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COMMUNICATION

Somatic Mutations of the Mixed-Lineage Leukemia 3 (*MLL3*) Gene in Primary Breast Cancers

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Abstract The mixed-lineage leukemia 3 (*MLL3*) gene, which encodes an important component of a histone H3 lysine 4 methyltransferase complex named the ASC-2- and Mll3-containing complex (ASCOM), has been implicated as a tumor suppressor gene due to its frequent mutations in multiple types of human tumors as well as tumor induction upon targeted inactivation of the gene in mice. The role of MLL3 in breast cancer, however, remains unknown. In this study, we sequenced all 59 exons of *MLL3* (14.7 Kb coding sequence) in 38 breast cancers from Chinese women, and found three somatic mutations in two of the cases, including one frameshift mutation (c.2687 ins A) that truncates the majority of the MLL3 protein, and two synonymous mutations. In addition to 24 known single nucleotide polymorphisms (SNPs), 5 novel SNPs were also detected in the 38 women; and interestingly, all the 5 novel SNPs alter amino acid sequences of MLL3 thus could have functional consequences. We also examined the expression of *MLL3* mRNA in 30 breast tumors and their matched normal breast tissues. While no associations were found between expression change and clinicopathologic parameters, 40% of the samples showed reduced expression in cancer tissues. These results suggest that mutation of *MLL3* plays a role in the development of breast cancer.

Keywords MLL3 · Mutation · Single nucleotide polymorphism · Expression · Breast cancer

Abbreviations

ASC-2	anterior suture cataract 2
MLL3	mixed-lineage leukemia 3
PAX2	paired box gene 2
PPAR gamma	peroxisome proliferator-activated receptor gamma
SNPs	single nucleotide polymorphisms
ER	estrogen receptor
PR	progesterone receptor
IDC-NOS	invasive ductal carcinoma not otherwise specified
ILC	invasive lobular carcinoma
DCIS	ductal carcinoma in situ
MC	medullary carcinoma

Introduction

Breast carcinoma is a common lethal malignancy. It is widely accepted that cancer is a genetic disease caused by

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sequential accumulation of DNA alterations that activate oncogenes and inactivate tumor-suppressor genes. The *MLL3* gene is a member of the mixed-lineage leukemia (MLL) family, homologous to the Drosophila gene trithorax (trx) that encodes a regulator of homeotic gene expression [1]. With histone H3 lysine-specific methylation activity [2], MLL3 is an important component of a histone H3 lysine 4 methyltransferase complex called the ASC-2- and Mll3-containing complex (ASCOM) [3], which associates with different proteins including PTIP (a protein with tandem BRCT domains) [4], UTX (a member of the Jumonji C family of proteins) [5], the DNA-binding protein PAX2 [6], and PPARgamma to effect different processes such as adipogenesis [7].

MLL3 is located at 7q36, a chromosome region that is frequently deleted in myeloid leukemia [1, 8]. Studies suggest that MLL3 has a role in carcinogenesis. While two studies found that somatic mutations of *MLL3* appear to be infrequent in colorectal carcinomas [9, 10], a systematic sequence analysis of well-annotated human protein coding genes or consensus coding sequences in colorectal cancers in other studies demonstrated that mutation of *MLL3* could be frequent (5 mutations in 11 cases) [11, 12]. Mutations of *MLL3* were also reported in several types of highly aggressive tumors including glioblastoma, melanoma, and pancreatic carcinoma, [13]. In addition, targeted inactivation of the *Mll3* in mice resulted in ureteral epithelial tumors, and this phenotype was exacerbated by a p53(+/−) background, indicating that MLL3 contributes to the DNA damage response pathway through p53 [14].

No mutations have been reported in breast cancer, but only 11 tumors have been examined [11, 12]. In this study, we sequenced all 59 exons of *MLL3* (14.7 kb coding sequence) in 38 breast cancers from Chinese women, and found three novel somatic mutations. We also compared the expression of *MLL3* mRNA in 30 breast tumors and their matched normal tissues, and found reduced *MLL3* expression in about 40% of the 30 tumors examined. These findings suggest that MLL3 could play a role in the development of breast cancer.

Materials and Methods

Tumor Specimens and Matched Noncancerous Tissues

Breast cancer specimens from 38 patients and adjacent noncancerous tissues from 31 of the patients were obtained from the Department of Breast Cancer Pathology and Research Laboratory, Cancer Hospital of Tianjin Medical University, Tianjin, China. Within 30 min after surgery, breast tissues were grossly examined by a pathologist, and both tumor and noncancerous tissues were separated after tumor tissues were taken for clinical diagnosis. Tissues were snap-frozen in liquid nitrogen and then stored in a freezer at −80°C.

