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Cytosine 5-Hydroxymethylation of the *LZTS1* Gene Is Reduced in Breast Cancer[1,2]

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Abstract

Change of DNA cytosine methylation (5mC) is an early event in the development of cancer, and the recent discovery of a 5-hydroxymethylated form (5hmC) of cytosine suggests a regulatory epigenetic role that might be different from 5-methylcytosine. Here, we aimed at elucidating the role of 5hmC in breast cancer. To interrogate the 5hmC levels of the *leucine zipper, putative tumor suppressor 1* (*LZTS1*) gene in detail, we analyzed 75 primary breast cancer tissue samples from initial diagnosis and 12 normal breast tissue samples derived from healthy persons. Samples were subjected to 5hmC glucosyltransferase treatment followed by restriction digestion and segment-specific amplification of 11 polymerase chain reaction products. Nine of the 11 5′*LZTS1* fragments showed significantly lower (fold change of 1.61-6.01, $P < .05$) 5hmC content in primary breast cancer tissue compared to normal breast tissue samples. No significant differences were observed for 5mC DNA methylation. Furthermore, both *LZTS1* and TET1 mRNA expressions were significantly reduced in tumor samples ($n = 75$, $P < .001$, Student's *t* test), which correlated significantly with 5hmC levels in samples. 5hmC levels in breast cancer tissues were associated with unfavorable histopathologic parameters such as lymph node involvement ($P < .05$, Student's *t* test). A decrease of 5hmC levels of *LZTS1*, a classic tumor suppressor gene known to influence metastasis in breast cancer progression, is correlated to down-regulation of *LZTS1* mRNA expression in breast cancer and might epigenetically enhance carcinogenesis. The study provides support for the novel hypothesis that suggests a strong influence of 5hmC on mRNA expression. Finally, one may also consider 5hmC as a new biomarker.

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Introduction

Cytosine methylation is a well-studied epigenetic mechanism, which is essential for silencing of repetitive elements, X-chromosome inactivation, or imprinting and is also involved in gene repression and chromatin remodeling [1] through recruitment of specific factors [2]. Changes in DNA methylation can be detected early during tumorigenesis [3], and recent data have allowed for tumor classification based on genome-wide DNA methylation signatures of different cancers [4]. Methylated cytosines can be subjected to hydroxymethylation (5hmC) by ten eleven translocation (TET) enzymes, which was discovered in mammalian genomes in 2009 by Kriaucionis and Heintz [5]. However, the function and underlying mechanisms of 5hmC DNA modification have not yet been fully elucidated and it is still unclear whether 5hmC serves solely as a precondition for DNA demethylation or has a regulatory role by itself [6]. Classic bisulfite deamination–based sequence analysis cannot distinguish 5hmC from 5mC [7], but recent advances have made it possible to determine 5hmC-modified sequences by introducing an oxidative step into the DNA bisulfite conversion procedure [8]. Most studies have relied on a global antibody-based determination of 5hmC levels in different cell and tissue types [9], which was correlated with increased mRNA expression of target genes [10] and increased expression of TET proteins [11]. 5hmC levels have been analyzed in different tissues, and high levels of this modification were found in brain, kidney, or liver [9,10,12]. Loss of 5hmC has been observed in different cancers [13] and linked to decreased TET expression and mutations in isocitrate dehydrogenase 1 (IDH1 and IDH2) [14]. In mouse embryonic stem cells, 5hmC was found to be associated with active promoters and correlated with increased expression, and silencing of TET1 and TET2 resulted in increased 5mC and decreased expression of a group of genes including pluripotency factors [15]. A recently published report suggests that 5hmC is lost, resulting from a replication-dependent passive process [16].

Leucine zipper, putative tumor suppressor 1 (*LZTS1*) is a tumor suppressor, which was found repressed in various cancers such as breast carcinoma [17], squamous cell carcinoma [18], uveal melanoma [19], and bladder cancer [20]. Furthermore, the reduced amounts of mRNA in breast cancer could be connected to high histologic grade lymph node metastasis and poor prognosis in breast cancer [21]. Loss of *LZTS1* could, until now, not be explained by genetic factors like mutations or loss of heterozygosity (LOH) [20]. However, epigenetic silencing of *LZTS1* by DNA methylation was described in breast cancer [17,21] and malignant mesothelioma of the lung where the methylation status correlated with patient's lymph node involvement [22].

In this study, we aimed at exploring the feasibility of 5hmC as a biomarker in breast cancer by application of a targeted microarray [23] interrogating 323 loci. We detected aberrant 5-hydroxymethylation as well as unaffected methylation levels of *LZTS1* of DNA from breast cancer samples compared to normal breast tissue, which correlated with the mRNA expression of the *LZTS1* gene and *TET1*. Further, the variances in 5hmC within the patients with breast cancer could be connected to patients' nodal involvement.

Patients and Methods

Samples and Patients

A total of 75 fresh frozen invasive breast cancer tissue samples derived from patients with breast cancer and 12 samples containing normal breast tissue were collected at the Department of Obstetrics and Gynecology, Medical University of Vienna (Vienna, Austria) between 1990 and 1999 (Table 1). Twenty-six patients with breast cancer (34%) did not have involved lymph nodes at the time of surgery, whereas 47 patients (52%) had one or more lymph nodes involved. More detailed information on patient characteristics is given in Table 2. An assay control female blood was obtained from the Austrian Red Cross Blood Center. The Blood Center provided anonymized samples after the testing was successfully completed in accordance with the institute's guidelines. The study was approved by the ethics committee of the Medical University of Vienna and was carried out in compliance with the Helsinki Declaration.

