Published in final edited form as:
Breast Cancer Res Treat. 2009 August ; 116(3): 471–478. doi:10.1007/s10549-008-0147-6.

Down-regulation of tumor suppressor gene FEZ1/LZTS1 in breast carcinoma involves promoter methylation and associates with metastasis

Ling Chen[1,#], **Zhengmao Zhu**[2,#], **Xiaodong Sun**[3], **Xue-Yuan Dong**[3], **Jia Wei**[1], **Feng Gu**[1], **Yu-Lan Sun**[1], **Jun Zhou**[2], **Jin-Tang Dong**[2,3,*], and **Li Fu**[1,*]

[1]Department of Breast Cancer Pathology and Research Laboratory, Key Laboratory of Breast Cancer Research, Cancer Hospital of Tianjin Medical University, Huan Hu Xi Road, Tianjin 300060, China

[2]Department of Genetics and Cell Biology, Nankai University College of Life Sciences, 94 Weijin Road, Tianjin 300071, China

[3]Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, 1365-C Clifton Road, Atlanta, Georgia, USA

Abstract

FEZ1/LZTS1 is a tumor suppressor gene located in chromosomal band 8p22, and methylation has been identified as a mechanism for its loss of function in tumors. Chromosomal deletion at 8p22 is also frequent in breast cancer. We therefore examined whether LZTS1 plays a role in breast cancer. We analyzed expression of LZTS1 at both the RNA and protein levels, and promoter methylation in a number of primary tumors and cell lines from breast cancer. We also examined the association between LZTS1 expression and different clinicopathological parameters of breast cancer. We found that the expression of LZTS1 mRNA was reduced in 25 of 50 (50%) primary tumors and 29 of 30 (97%) breast cancer cell lines. Immunohistochemical staining showed that LZTS1 protein was absent or down-regulated in 72 (72%) of 100 primary breast carcinomas. Reduced expression of LZTS1 at either the RNA or protein level was significantly correlated with lymph node metastases ($P<0.05$). DNA methylation analysis revealed that the LZTS1 gene was frequently methylated in both cell lines and primary tumors from breast cancer, and the extent of DNA methylation was correlated with reduced expression of the gene. These findings suggest that LZTS1 plays a role in the development and progression of breast cancer at least through promoter methylation-mediated transcriptional downregulation.

Keywords

FEZ1/LZTS1; breast cancer; tumor suppressor gene; promoter methylation

Background

Breast carcinoma is a common malignancy with increasing morbidity. The molecular pathways underlying breast cancer development are still not well understood. It is widely accepted that cancer is a genetic disease caused by sequential accumulation of DNA

[*]To whom correspondence should be addressed: Li Fu, MD, Ph.D., Tel: +86-22-23340123-5221, Fax: +86-22-23359337, fulijyb@hotmail.com. Jin-Tang Dong, Ph.D., jtdong@nankai.edu.cn.
[#]These two authors contributed equally to this work.

alterations that activate oncogenes and inactivate tumor-suppressor genes. Deletion of the short arm of chromosome 8 is frequent in many types of human tumors including breast cancer, indicating the involvement of a tumor suppressor gene at this locus in breast cancer.

The FEZ1/LZTS1 gene was identified as a candidate for the tumor suppressor gene at 8p22 by Ishii et al in 1999 (1). Ubiquitous expression of LZTS1 was detected in normal tissues, but its expression is often much lower in tumor cells (1-4). In addition, introduction of LZTS1 into LZTS1-negative cancer cells resulted in suppression of tumorigenicity and reduced cell growth with accumulation of cells at the late S-G2/M stage of the cell cycle (5). Using YAC retrofitting, LZTS1 was also identified as a candidate suppressor gene (6). Germline alterations in this gene have also been linked to prostate cancer risk (7). On the other hand, only three mutations were detected in almost 200 tumor samples (5). LZTS1 has been examined in different cancers including gastric, lung, bladder, oral and kidney, and frequent reduction in expression and infrequent mutations were reported (2-4,8-11). Hypermethylation of a CpG island in the LZTS1 promoter appeared to be frequent and could be responsible for the reduced expression of LZTS1 in cancer cells (2,3).