Preparation of DNA and RNA for Analysis

Genomic DNA was isolated from both tumor and noncancerous tissues using the DNeasy Blood & Tissue Kit (Qiagen China, Shanghai, China) according to the manufacturer's instructions. Total RNA was extracted using the RNeasy Fibrous Tissue Kit (Qiagen China) following the manufacturer's instructions.

Mutation Analysis

All 59 coding exons of *MLL3* and their splicing junctions were amplified from each DNA sample by polymerase chain reaction (PCR) using previously reported primers [11]. PCR products were purified using nucleotide removal columns (Qiagen) and then subjected to sequencing (Invitrogen, Beijing). Sequences were compared to the wildtype sequences of *MLL3* in GenBank to identify any sequence alterations. All sequence alterations were then compared to the HAPMAP SNP database to identify known SNPs, which were then excluded from further analysis. For each non-SNP sequence alteration identified in a tumor, PCR and sequencing were repeated in the same tumor DNA sample to confirm the alteration. PCR and sequencing were also applied to the DNA sample from the matched noncancerous tissue to determine whether a sequence alteration was somatic or germline.

Measurement of Gene Expression by Real-Time PCR

Reverse transcription was performed using 2 μg total RNA with the Reverse Transcription System (Promega Biotech, Beijing, China). Real-time PCR reactions were conducted in a volume of 25 μl using the SYBR Premix Ex Taq Kit (TaKaRa Biotechnology, Dalian, China) on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Beijing, China). The expression level of *MLL3* in each sample was indicated by the ratio of *MLL3* reading to *GAPDH* reading, and the ratio for each sample was normalized against that of a placenta sample to determine the relative level of *MLL3* among different samples. The PCR primers used for *MLL3* were MLL3-F: 5′-AACCTGCACGTTTTAAT GGAGT-3′ and MLL3-R: 5′- TGGTCACTAGGAGTATGCCAGA-3′ and those for GAPDH were GAPDH-F: 5′-GGTGGTCTCCTCTGACTTCAACA-3′ and GAPDH-R: 5′-GTTGCT GTAGCCAAATTCGTTGT-3′.