Nucleic Acid Extraction

Approximately 15 mg of breast tissue was applied per sample for tissue lysis. Lysis was performed in Lysis Matrix A tubes (MP Biomedicals, Eschwege, Germany) combining the tissue and prechilled RLT plus buffer (Qiagen, Hilden, Germany) including 2-mercaptoethanol (Sigma, St Louis, MO). To achieve tissue extraction, a repeated bead whirling procedure was applied on a Fast Prep 24 (MP Biomedicals) at a speed setting of 5.5 for 30 seconds, twice per sample. The lysate was subsequently centrifuged at 13,000 rpm for 3 minutes to remove cell debris. The resulting 600 µl of cell lysate was applied to a Qiagen All Prep procedure, which was performed according to the manufacturer's instructions. DNA concentrations were determined through absorbance measurements. The quality and quantity of RNA were appointed with absorbance measurement and Agilent RNA 6000 Nano microfluidic chip.

Analyses of 5-Hydroxymethylcytosine

We applied a method that enables specific screening of 5hmC in DNA samples with quantitative polymerase chain reaction (qPCR) or

Table 1. Clinical Features of Breast Cancer Patients.

Characteristics	Patients (n = 75)
Nodal involvement	
LN0	n = 26 (34%)
LN1	n = 39 (52%)
LN2	n = 8 (10%)
NA	n = 2 (3%)
Tumor metastasis	
No	n = 29 (39%)
Yes	n = 46 (61%)
Histologic type	
Invasive lobular	n = 13 (17%)
Invasive ductal	n = 56 (74%)
Other	n = 4 (5%)
NA	n = 2 (3%)
Progesterone receptor status	
Negative	n = 47 (62%)
Positive	n = 28 (37%)
Estrogen receptor status	
Negative	n = 30 (40%)
Positive	n = 45 (60%)
Tumor grade	
1 + 2	n = 35 (47%)
3 + 4	n = 35 (47%)
NA	n = 5 (6%)
Tumor size	
1	n = 19 (25%)
2	n = 45 (60%)
3 + 4	n = 9 (12%)
NA	n = 2 (3%)

NA indicates patients, where the specific annotation was not available.
Characteristics reflect the analyzed subsets within the 75 patients with breast cancer. "Patients" indicate the number of patients within each clinical subset, also given in a percentage. The clinical annotation was not available for all 75 tested patients with cancer.

a targeted microarray. The principle of this approach is based on the selective glucosylation of 5hmC with T4-β-glucosyltransferase, which transfers a glucose moiety from uridine diphosphoglucose onto 5hmC, thus modifying it to glucosyl-5′-hydroxymethylcytosine (glu-5hmC), and the inability of glucosyl-sensitive restriction enzymes (GSREs) like *Msp*I to digest glu-5hmC DNA. *Msp*I is not sensitive to methylated or hydroxymethylated DNA and therefore enables a differentiation between glucosylated (i.e., hydroxymethylated) DNA and unmodified or methylated DNA. The initial screening for 5hmC markers was performed on a targeted microarray analyzing 323 genetic loci for their 5hmC status (Table W1).

Glucosylation of 5-Hydroxymethylcytosine

Glucosylation of 5hmC was performed using the Quest 5hmC Detection Kit (Zymo Research, Irvine, CA), according to manufacturer's instructions. Five hundred nanograms of genomic DNA from patient samples, standardized controls, and blood was applied to the Quest 5hmC Detection Kit. To distinguish between 5-hydroxymethylated DNA and methylated or unmethylated DNA, each reaction was split into two reactions containing the 5hmC glucosyltransferase enzyme or no enzyme, respectively. The reactions were incubated for 2 hours at 37°C in a total volume of 50 μl.

Glucosylation-Dependent Restriction Digestion (GSRE)

To detect differences between 5hmC-glucosylated DNA and mock-treated DNA, a restriction digestion with *Msp*I endonuclease was performed. Thirty units of *Msp*I were applied to each reaction and incubated at 37°C overnight. Each reaction was heat inactivated at 65°C for 20 minutes and subsequently purified with DNA Clean & Concentrator (Zymo Research). Performance of GSRE was tested using control DNA and a concordant primer (Figure W1).

Analyses of Methylcytosine

Similar to GSRE-dependent analysis of 5hmC content, methylation levels were assessed using the *Msp*I isoschizomer *Hpa*II, which is blocked by methylated cytosines. Two hundred nanograms of genomic DNA was subjected to methyl-sensitive restriction digestion (MSRE) in a reaction volume of 30 μl using 0.2 units *Hpa*II (Fermentas, Burlington, CA) per μl. The reaction was incubated at 37°C for 16 hours followed by 20 minutes at 65°C to inactivate the enzyme. Aliquots (2 μl) of reaction were subjected to qPCR. Methylation-specific qPCR was performed on all *LZTS1* fragments and the internal control (Table W2).

Quantitative Polymerase Chain Reaction

We performed qPCRs to measure the difference between hydroxymethylated DNA, which was glucosylated and therefore the restriction enzyme was blocked, and non-hydroxymethylated DNA (where the restriction enzyme cuts both positive and negative reactions of the DNA samples without glucosyltransferase enzyme). The qPCR setup, LC480 instrument settings, and cycle threshold (C_t) values were published previously [24]. The primer for genomic DNA amplification and that for reverse transcription (RT)–qPCR were optimized and pretested according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines. PCR annealing temperatures and primer sequences are given in Table W2.

Primer Optimization

To exclude experimental bias in the analyzed data sets, primers were tested before use as suggested in MIQE guidelines by Bustin et al. [25]. A serial dilution ranging from 50 ng per qPCR reaction to 0.05 ng per qPCR was analyzed with every primer pair. This enabled us to create a five-point standard curve for every primer. The PCR efficiency, slope, intercepting point with *y*-axis, and correlation coefficient were then calculated (Table W2).

cDNA Generation and RT-PCR

RNA samples showing an RNA integrity number greater than six were used for cDNA synthesis with 100 ng of total RNA (SuperScript VILO; Invitrogen, Carlsbad, CA). Before qPCR, the cDNA was diluted 1:3 where 2 μl of cDNA was used for each RT-qPCR. The PCR was performed as described above with an annealing temperature of 60°C.