In breast cancer, only 15 cell lines and 10 primary tumors were analyzed to show frequent downregulation of LZTS1 in Ishii et al's initial study (5). The mechanism of LZTS1 loss of expression in breast cancer has not been evaluated. In this study, we examined the expression and promoter methylation of LZTS1 in a large number of breast cancer samples including cell lines and tumor specimens to clarify the role of LZTS1 in breast cancer. We detected frequent downregulation of LZTS1, which involves promoter methylation.

Methods

Breast cancer cell lines and primary tumor specimens

Thirty breast cancer cell lines (Hs.578T, T-47D, MDA-MB-453, MCF7, HCC-70, HCC-1500, MDA-MB-361, HCC-202, BT-474, HCC-1937, HCC-1599, MDA-MB-231, DU-4475, HCC-1143, ZR-75-30, ZR-75-1, SK-Br-3, BRF-71T1, HCC-38, HCC-2218, BT-20, HCC-1395, BT-483, MDA-MB-157, MDA-MB-415, MDA-MB-134, MDA-MB-468, SW527, MDA-MB-175, BT-549) were purchased from American Type Culture Collection (Manassas, VA). The immortalized non-neoplastic breast epithelial cell line BRF-97T was purchased from Biological Research Faculty & Facility (BRFF, Ijamsville, MD, USA), while the primary culture of human mammary epithelial cells (HMEC) was purchased from Cambrex, East Rutherford, NJ. Cells were propagated following standard protocols from the providers.

All primary breast cancer specimens were obtained from patients who underwent surgical resection for clinical treatment of breast cancer from October to December of 2006 in the Cancer Hospital of Tianjin Medical University, Tianjin, China. After the standard procedure for clinical diagnosis was completed, additional tissues were cut into small pieces, snap frozen in liquid nitrogen, and stored in a -80°C freezer until use. Both tumor and adjacent normal tissues from 50 women diagnosed with IDC-NOS (invasive ductal carcinoma, not otherwise special) were used. All patients were females aged from 34 to 83 years old (mean age 54). Use of human tissues in this study was approved by the Ethics Committee of the Tumor Hospital of Tianjin Medical University. Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Concentrations of DNA and RNA were measured following standard protocols. For immunohistochemistry assay, 100 cases of formalin-fixed breast cancer were used.

Semi-quantitative RT-PCR and quantitative real-time RT-PCR

Expression of LZTS1 mRNA in primary breast cancer and cell lines was determined by regular and real-time RT-PCR. The first strand cDNA was synthesized from total RNA using the Iscript cDNA synthesis kit (Bio-Rad Laboratories, CA, USA) or the oligodeoxythymidylic acid primer system (TaKaRa, Japan). An RNA sample from a pool of normal human breast tissues (Clontech, Palo Alto, CA) was used as the normal control. Primer sequences for regular RT-PCR are 5'-AGGGCCTGGAGCTGGAGGTCT-3' and 5'-AGCCCGAGGACATCTGGTCA T-3', and those for real-time PCR are 5'-TTTGGACTGCTTCTCTCAGTTCCT GC-3' and 5 '-TTTGACAATGTGTTGCCCAACCAAAG-3'. To avoid contamination of genomic DNA, each pair of PCR primers spanned at least one intron of the LZTS1 gene. GAPDH was used as an internal control. Its primer sequences are 5'-ATCACTGCCACCCAGAAGAC-3' and 5'-ATGAGGTCCACCACCCTGTT-3'. PCR products were analyzed on a 2% agarose gel and visualized after staining with ethidium bromide. Each RT-PCR assay was repeated at least once for confirmation. The intensity for a band was quantitated with image analysis software (Gel - Pro 4400 image System, China), and the ratio of LZTS1 signal to GAPDH signal was calculated for each normal and tumor sample. The ratio for a tumor was then divided by the ratio for matched normal tissue to indicate the relative level of expression in a tumor, and a reduced expression was defined when such a value was smaller than 0.5. In real-time PCR analysis, expression of LZTS1 in a sample was indicated by the ratio of LZTS1 signal to GAPDH signal. Each signal was the average of readings from triplicate reactions, and the ratio for each sample was normalized against the ratio from the normal control.