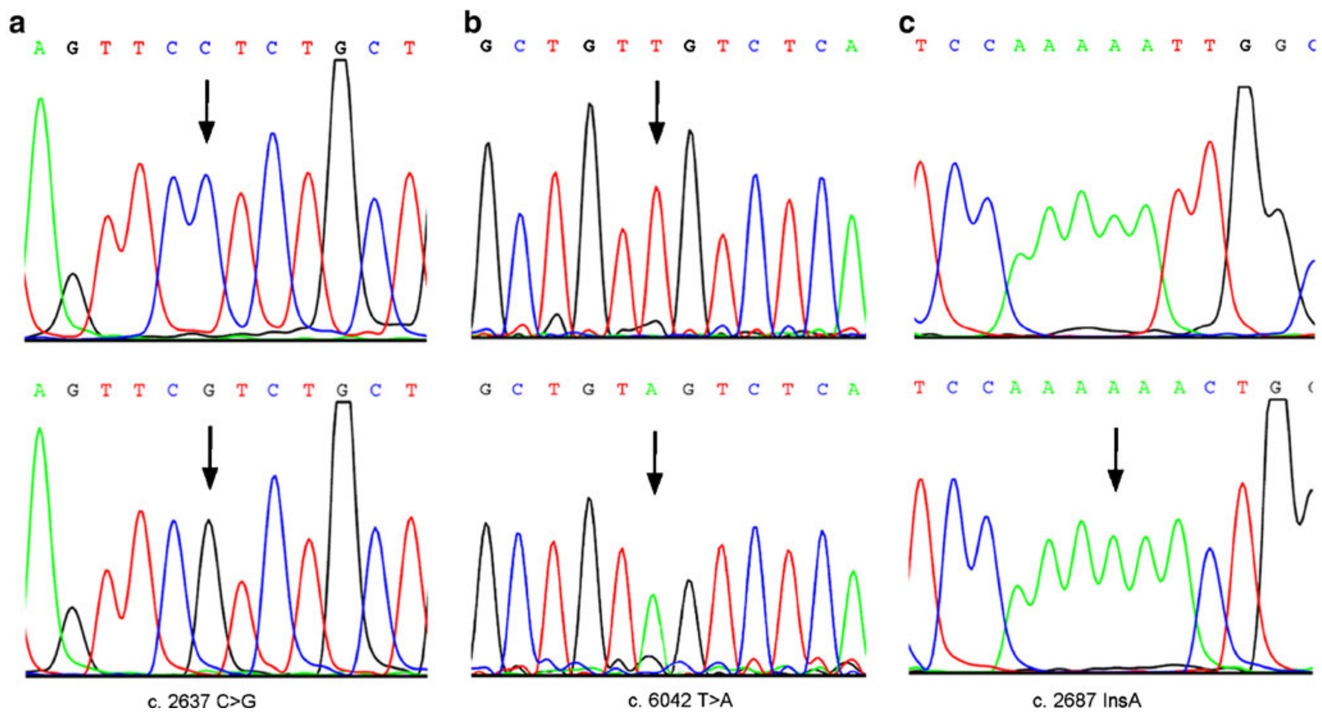

Fig. 1 Detection of three somatic mutations of *MLL3* in 2 breast cancer samples. In each panel, the chromatogram at the top is for matched normal cells, while that at the bottom is for tumor cells. Arrows point to the locations of mutations. The synonymous mutation in panel A and the frameshift mutation in panel C were from the same patient, while the synonymous mutation in panel C was from another patient

Statistical Analysis

The Mann-Whitney U test was used to analyze the correlation between clinicopathologic characteristics and *MLL3* mRNA expression levels. A 2-sided $p<0.05$ was considered statistically significant.

Results

Somatic Mutations of MLL3 in Breast Cancer

Sequencing all 59 exons of *MLL3* (14.7 kb coding sequence) in 38 breast cancer specimens and matched noncancerous tissues revealed three somatic mutations of *MLL3* in two tumors. Two of the mutations were heterozygous synonymous mutations that did not change amino acid sequences (c.C2637G and c.T6042A, referring to GenBank sequence NM_170606.2) (Fig. 1a, b). The third mutation was a heterozygous frameshift mutation (c.2687 ins A) (Fig. 1c), which resulted in the truncation of MLL3 protein from residues 827 to 4911 including the 3 PHD zinc fingers, HMG (high mobility group)-box, and PTA1 (Topoisomerase II-associated protein 1) and catalytic SET domains. The c.C2637G synonymous mutation and the frameshift mutation occurred in the same sample.

We also found a number of SNPs of *MLL3* in the 38 women, including 24 known SNPs and 5 novel SNPs. The

Table 1 Location, allelic nucleotide and amino acids involved, and allele frequencies of five novel single nucleotide polymorphisms (SNPs) that alter amino acid sequences of MLL3 identified from 38 patients with breast cancer

Exon #	Position in mRNA	Allelic change	Amino acid position	Amino acid change	Allele frequency
9	1478	C/G	420	Pro/Arg	C:50.0%
14	2339	T/C	707	Ile/Thr	T:50.0%
16	2945	G/A	909	Arg/Lys	G:30.2%
37	7526	G/A	2436	Pro/Leu	G:69.7%
38	8591	C/T	2791	Ser/Phe	C:50.0%

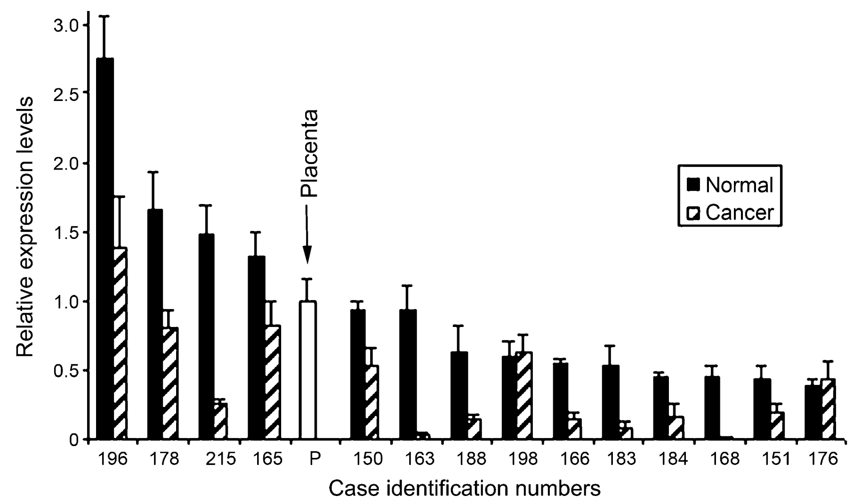

Fig. 2 Analysis of mRNA expression of *MLL3* in representative breast cancer specimens and their matched noncancerous tissues. The level of *MLL3* expression in placenta tissue was defined as 1, and those in other samples were adjusted accordingly

identification numbers for the 24 known SNPs in the dbSNP database are: rs6464211, rs10252263, rs3868462, rs4004634, rs4004631, rs9712674, rs4004633, rs4004631, rs7369633, rs4060384, rs56771269, rs4024419, rs4024420, rs3888469, rs4639425, rs4024453, rs3896406, rs2838171, rs3735156, rs2360887, rs2740327, rs62478356, rs56850341, and rs10454320. The 5 novel SNPs, which altered the amino acid sequences of *MLL3*, are listed in Table 1.