Data Analysis

For the detection on the targeted microarray of gene loci with a significant difference between glucosylated (corresponding to gene loci with 5hmC) and unglucosylated (no 5hmC present) samples, the median signal intensity of the microarray data of each spot was $\log_2$ transformed and statistically analyzed with BRB array tools [26]. On the basis of the *P* value (by Student's *t* test) and the mean of the $\log_2$ transformed median intensities of the samples, the markers were selected.

To exclude the influence of different DNA concentrations on qPCR, all qPCR results were normalized to a control fragment or housekeeping gene (Table W2), yielding ΔC_t values. Differential hydroxymethylated DNA fragments are displayed as mean $\log_2$ fold change + SD. Fragments with higher C_t values for glucosylated samples than untreated controls were scored as 0. Statistical tests were performed in GraphPad Prism5 (GraphPad Software Inc, San Diego, CA). Calculation of the $\log_2$ fold change was based on $\Delta\Delta C_t$ differences between the negative reactions of each sample and the positive (glucosylated) sample (Figure 1). These ΔC_t values were then compared between the sample groups (i.e., subtraction of ΔC_t values of normal control samples from ΔC_t values derived from cancerous tissue) yielding a "fold change value." For calculation of methylation levels, $\Delta\Delta C_t$ values were calculated in the same way as for 5hmC levels. Subsequently, the $\Delta\Delta C_t$ values reflecting the 5hmC levels were subtracted from the $\Delta\Delta C_t$ values gained from methylation analysis, to yield methylation levels that reflect solely methylation changes and not a mixture of methylation changes and 5hmC changes.

Results

Identification of LZTS1 *as a Target for Altered 5-Hydroxymethylation in Primary Tumors of Patients with Breast Cancer*

The method for 5hmC detection of DNA samples performed in this study relies on *in vitro* glucosylation of 5hmC and subsequent restriction digestion using *Msp*I, which is blocked by glucosylated cytosine in 5′-C^CGG-3′, whereas unmodified or methylated cytosine can be digested. We initially established the assay to detect 5hmC using qPCR comparing glucosylated and unglucosylated control DNA (Figure W1). To detect differences in 5hmC levels in peripheral blood mononuclear cells of healthy donors and breast cancer tissues, we used a targeted microarray containing 323 cancer-associated genetic loci. Except for three candidate genes, the 5hmC levels were generally low and comparable between breast cancer tissues and peripheral blood mononuclear cells (Table W1). We were able to validate higher 5hmC

levels in breast tissue than in peripheral blood for the *LZTS1* gene (Figure W2).

Next, we aimed at analyzing 5hmC levels of the *LZTS1* gene in more detail in a larger patient cohort including the screening set samples. PCR amplicons (A-K) were designed for analyzing a region from −98 to +2567 relative to the transcription start site of the *LZTS1* gene. We performed qPCR analyses of 75 breast cancer samples and 12 normal control breast samples (Figure 1). We used the $\Delta\Delta C_t$ values (glucosylated − unglucosylated) to assess the 5hmC levels in the different samples with significant values for breast cancer and normal tissues for all PCR fragments tested ($P < .01$). A mean $\Delta\Delta C_t$ of 3.5 ± 1.4 was calculated for normal breast tissue samples and a mean $\Delta\Delta C_t$ of 1.95 ± 0.95 for cancerous samples.

Furthermore, our analyses demonstrated a significant decrease of 5hmC levels in fragments A to I in breast cancer *versus* healthy control breast tissues with a mean difference (i.e., $\log_2$ fold change) of 1.23 ± 0.54 (Figure 1*B*). The $\log_2$ fold changes ranged from 2.6 ± 0.57 (i.e., fold change of 6.01) for fragment C to 0.7 ± 0.3 (i.e., fold change of 1.72) for fragment B. All numerical fold change values as well as area under curve (AUC) values derived from receiver operating characteristic (ROC)-curve analysis of fragments distinguishing between normal tissue and breast cancer tissue are given in Table 2.