Immunohistochemistry

Tissue sections were deparaffinized and rehydrated with xylene and a series of grades of alcohol. Antigen retrieval was carried out in 5 mM citrate buffer (pH 6.0) for 1.5 min in an autoclave, followed by cooling at room temperature for 45 min. After inactivation of endogenous peroxidase with 3% H_2O_2, sections were blocked with 10% normal goat serum for 10 min and then incubated with polyclonal goat anti-LZTS1 antibody at a 1:75 dilution (Santa Cruz, USA) at 4°C overnight. The preimmune serum was used as the negative control. Sections were then reacted with biotinylated anti-goat antibody and streptavidin-biotin-peroxidase (Zhongshan Golden Bridge, China). Diaminobenzidine was used as a chromogen substrate. Finally sections were washed in distilled water and weakly counterstained with hematoxylin. All sections were examined independently by two pathologists (L.F. and L.C.), and agreement was reached for the grade of LZTS1 staining. LZTS1 immunoreactivity was classified into four groups using a previously reported scoring method (4): +/+ or strong (96-100% positive cells), +/- or moderate (51-95% positive cells), -/+ or weak (2-50% positive cells), and -/- or absent (<2% positive cells).

Bisulfite sequencing

We treated DNA with bisulfite as previously described (12). Briefly, 2 μg of genomic DNA was diluted into 50 μl with distilled water, 5.5 μl of 2 M NaOH was added, and the mixture was incubated at 37°C for 10 minutes. Thirty μl of freshly prepared 10 mM hydroquinone (Sigma, USA) and 520 μl of freshly prepared 3 M sodium bisulfite (pH 5.0) (Sigma, USA) were added to each tube. After thorough mixing, mineral oil was added to each tube and incubated at 50°C for 16 hours. Then 1 ml of DNA wizard cleanup resin (Promega, USA) was added and DNA was purified and eluted in 50 μl of water following the instructions in the kit. After adding 5.5 μl of 3 M NaOH and incubation at room temperature for 5 minutes, 1 μl glycogen was added to each sample (Roch, USA), with 33 μl of 10 M NH_4Ac (Sigma, USA) and 3 volumes of ethanol to precipitate DNA. DNA was resuspended in 20 μl distilled water and stored at -20°C until use.

To analyze the CpG methylation status of LZTS1 promoter, two pairs of primers were designed from the CpG-enriched region within LZTS1 promoter: 2263/2662 (400 bp), 5'-GGTTTTGTGAGGGTTTTGTTATG-3'/5'-AA CCTAACCCCCTAAATCCC-3'; 2651/2967 (317 bp), 5'-GGGGGTTAGGTTGG GGTGGA-3'/5'-ACCTAACCCAAAACTTCTACTTCA-3'. PCR reaction was performed in a volume of 50 μl containing 1 μl of bisulfite-treated DNA. The PCR cycling profile consisted of an initial denaturing at 95°C for 5 min, followed by 4 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s; 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; and an additional incubation at 72°C for 10 mins. PCR products from cell lines were sequenced directly, whereas PCR products from primary tumors were cloned into the pGEM-T Easy Vector (Promga, USA), and then subjected to sequencing (Invitrogen, China).

