients who subsequently developed invasive breast cancer [9]. These findings suggest that some methylation abnormalities associated with invasive disease may already be present at the DCIS stage and that the extent of these alterations may be related to subsequent development of invasive cancer.

The present analysis therefore considered both the direction of methylation changes across lesion groups and the consistency of these changes within matched patients. Nevertheless, because statistical significance was not used as a selection criterion in the stepwise analysis, the 419 CpG sites identified here should be regarded as hypothesis-generating candidates rather than validated biomarkers. Further studies are required to determine whether these alterations are reproducibly associated with invasive potential.

Limitations and future directions

Several limitations should be considered when interpreting the present findings. First, this was a very small exploratory study involving only eight specimens from five patients. Consequently, the estimated effect sizes are highly uncertain, and both false-positive findings and insufficient statistical power must be considered. The absence of DMPs or DMRs that remained significant after multiple-testing correction in the matched mixDCIS–mixIDC comparison does not exclude biologically meaningful methylation differences and may partly reflect the limited sample size.

Second, the pureDCIS and mixDCIS specimens were obtained from different patients and differed in tumor size and histological characteristics. Thus, the pureDCIS–mixDCIS comparison confounds the presence or absence of an invasive component with interindividual and histological differences. Furthermore, the subsequent clinical course of the two pureDCIS cases was not evaluated. Accordingly, the three groups cannot be considered to represent a temporal sequence within the same lesions.

Third, normal breast epithelium was not included in the analysis. Therefore, we could not determine whether the methylation alterations observed in DCIS were already established before the in situ stage or were acquired during the transition from DCIS to IDC.

Fourth, the restriction of the invasive component to less than 1 cm may have introduced selection bias. Although this criterion was intended to minimize secondary molecular alterations associated with tumor growth, lesions with rapidly progressive invasive growth may have been underrepresented.

Fifth, germline *BRCA1* and *BRCA2* status was unavailable for all five patients. Therefore, hereditary and sporadic cases could not be distinguished. In addition, *BRCA1* promoter methylation was not specifically assessed in the present study.

Several technical limitations should also be acknowledged. Although laser capture microdissection was used, contamination by non-neoplastic cells, differences in tumor cell content between DCIS and IDC components, and intralesional heterogeneity could not be completely eliminated. No cell-type deconvolution or adjustment for tumor

purity was performed. Because β values can be influenced by tumor cell content, this issue is particularly relevant when comparing an in situ component embedded within stroma with an invasive component [35,36].

Furthermore, DNA extracted from formalin-fixed, paraffin-embedded tissue is fragmented and requires restoration before hybridization, which may introduce additional measurement variability[37]. The small number of arrays also prevented a formal assessment of batch effects. The directional-concordance criterion used in the stepwise analysis only partially addresses this issue.

Regions represented by a single probe, such as the *CARHSP1-DT*-related region, should therefore not be interpreted as definitive region-level methylation alterations. In addition, EPIC v2 probe annotation continues to be refined, and the positional relationship between the *RASSF1*-related region identified here and the *RASSF1A* promoter and CpG island should be confirmed [38].

Finally, no orthogonal validation, gene expression analysis, or independent cohort validation was performed. Therefore, none of the candidates identified in this study should currently be considered a validated biomarker or used to inform clinical decision-making.

These limitations also define the priorities for future research. First, the present dataset should be reanalyzed using statistical models that appropriately account for the matched patient structure and differences in tumor cell content. The *RASSF1A*-related region and leading stepwise candidates should then be evaluated using targeted bisulfite sequencing or pyrosequencing. A direct analysis of *BRCA1* promoter methylation would also be informative.

Second, the reproducibility of these candidates should be examined using publicly available DNA methylation datasets, including The Cancer Genome Atlas and datasets from previously published studies of DCIS and invasive breast carcinoma [8,9,11]. Because methylation differences between DCIS and invasive carcinoma may depend strongly on molecular subtype, these analyses should focus on, or at least be stratified by, TNBC and basal-like lesions [11].

Finally, prospective studies are required to determine the clinical significance of these methylation alterations. In particular, the methylation profile of DCIS at diagnosis should be linked to subsequent clinical outcomes to determine whether the identified alterations are associated with future development of invasive carcinoma. Such a design is essential for distinguishing methylation changes that merely accompany invasive disease from alterations that can predict future invasive potential.

Conclusion

In this study, we compared matched mixDCIS and mixIDC components from patients with triple-negative breast cancer to explore DNA methylation alterations potentially associated with invasion. Although no differentially methylated positions or regions remained significant after correction for multiple testing in the mixDCIS–mixIDC comparison, a *RASSF1*-related region was identified as an exploratory candidate. We also identified 419 CpG sites showing stepwise methylation patterns across the three groups. Because a proportion of DCIS lesions managed without immediate surgery prove to harbour or to develop invasive disease, and because clinicopathological criteria alone identify these lesions imperfectly, subtype-specific molecular characterization of the in situ component is a rational complementary line of investigation. These exploratory findings require validation through targeted methylation analyses in independent cohorts. All of the findings presented here are exploratory. They require, in order, re-analysis accounting for the matched study structure, validation against publicly available methylation datasets restricted to or stratified by triple-negative and basal-like lesions, and ultimately prospective evaluation in cohorts in which the methylation profile of DCIS at diagnosis is linked to subsequent invasive events.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital (approval no. KEN 2606-040). The requirement for individual informed consent was waived because of the retrospective nature of the study and the use of archived formalin-fixed, paraffin-embedded tissue specimens, in accordance with institutional regulations.

Consent for publication

Not applicable.

Availability of data and materials

The DNA methylation datasets generated and analyzed during this study are available from the corresponding author upon reasonable request. The list of 419 CpG sites showing stepwise methylation patterns is provided in Additional file 1.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TT conceived and designed the study and contributed to the interpretation of the data. NY collected the clinical and pathological data, prepared the samples, and performed the DNA methylation and statistical analyses. ST critically reviewed the manuscript for important intellectual content. All authors read and approved the final manuscript.

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