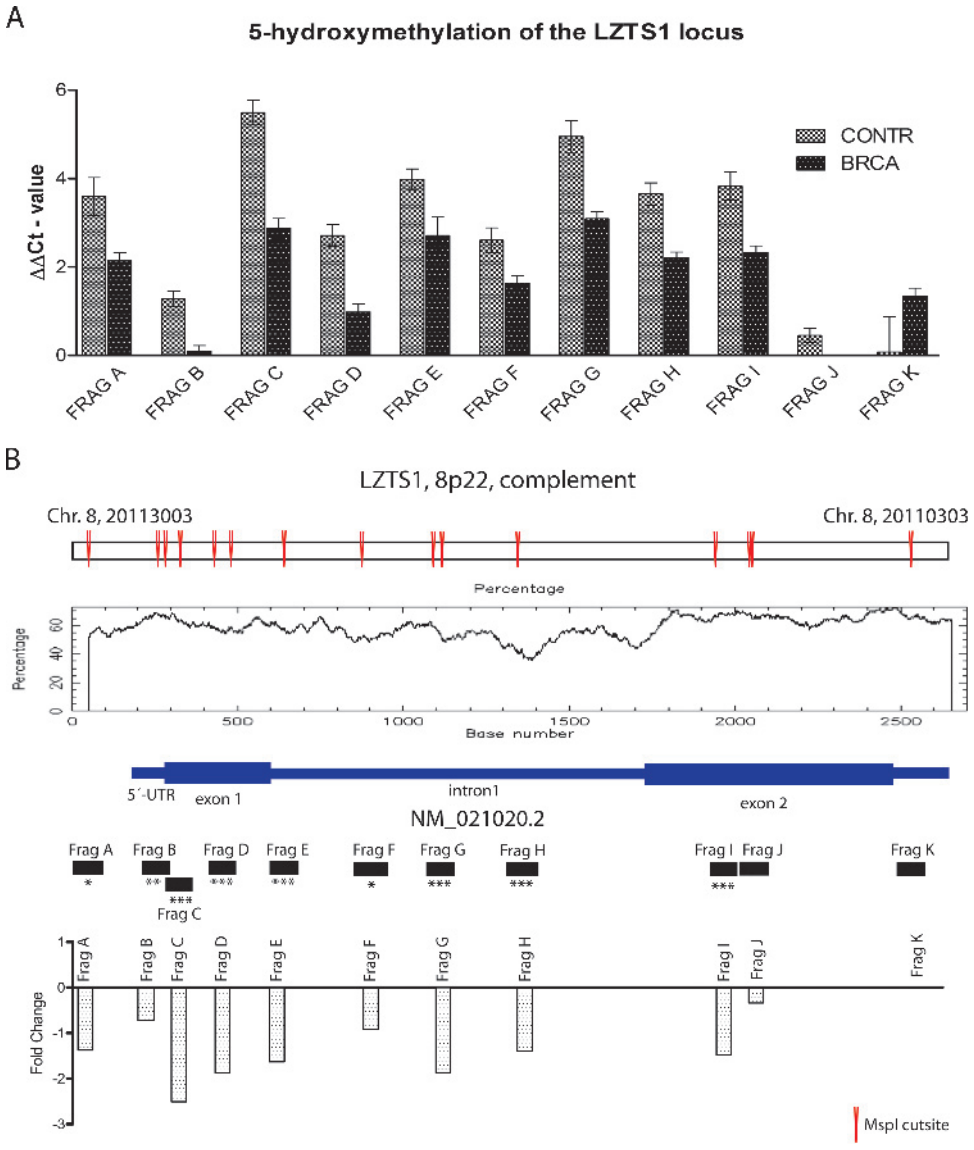

Figure 1. (A) $\Delta\Delta C_t$ values of qPCR-tested fragments of the *LZTS1* locus on all sample groups. ΔC_t methodology was applied to minimize the experimental bias and varying DNA concentrations. $\Delta\Delta C_t$ values were obtained by qPCR, and the negative reaction of each sample was then subtracted from the positive (glucosylated) sample and plotted as the mean difference of each sample group. Fragments A to K reflect the different qPCR-tested regions of the *LZTS1* locus. BRCA, breast cancer patients; CONTR, healthy controls were derived from healthy breast tissue; FRAG, fragment. (B) Chromosomal position of *LZTS1* including *Msp*I cut sites is given. CpG density plot indicates the percentage of CpG in genomic DNA of the tested region. Blue drawing indicates *LZTS1* mRNA including RefSeq.; fragments A to K reflect the different qPCR-tested regions of the *LZTS1* locus, which were plotted according to their chromosomal positions. Asterisks indicate significance levels of fragments distinguishing healthy controls and patients with breast cancer. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$; $\log_2$ fold changes were calculated between healthy controls and patients with breast cancer on the basis of $\Delta\Delta C_t$ values.

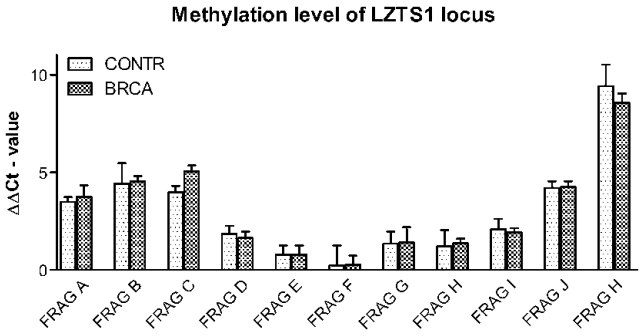

Figure 2. Methylation levels of *LZTS1* locus; identical qPCR assays (fragments A-K) as for 5hmC assessment were used. ΔC_t methodology was applied to minimize the experimental bias and varying DNA concentrations. $\Delta\Delta C_t$ values were obtained by subtraction of the negative untreated sample from the *Hpa*II digested sample values. To remove the effect of the 5-hydroxymethylation on the data, the $\Delta\Delta C_t$ value of the 5hmC levels of each fragment was subsequently subtracted from the methylation values. Bars reflect the mean difference of each sample group.

To get deeper insight into the epigenetic regulation of *LZTS1*, the 5mC levels within this region were also assessed, testing the same PCR fragments (A-J) as for 5hmC determination after MSRE digest with the methylation-sensitive *Msp*I isoschizomer *Hpa*II. As depicted in Figure 2, comparable low levels of methylation were observed after subtraction of 5-hydroxymethylated DNA from the methylated fraction in the respective regions. Fold changes ranged from 0.01 for fragment E to a methylation increase in patients with cancer of 2.1 for fragment C.

Altered LZTS1 *and* TET1 *mRNA Expression in Primary Tumors of Patients with Breast Cancer*

High levels of 5hmC were correlated with active transcription in genome-wide approaches before [10,15]. Thus, we analyzed the mRNA expression levels of *LZTS1* in normal breast *versus* breast tumor samples (Figure 3*A*). Our data demonstrate a significant downregulation ($P < .0001$, Student's *t* test) of *LZTS1* mRNA expression in breast cancer samples, which correlated with decreased levels of 5hmC across the *LZTS1* locus. Furthermore, we observed a significant reduction of *TET1* expression in cancerous breast tissue, showing a difference in ΔC_t values of 2.8 (fold change = 6.96; Figure 3*A*). A sample-wise correlation between gene expression and 5hmC levels of defined fragments (C-I) indicated a link between 5hmC and *LZTS1* expression as well as 5hmC levels and TET1 expression (Spearman correlation of $P < .01$; Figure 3*B*). These data suggest a disturbed 5hmC conversion of 5mC in tumors, which might result in decreased expression of target genes such as *LZTS1*.

5hmC Levels of LZTS1 *in Primary Tumors with Respect to Breast Cancer Pathology*

In a next step, we raised the question of whether this decreased level of 5hmC may be linked to any clinical characteristics of the tested patients. The comparison of patients with no metastasis ($n = 29$) and patients with breast cancer with distant metastases ($n = 46$) did not yield any significant correlation to altered 5hmC levels in tissues of primary tumors of the tested patients. When patients were grouped according to nodal involvement (LN0, LN1, and LN2), the 5hmC levels of six fragments were significantly associated with nodal involvement (five of six presenting a decrease in LN2; Figure W3 and Table 2). Interestingly, a robust decrease in 5hmC levels was apparent between stages LN1 and LN2 (1.37 ± 0.47; fold change = 2.58), whereas solely for one fragment significant changes between LN0 and LN1 were observed.