Results

LZTS1 mRNA was frequently down-regulated in breast cancer

Semi-quantitative RT-PCR was performed to evaluate the expression of LZTS1 mRNA in 50 pairs of breast cancer specimens and matched normal breast tissues. The results showed that the RNA level of LZTS1 was reduced in 25 of the 50 (50%) samples, including down-regulation in 18 of the 50 (36%) and absence of expression in 7 of the 50 (14%) samples, compared to matched normal breast tissues. Representative RT-PCR results from 6 pairs of breast cancer samples are shown in Figure 1A. Statistical analysis showed that LZTS1 mRNA expression was significantly reduced in breast cancer ($\chi^2 = 33.333$, $P < 0.001$) compared to matched normal tissue. In addition, the absence or down-regulation of LZTS1 was significantly correlated with lymph node metastasis ($\chi^2 = 4.160$, $P<0.05$, Table 1), but not with age, tumor size, or expression status of ER, PR, CerbB-2 and p53 expression (data not shown). Consistently, real-time RT-PCR analysis showed that LZTS1 mRNA expression was significantly reduced in breast cancer cell lines. When compared to a normal breast control, all breast cancer cell lines but Hs.578T displayed reduced LZTS1 mRNA expression by at least 60% (Fig. 1B).

LZTS1 protein was frequently down-regulated in breast cancer

To further evaluate whether down-regulation of LZTS1 in breast cancer also occurs at the protein level, immunohistochemical staining was performed in 100 pairs of breast cancer and matched normal tissue sample. The results showed that LZTS1 protein was expressed in normal breast ductal epithelial cells with a granular location in cytoplasm (Fig. 2A, 2B). In the 100 breast cancer samples examined, 72 (72%) showed absence or down-regulation of LZTS1 protein (28 +, 29 +/-, 29 -/+, and 14 -). LZTS1 protein expression in each pathology type is shown in Table 2. LZTS1 protein was absent or down-regulated in 65 of 88 (74%) invasive ductal carcinomas (IDCs) (23 +, 25 +/-, 26 -/+, and 14 -) (Fig. 2C-2F). Furthermore, absence or down-regulation of LZTS1 protein was correlated with lymph node metastasis ($\chi^2 = 9.631$, $P<0.05$), since the rate of lymph node metastasis in the group of tumors with a status of "-" or "-/+ expression was significantly higher than that in the group of tumors with an expression status of "+" or "+/-" ($\chi^2 = 9.423$, $P<0.05$) (Table. 1).

DNA methylation of LZTS1 promoter was associated with reduced expression in breast cancer

To quantitatively evaluate the status of promoter methylation of LZTS1 in cell lines and primary tumors from breast cancer, bisulfite-treated genomic DNA was subjected to PCR and sequenced to determine the level of promoter methylation. In total, we analyzed 11 breast cancer cell lines that showed down-regulation of LZTS1, 49 primary tumors of breast cancer, and 9 normal breast tissues.

Methylation of CpG islands surrounding the transcription initiation site of LZTS1 was clearly increased in 8 breast cancer cell lines. Three breast cancer cell lines, HCC-202, BT-483 and HCC2218, did not show obvious promoter methylation in LZTS1 (Fig. 3, Table 3).

For primary tumors, PCR products were cloned into pGEM-T Easy Vector and three clones were randomly picked and sequenced for each tumor sample (Fig. 4A, 4B). We defined the status of methylation at each CpG site as 1 when methylation was detected in all three clones (full methylation); 0.5 when methylation was detected in at least one of the three clones (partial methylation); and 0 when none of the clones showed methylation (no methylation). The methylation level for each of the 18 CpG dinucleotides within the PCR product is shown in Table 4. While normal tissues had an average methylation level (AML) of 0.46 per CpG, that for the 49 tumors was 0.70 per CpG, which was significant higher than 0.46 ($P < 0.001$, *t* test, Table 4). Figure 4B shows sequencing results for PCR products from a pair of normal and cancer tissues from the same patient, in which all three CpGs are methylated in the tumor but not in the normal tissue.