Expression of MLL3 Mrna in Breast Cancer

In general *MLL3* is expressed at higher levels in the placenta, peripheral blood, and testes, and is weakly expressed in the heart, brain, lung, liver and kidney [1]. Using the placenta as a reference, we performed real-time PCR to evaluate the mRNA expression of *MLL3* in 30 pairs of breast cancer tissues and matched noncancerous breast tissues. Relative to the placenta, expression of *MLL3* varies among normal breast tissues, with levels higher than in the placenta in some cases (Fig. 2). Compared to matched normal tissue, expression of *MLL3* was reduced by at least half in 13 of the 30 (43%) samples, and remained the same in 11 of the 30 (37%) samples (Fig. 2). In the remaining 6 samples (20%), expression of *MLL3* was increased by at least one fold in a tumors compared to its matched normal (>2 fold). However, expression changes of *MLL3* did not correlate with age at diagnosis, tumor size, lymph node metastasis, or the ER, PR and HER2 status of breast cancer patients (Table 2).

Table 2 Correlation analysis between clinicopathologic characteristics and *MLL3* mRNA expression

Clinical pathological character[a]	*MLL3* expression			Z value	P value
	T/N<=0.5[b]	T/N>=2	No change		
Tumor size					
<2 cm	5	1	4		
>=2 cm	7	5	7	−0.64	0.522
Lymph node metastasis					
−	8	3	5	–	
+	5	3	6	−0.381	0.704
ER					
−	7	1	3		
+	6	5	8	−0.046	0.963
PR					
−	8	1	4		
+	5	5	7	−0.27	0.787
HER2					
−/+	10	5	8		
++/+++	3	1	3	0.475	0.635

[a] With regard to the histological subtype, 26 of the 30 tumors were invasive ductal carcinoma—not otherwise specified (IDC-NOS), while only two were invasive lobular carcinoma (ILC), one was ductal carcinoma in situ (DCIS), and one was medullary carcinoma (MC). It was not possible to correlate *MLL3* expression with the subtypes

[b] T, tumor tissue; N, matched normal tissue

Discussion

In this study, we analyzed the complete coding sequence of the *MLL3* gene in primary breast cancers for mutations in breast cancer. In total, we detected 3 somatic mutations in 38 breast cancer samples (8%). Mutations in tumors can be categorized into "driver mutations" and "passenger mutations", with the former providing selective growth advantage, thus playing a causal role in carcinogenesis, and the latter being a consequence of carcinogenesis [15]. The frequency of passenger mutations in breast cancer has been estimated to be approximately 0.33 per megabases (Mb) of genome [16]. We detected three somatic mutations in 559 Kb of tumor genome or 5.4 mutations per Mb genomic DNA (14.7-Kb coding region of *MLL3* per tumor times 38 tumors), which is 16 times the passenger mutation rate. In addition, the insertion mutation truncated the majority of the protein. Therefore, while the mutation frequency of *MLL3* in breast cancer does not appear to be as high as in colon cancer, glioblastoma multiforme and pancreatic ductal adenocarcinoma [11–13], it is mutated in breast cancer and may play a role in breast cancer development.

Detection of 5 novel SNPs that alter amino acid sequences of *MLL3* in 38 cases is rather unusual, as the current SNP database is quite well established. Unless these SNPs are common to Chinese women and are under-represented in the current SNP database, some of these novel SNPs could be germline mutations that affect breast cancer development in women who carry them.

Our real-time RT-PCR analysis showed that *MLL3* mRNA expression was reduced in 43% of breast cancers examined. However, expression changes in the 30 cases did not correlate with age, tumor size, lymph node metastasis, or the ER, PR and HER2 status. It is not clear whether changes in the expression of *MLL3* have any significance in breast cancer.

In summary, our analyses of 38 breast cancers revealed three somatic mutations in *MLL3*, including one that truncates the majority of the MLL3 protein. Relative to the placenta, *MLL3* is expressed in both normal and cancer tissues, although 43% of 30 tumors showed reduced expression compared to their matched noncancerous tissues. These results indicate that *MLL3* is expressed in the breast, and its mutation may play a role in breast cancer development.

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