Discussion

5-Hydroxymethylation of cytosines is a dynamic process, which makes 5hmC an appropriate target for biomarker development in the field of cancer diagnostics and prognostics. Recently published data relying on sequencing-based methods show a decrease of 5hmC in neoplastic tissue for both a global reduction and a decrease in the promoter region of genes [27]. Immunohistochemical analysis further indicated a global decrease of 5hmC in many cancerous tissues such as lung and brain [28], and by the application of immunohistochemical methods and tiling microarrays, Nestor et al. [12] showed that both global 5hmC content and the 5hmC content of specific gene clusters are strongly dependent on the tissue type, with highest levels in brain tissue and very low levels in blood cells. These observations might explain that solely 3 regions of 323 tested showed detectable levels of 5hmC in patients with breast cancer and none could be identified in the blood samples tested in our study (Table W1). *LZTS1*, which we validated by qPCR, showed a reduced level of 5hmC in DNA from primary tumors of patients with breast cancer *versus* normal breast tissue from healthy individuals. Recently published data concerning 5hmC levels in patients with melanoma are in line with our finding of reduced 5hmC levels in malign neoplasms [27]. The authors further suggest that loss of 5hmC serves as a hallmark in malignant transformation and 5hmC might be taken into consideration as a biomarker. However, quantitative determination of 5hmC content for a specific locus is still rare [12].

LZTS1 is a classic tumor suppressor gene, and its altered expression in tumor samples has been proven for many cancer types [17]. In this study, we show a correlation between decreased 5hmC levels and lower mRNA expression for *LZTS1*, which is in coherence with recent reports demonstrating a correlation between increased mRNA expression and increased 5hmC levels in gene bodies [10,29]. Loss of *LZTS1* expression has been associated with tumor progression,

Table 2. LZTS1 5-Hydroxymethylation in Cancerous and Normal Breast Tissues.

Fragment	BRCA *versus* CONTR			LN0 *versus* LN1			LN1 *versus* LN2		
	FC	*P* Value	AUC	FC	*P* Value	AUC	FC	*P* Value	AUC
A	−1.35	**	0.75	−0.41	NS		1.12	NS	
B	−0.69	*	0.73	−0.34	NS		0.83	*	0.82
C	−2.47	***	0.87	−0.81	NS		1.88	**	0.82
D	−1.84	***	0.83	−0.54	NS		1.12	*	0.72
E	−1.61	***	0.87	−0.39	NS		1.27	NS	
F	−0.88	*	0.7	−0.17	NS		1.58	**	0.87
G	−1.85	***	0.84	−0.56	NS		1.45	**	0.81
H	−1.37	***	0.82	−0.51	NS		1.02	NS	
I	−1.45	***	0.83	−0.78	*	0.7	0.58	NS	
J	−0.32		NS	0	NS		0	NS	
K	0		NS	0	NS		0	NS	

P values and fold changes of qPCR-tested fragments of BRCA *versus* healthy controls and within the clinical subgroups of patients with breast cancer. BRCA, breast cancer; LN, lymph node affection as part of tumor grading; fragments A to K reflects the different qPCR-tested regions of the *LZTS1* locus. Significance was determined by Student's *t* test; NS, not significant; $^*P < .05$, $^{**}P < .01$, and $^{***}P < .0001$; FC indicates the $\log_2$ fold change; AUC refers to area under curve calculation (ROC curve analysis) of all significant rated fragments.