According to their AMLs per CpG, we divided the tumors into three groups: higher level of methylation (AML ≥ 0.75), median level (0.75 > AML ≥ 0.65), and lower level (AML < 0.65). Chi square analysis showed that AML in the LZTS1 promoter inversely correlated with reduced expression (absence or down-regulation) of LZTS1 mRNA ($\chi^2 = 22.539$, $P < 0.005$, Table. 5). Consistently, correspondence analysis showed that low AMLs for LZTS1 promoter and normal levels of LZTS1 expression were located closely, whereas high AMLs located closely with reduced or absent expression of LZTS1 (Fig. 4C). In the correspondence analysis, the shorter the distance between the AML spot and the expression spot, the stronger the correlation between the two parameters. Therefore, hypermethylation of CpGs in the LZTS1 promoter significantly correlated with absent or reduced expression of LZTS1 mRNA in breast cancer (Fig. 4C). As expected, RNA expression of LZTS1 significantly correlated with its protein expression ($\chi^2 = 29.965$, $P < 0.005$, Table 5).

Discussion

In this study, we found that the expression of LZTS1 was frequently reduced or even absent at both RNA and protein levels in primary tumors and cell lines from breast carcinoma. In addition, promoter methylation of LZTS1 was also frequently detected, and was found to correlate with downregulation of LZTS1 in breast cancer, indicating that promoter methylation is at least partially responsible for the downregulation of LZTS1 in breast cancer. Furthermore, downregulation and promoter methylation status were associated with metastasis in breast cancer.

Promoter methylation is a common mechanism for transcriptional inactivation of tumor suppressor genes in human cancer, and tumor-specific promoter methylation and associated downregulation suggest a tumor suppressor function for a gene (13). While downregulation of LZTS1 was frequent in human tumors, occurrence of promoter methylation in LZTS1 has been detected in gastric cancer (3) and treatment of cells with the demethylating agent 5-aza-2'deoxycytidine restored the expression of LZTS1 in cancer cell lines (10), supporting the notion that LZTS1 is a tumor suppressor gene (1). Transfer of a large fragment of genomic DNA into cancer cells also demonstrated a tumor suppressor function of LZTS1 in tumor growth (6). More definitively, knockout of LZTS1 in mice significantly increased incidence of both spontaneous and carcinogen-induced cancers (14,15), likely through inhibition of cell cycle progression by regulating cell cycle regulators CDC25C and cdk1 (1,14). Consistent with these results, our findings of frequent downregulation and promoter

methylation further support the status of LZTS1 as a tumor suppressor gene in human cancer.

The role of LZTS1 as a tumor suppressor has been suggested by downregulation, promoter methylation, or mutation in different types of tumors, including gastric, lung, bladder, oral, prostate, and kidney cancers (2-4,7-11). In breast cancer, previous analysis of a smaller number of samples (15 cell lines and 10 primary tumors) showed frequent downregulation of LZTS1 (5). Our analysis of a larger number of samples and detection of both downregulation and promoter methylation indicate that LZTS1 also plays a role in the development of breast cancer as a tumor suppressor gene.

In the present study, we found a significant correlation between reduced expression of LZTS1 and lymph node metastases in breast cancer, suggesting that loss of LZTS1 plays a role in tumor progression. Consistently, reduced expression of LZTS1 was positively associated with higher tumor grade in lung cancer (8) and presence of lymph node metastases in collecting duct carcinoma of the kidney (11). Loss of expression and/or promoter methylation were also associated with poorer survival of patients with cancer. For example, in malignant mesothelioma, the methylation status of LZTS1 was associated with worse patient survival (16) and in non-small cell lung cancer, which is more malignant and has worse survival than small cell lung cancer, the expression of LZTS1 was more frequently downregulated (2). Collectively, these findings suggest that reduced expression of LZTS1 could be a prognostic marker predicting patient survival in different cancers.

In summary, LZTS1 was frequently down-regulated in breast cancer, which involves promoter methylation. Downregulation of LZTS1 was significantly associated with lymph node metastasis in breast cancer. These results not only indicate a role of LZTS1 in breast carcinogenesis, they also suggest that LZTS1 could become a biomarker predicting clinical outcome in breast cancer.