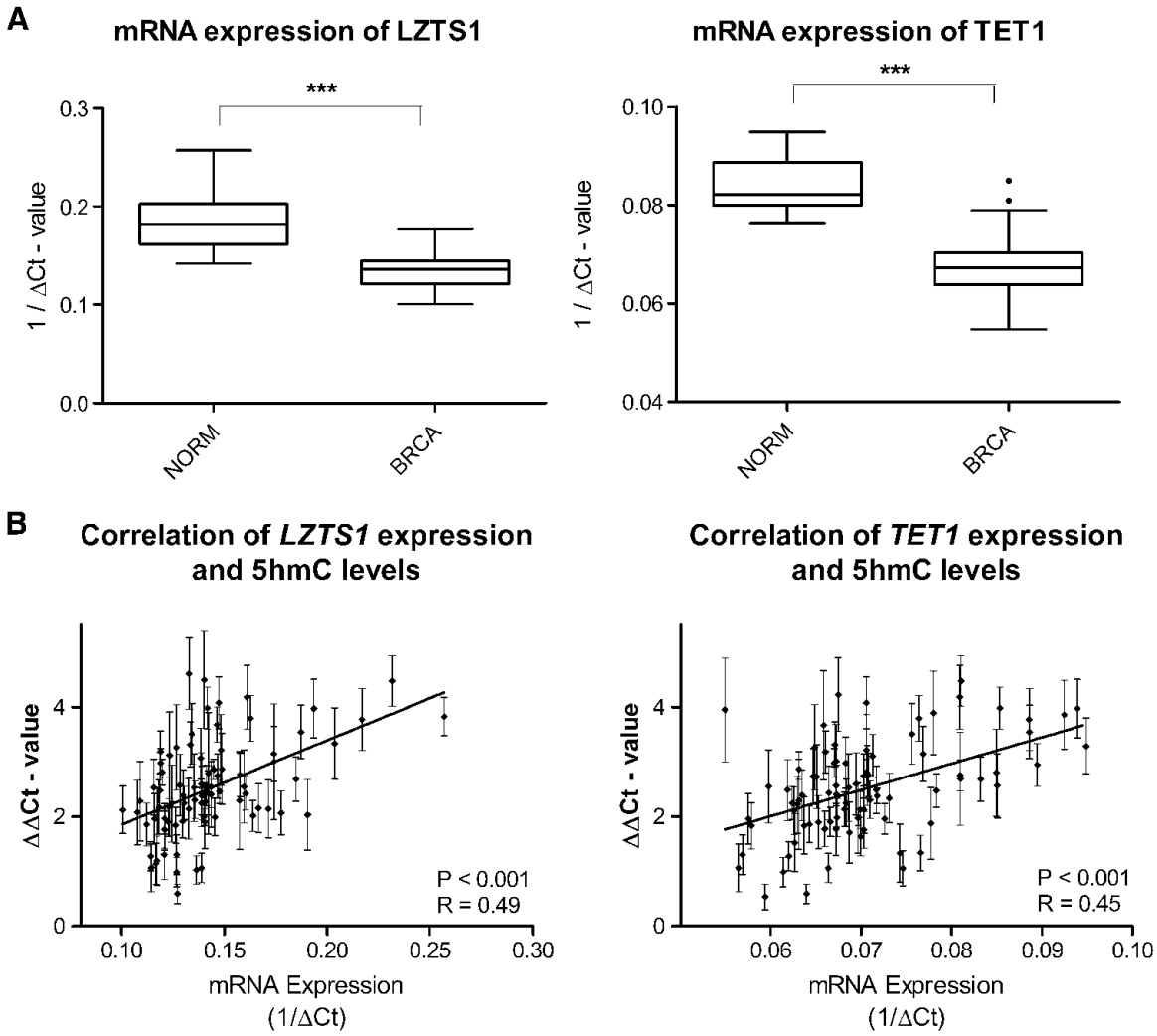

Figure 3. (A) Decreased mRNA expression of *LZTS1* and TET1 in patients with breast cancer and their correlation to 5hmC levels. Upper panel reflects mRNA expression of *LZTS1* and TET1. ΔC_t method was applied on qPCR data followed by division to gain a straightforward illustration of expression direction. As housekeeping gene, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured; ***$P < .001$. (B) Correlation between 5hmC levels given as $\Delta\Delta C_t$ values and mRNA expression. The mRNA expression of *LZTS1* was connected to 5hmC levels in a way that each sphere represents one patient sample. Fragments showing a significant Spearman correlation (fragments C-I) were plotted; for correlation between TET1 mRNA expression and 5hmC levels, the same fragments showed a significant Spearman correlation. Error bars indicate variance between 5hmC levels (i.e., $\Delta\Delta C_t$ values) for each sample.

metastasis, and poor prognosis in mouse models and human cancer [30]. Although, we could not detect significant associations of 5hmC levels with metastasis status, we demonstrated a significant correlation between 5hmC levels to nodal involvement, which might represent a group of more progressed tumors.

The observed lower levels of 5hmC in the *LZTS1* gene in breast cancer samples might be due to lower levels of *TET1* expression, which is essential for the conversion of 5mC to 5hmC, and was also described in other recently published reports [11,31]. The downregulation of TET family DNA hydroxylases and that of IDH2 [27], which both are necessary to transfer an OH– group to methylated cytosines, are the key players leading to the dynamic changes of 5hmC in neoplastic transformation [32] and during embryonic development [33]. One might also speculate that reduced levels of TET1 might yield an accumulation of 5mC and repression of the underlying gene, which is in line with reports demonstrating hypermethylation of the *LZTS1* promoter in breast cancer [17].

Regarding 5mC DNA methylation analysis performed in this study, we did not find a sample-wise correlation between 5hmC and 5mC levels. If 5mC values were not corrected for hydroxymethylation by subtraction, they mirrored the changes of the 5-hydroxymethylation to a lower extent (Figure W4), a fact that is in line with DNA methylation data available from the ENCODE consortium [34,35], showing lower DNA methylation levels of the *LZTS1* promoter and gene body in cell lines derived from a ductal carcinoma compared to normal breast tissue both in data sets generated by use of Illumina 450K bead arrays and reduced representation bisulfite sequencing by Dr Richard Myers Lab and Dr Devin Absher Lab at the HudsonAlpha Institute for Biotechnology.

After normalization of the 5mC data for the hmC effect (Figure 2), no significant changes of 5mC levels were observed. Solely for fragment C, where the highest portion of hydroxymethylation was observed, a clear, but not significant, change of methylation in line with lower hydroxymethylation was observed [36]. However, residual

fragments showed no or marginally changed methylation levels in patients with breast cancer compared to normal controls.

In sum, this study shows quantitative locus-specific measures of 5hmC and 5mC levels in a clinical sample cohort, where the reduced expressions of both the *LZTS1* and *TET1* genes are correlated with decreased 5hmC levels in patients with breast cancer. Further, a significant change in LTZS1 5hmC content was found in primary breast cancer tissue from initial diagnosis dependent on their lymph node involvement stage. Our detailed analysis of the *LZTS1* hydroxymethylation status provides new insights into the dynamics of 5hmC levels in malignant neoplasms and may also provide new impulses for the determination of the exact function of 5hmC.

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Supplementary Materials and Methods

Testing of Quest 5-hmC Detection Kit (Zymo Research)

Before experiments were performed on clinical sample materials, the Quest 5-hmC Detection Kit was tested on test DNA included in the kit and *in vitro* hydroxymethylated DNA of the BRCA1 locus. For *in vitro* hydroxymethylation, a PCR was performed using a primer pair (forward: gccccctgtccctttcccgggact; reverse: aaactgcgactgcgcggcgtgag) for the BRCA1 locus. This PCR was performed by adding hydroxymethylated cytosines (hydroxymethyl dCTP; Bioline, Berlin-Brandenburg, Germany) to the reaction. Subsequently, we used equal amounts of DNA as control reactions without any treatment and as a reaction performed according to the manufacturer's instructions. We yielded a mean difference between negative reaction and positive reaction, inclusive 5hmC glucosyltransferase enzyme of 3.06 ± 0.06 using the control DNA supplied by Zymo Research, and a mean difference of 1.84 ± 0.18 of *in vitro* hydroxymethylated DNA.