Acknowledgments

This work was supported by grants 30470667 and 30625032 from the China Natural Science Foundation.

References

1. Ishii H, Baffa R, Numata SI, Murakumo Y, Rattan S, Inoue H, Mori M, Fidanza V, Alder H, Croce CM. The FEZ1 gene at chromosome 8p22 encodes a leucine-zipper protein, and its expression is altered in multiple human tumors. Proc Natl Acad Sci U S A 1999;96:3928–3933. [PubMed: 10097140]
2. Toyooka S, Fukuyama Y, Wistuba II, Tockman MS, Minna JD, Gazdar AF. Differential expression of FEZ1/LZTS1 gene in lung cancers and their cell cultures. Clin Cancer Res 2002;8:2292–7. [PubMed: 12114433]
3. Vecchione A, Ishii H, Shiao YH, Trapasso F, Rugge M, Tamburrino JF, Murakumo Y, Alder H, Croce CM, Baffa R. Fez1/lzts1 alterations in gastric carcinoma. Clin Cancer Res 2001;7:1546–52. [PubMed: 11410489]
4. Vecchione A, Ishii H, Baldassarre G, Bassi P, Trapasso F, Alder H, Pagano F, Gomella LG, Croce CM, Baffa R. FEZ1/LZTS1 is down-regulated in high-grade bladder cancer, and its restoration suppresses tumorigenicity in transitional cell carcinoma cells. Am J Pathol 2002;160:1345–52. [PubMed: 11943719]
5. Ishii H, Vecchione A, Murakumo Y, Baldassarre G, Numata S, Trapasso F, Alder H, Baffa R, Croce CM. FEZ1/LZTS1 gene at 8p22 suppresses cancer cell growth and regulates mitosis. Proc Natl Acad Sci U S A 2001;98:10374–10379. [PubMed: 11504921]

6. Cabeza-Arvelaiz Y, Sepulveda JL, Lebovitz RM, Thompson TC, Chinault AC. Functional identification of LZTS1 as a candidate prostate tumor suppressor gene on human chromosome 8p22. Oncogene 2001;20:4169–4179. [PubMed: 11464283]
7. Hawkins GA, Mychaleckyj JC, Zheng SL, Faith DA, Kelly B, Isaacs SD, Wiley KE, Chang BL, Ewing CM, Bujnovszky P, Bleecker ER, Walsh PC, Meyers DA, Isaacs WB, Xu J. Germline sequence variants of the LZTS1 gene are associated with prostate cancer risk. Cancer Genet Cytogenet 2002;137:1–7. [PubMed: 12377406]
8. Nonaka D, Fabbri A, Roz L, Mariani L, Vecchione A, Moore GW, Tavecchio L, Croce CM, Sozzi G. Reduced FEZ1/LZTS1 expression and outcome prediction in lung cancer. Cancer Res 2005;65:1207–12. [PubMed: 15735004]
9. Knowles MA, Aveyard JS, Taylor CF, Harnden P, Bass S. Mutation analysis of the 8p candidate tumour suppressor genes DBC2 (RHOBTB2) and LZTS1 in bladder cancer. Cancer Lett 2005;225:121–30. [PubMed: 15922864]
10. Ono K, Uzawa K, Nakatsuru M, Shiiba M, Mochida Y, Tada A, Bukawa H, Miyakawa A, Yokoe H, Tanzawa H. Down-regulation of FEZ1/LZTS1 gene with frequent loss of heterozygosity in oral squamous cell carcinomas. Int J Oncol 2003;23:297–302. [PubMed: 12851677]
11. Vecchione A, Galetti TP, Gardiman M, Ishii H, Giarnieri E, Pagano F, Gomella LG, Croce CM, Baffa R. Collecting duct carcinoma of the kidney: an immunohistochemical study of 11 cases. BMC Urol 2004;4:11. [PubMed: 15357873]
12. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–9826. [PubMed: 8790415]
13. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 1998;72:141–196. [PubMed: 9338076]
14. Vecchione A, Baldassarre G, Ishii H, Nicoloso MS, Belletti B, Petrocca F, Zanesi N, Fong LY, Battista S, Guarnieri D, Baffa R, Alder H, Farber JL, Donovan PJ, Croce CM. Fez1/Lzts1 absence impairs Cdk1/Cdc25C interaction during mitosis and predisposes mice to cancer development. Cancer Cell 2007;11:275–89. [PubMed: 17349584]
15. Baffa R, Fassan M, Sevignani C, Vecchione A, Ishii H, Giarnieri E, Iozzo RV, Gomella LG, Croce CM. Fez1/Lzts1-deficient mice are more susceptible to N-butyl-N-(4-hydroxybutil) nitrosamine (BBN) carcinogenesis. Carcinogenesis 2008;29:846–8. [PubMed: 18192690]
16. Tsou JA, Galler JS, Wali A, Ye W, Siegmund KD, Groshen S, Laird PW, Turla S, Koss MN, Pass HI, Laird-Offringa IA. DNA methylation profile of 28 potential marker loci in malignant mesothelioma. Lung Cancer 2007;58:220–30. [PubMed: 17659810]

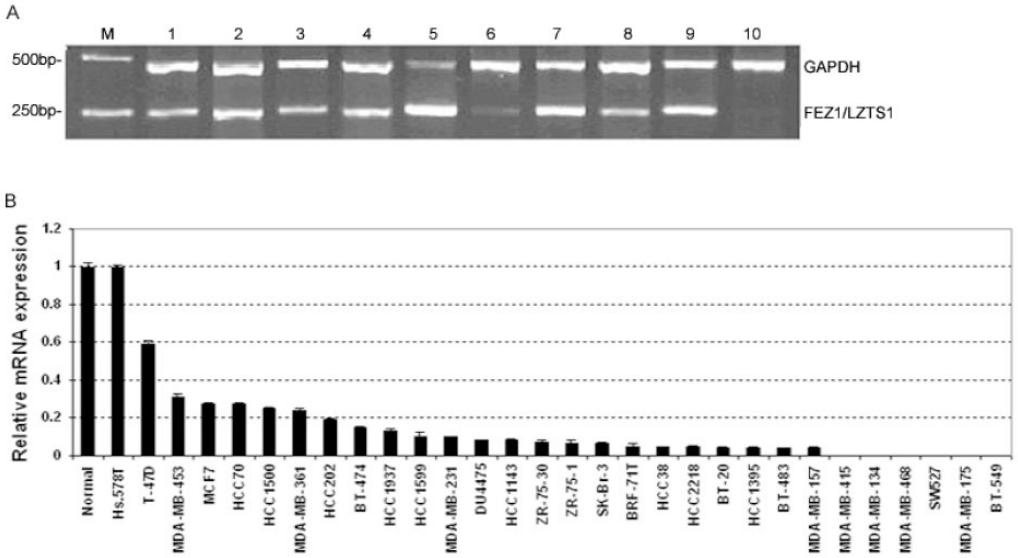

Figure 1.

Reduced expression of LZTS1 in primary tumors and cell lines from breast cancer. (A) Representative results of RT-PCR analysis of LZTS1 expression in primary tumors. Lanes 2, 4, 6, 8, and 10 are cancer samples; lanes 1, 3, 5, 7 and 9 are their matched adjacent normal breast tissues. GAPDH (246 bp) serves as an internal control. (B) Real time RT-PCR analysis of LZTS1 in 30 breast cancer cell lines. The relative level of LZTS1 expression was normalized using a pool of normal breast tissues.