Identification of LZTS1 on Targeted Microarray

DNA samples from breast cancer tissue (n = 6) and blood samples from healthy patients (n = 6) were used to determine 5hmC levels of 323 regions on a targeted microarray. To this end, 500 ng of DNA input of each sample was glucosylated with 5hmC glucosyltransferase to glu-5hmC for 6 hours at 37°C and digested with the GSRE *Msp*I, according to the manual of the Quest 5-hmC Detection Kit (Zymo Research). The control reaction of each sample was only treated with *Msp*I without prior glucosylation. The amplification products of the multiplex PCR with 360 target DNA fragments from each sample were pooled in a 0.2-ml PCR tube and hybridized onto the CpG-360 microarray for analysis of 5hmC in 323 gene loci and 5mC in all gene loci. Due to the fact that all samples were amplified with biotinylated primers, the detection of the hybridized amplicons on the microarray was performed with streptavidin Cy3 conjugate (CALTAG Laboratories, San Diego, CA), which binds biotin with high affinity. The Cy3 signals were scanned with GenePix 4000A (Axon Instruments) at an excitation wavelength of 532 nm and an emission wavelength of 560 nm at a photomultiplier tube setting of 800 PMT. The microarrays were analyzed and translated into data files by the GenePix 4000A software. For the detection of gene loci with a significant difference between glucosylated and unglucosylated samples and gene loci with 5mC, the median signal intensity of the microarray data of each spot was $\log_2$ transformed and statistically analyzed with BRB array tools for Microsoft Excel [1].

On the basis of the P value (by Student's t test) and the mean of the $\log_2$ transformed median intensities of the samples, we selected three gene loci with a significant difference between the glucosylated and unglucosylated groups for detection of 5hmC markers.

As depicted in Table W1, the three genes show an altered 5hmC breast cancer patients. All three markers were validated by qPCR, where one gene (*LZTS1*) showed an altered level of 5hmC after qPCR in the preliminary sample set. The described breast cancer samples (Table W1) were then processed and analyzed again within the main study.

qPCR Validation of LZTS1

Primer optimization. To exclude experimental bias in the analyzed data sets, primers were tested before use as suggested in MIQE guidelines by Bustin et al. A serial dilution ranging from 50 ng per qPCR reaction to 0.05 ng per qPCR was analyzed with every primer pair. This enabled us to create a five-point standard curve for every primer. The PCR efficiency, slope, intercepting point with y-axis, and the correlation coefficient were then calculated.

Table W1. Microarray Results Leading to the Identification of Altered 5hmC Level of LZTS1.

Gene	Mean Intensities in Glucosylated Sample Group	Mean Intensities in Unglucosylated Sample Group	*P* Value
LZTS1	410.57	112.38	.0148
SOCS1	200.02	68.91	.0369
TP53	3564.11	905.54	.0404

The absolute signal intensities and *P* values of three candidate genes for qPCR validation are given in Table 1.

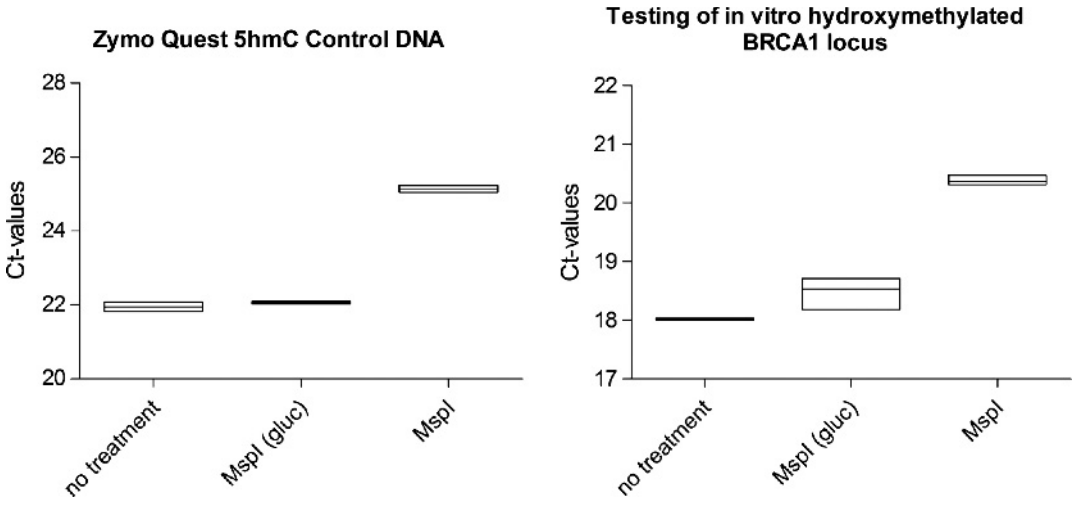

Figure W1. Quest 5-hmC Detection Kit testing using DNA supplied by Zymo Research and *in vitro* hydroxymethylated DNA of the BRCA1 PCR product. C_t values, unnormalized raw data; *Msp*I (gluc) indicates positive reaction, where 5-hmC glucosyltransferase enzyme was added to the reaction; *Msp*I indicates reactions with the same input DNA (*in vitro* hydroxymethylated) but without the enzyme.

Table W2. PCR Products plus Primer Sequences and PCR Efficiency.

PCR Product	Target	Left Primer	Right Primer	Tm	Slope	*d*	R^2	PCR Efficiency
LZTS1_frag_A	DNA	cccagtgaatgtttgttgaat	tctgggcagtagagaaacaca	65	−3.20	26.55	0.997	105.20
LZTS1_frag_B	DNA	cttgctgccacagcctttt	ccggagatgaggctactgac	65	−3.80	29.30	0.996	83.38
LZTS1_frag_C	DNA	ggcttcgcagtacaagctg	agtcctgggagaagccaaac	65	−4.26	35.41	0.981	71.58
LZTS1_frag_D	DNA	gcgtcagtagcctcatctcc	tcttgaggtgggaggacttg	65	−3.95	30.65	0.992	79.20
LZTS1_frag_E	DNA	atgggcaagagcgaagact	ctaaatccccgctggacagt	65	−3.34	26.77	0.997	99.28
LZTS1_frag_F	DNA	cagactcctcaaaaccagagc	acttctgcttcaggggact	65	−4.01	35.35	0.992	77.68
LZTS1_frag_G	DNA	ctggaagcacagatgaagagg	agggcagcaaatgagaagac	65	−3.46	27.38	0.999	94.43
LZTS1_frag_H	DNA	attcagtcctctgccccttg	gccccttaatttgaaaagctg	65	−3.41	25.64	0.999	96.40
LZTS1_frag_I	DNA	caggagccatcctgcact	gcttcagctcctgctcctt	65	−3.95	29.93	0.996	79.07
LZTS1_frag_J	DNA	caaggagcaggagctgaag	gctgctggtgctgtgtgt	65	−3.61	27.17	0.999	89.22
LZTS1_frag_K	DNA	ctgcagcttcagcaggaga	ctcgtaggacctgagcttgg	65	−4.24	35.11	0.961	72.16
control	DNA	gctctgcccatagatgcctttg	tccctggttttgacctgggggga	65	−3.48	29.29	0.998	93.97
LZTS1	mRNA	gactgcttctctcattcctgc	acaatgtgttgcccaaccaaag	60	−4.14	34.76	0.996	74.21
TET1	mRNA	ccggcgcgagttggaaagtt	aaggtttttggtcgctggccg	60	−3.81	32.52	0.994	82.86
GAPDH	mRNA	atcactgccacccagaagac	atgaggtccaccaccctgtt	60	−3.63	30.04	0.993	88.41

"Target" indicates if primer is specific to mRNA of DNA; "Tm" reflects the annealing temperature that was applied to perform qPCR; "slope" represents slope of standard curve; *d* gives the intercept point of the standard curve and the *y*-axis; R^2 indicates correlation coefficient.

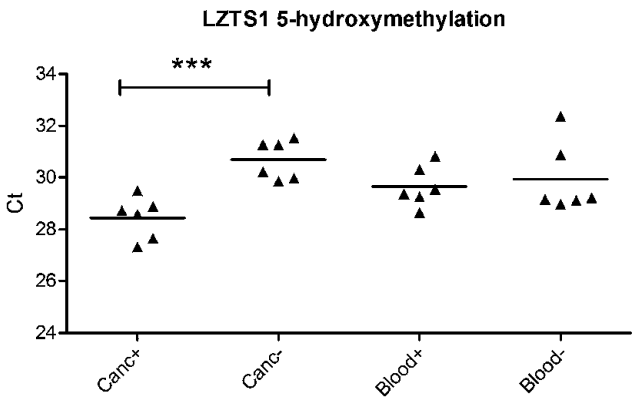

Figure W2. qPCR validation of LZTS1 on the discovery sample set (previous section) shows changed 5hmC levels in cancer compared to no change in 5hmC content in blood of healthy donors. Canc+ indicates positive reaction inclusive of glucosyltransferase enzyme; canc− indicates negative reaction without the enzyme.

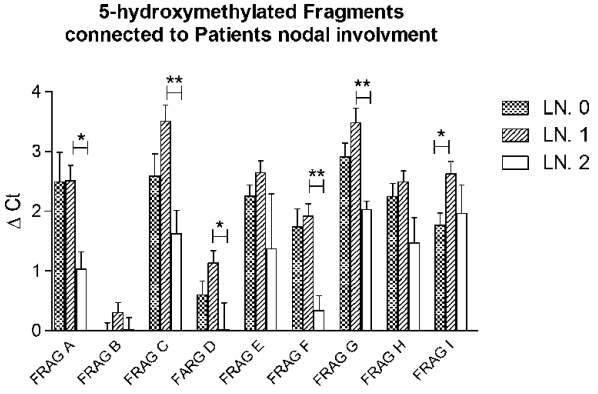

Figure W3. LZTS1 5-hydroxymethylation in cancerous breast tissue. 5-Hydroxymethylated fragments correlate to patient's lymph node affection. ΔC_t method was applied to minimize the experimental bias and varying DNA concentrations. ΔC_t values were obtained by qPCR. The negative reaction of each sample was then subtracted from the positive (glucosylated) sample and plotted as the mean difference of each sample group. Fragments A to I reflect the different qPCR-tested regions of the LZTS1 locus. LN0, patients with no lymph node involvement detected ($n = 26$); LN1, patients with lymph node affection of clinical grade 1 ($n = 39$); LN2, patients with local lymph node affection of grade 2 ($n = 8$); error bars indicate the SEM; $^*P < .05$ and $^{**}P < .01$.

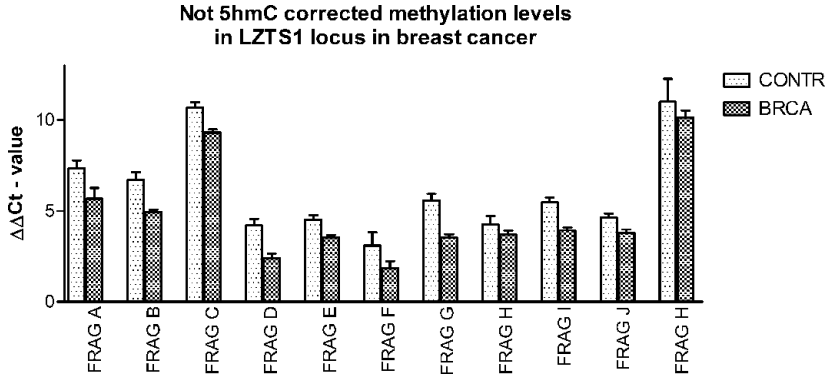

Figure W4. Methylation levels of LZTS1 locus; identical qPCR assays (fragments A-K) such as for 5hmC assessment were used. ΔC_t methodology was applied to minimize the experimental bias and varying DNA concentrations. $\Delta\Delta C_t$ values were obtained by subtraction of the negative untreated sample from the *Hpa*II digested sample values. The effect of 5hmC was not considered. The plot reflects a combination of 5hmC and 5mC of the LZTS1 locus.