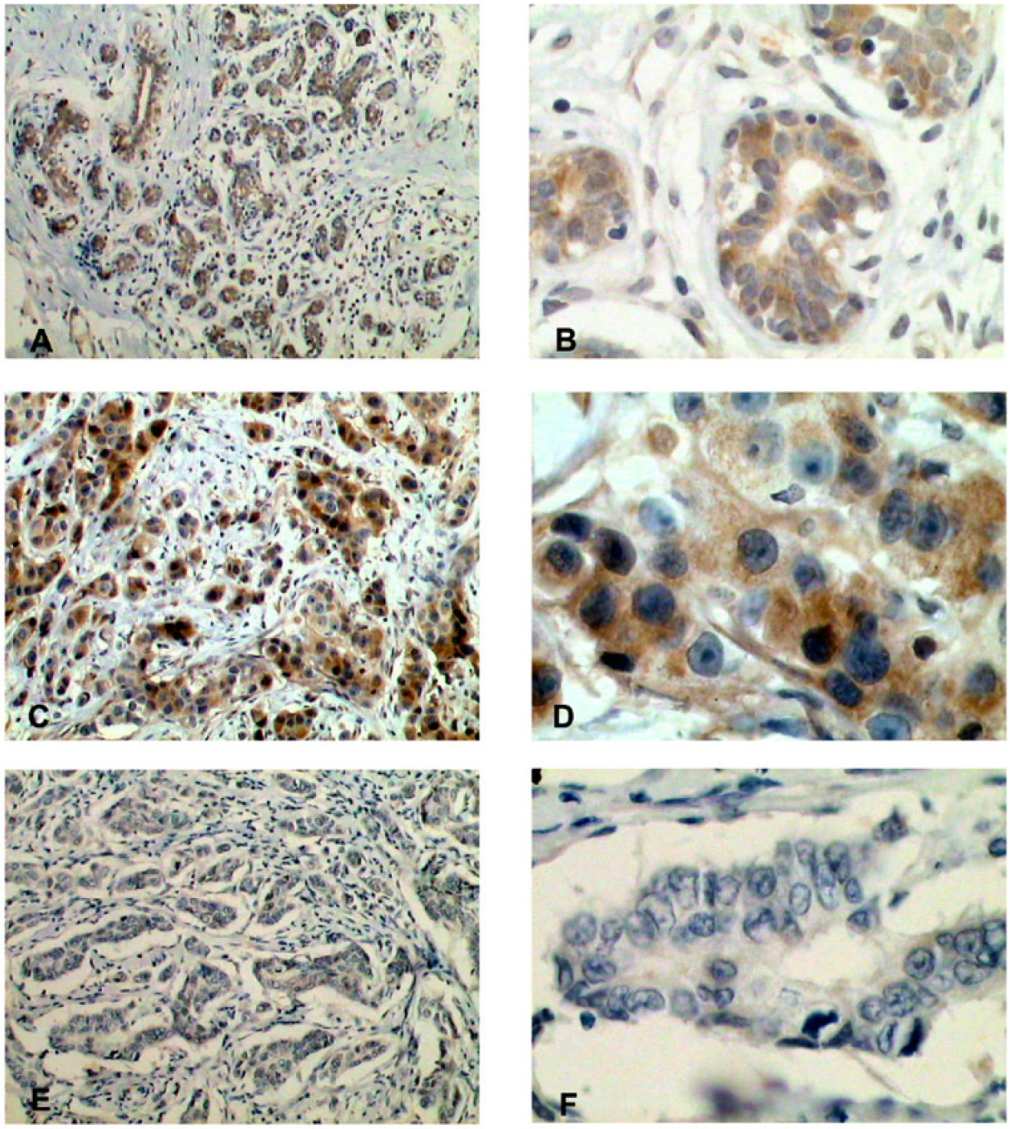

Figure 2.

Immunohistochemical detection of LZTS1 protein in normal breast tissue and breast carcinoma. (A, B) LZTS1 protein is located in the cytoplasm of normal breast ductal epithelia. (C, D) LZTS1 protein expression is strongly positive (+) in a breast carcinoma (IDC-NOS). (E, F) Absence of LZTS1 protein in a breast carcinoma (IDC-NOS). A, C, and E, × 100; B, D, and F, × 400.

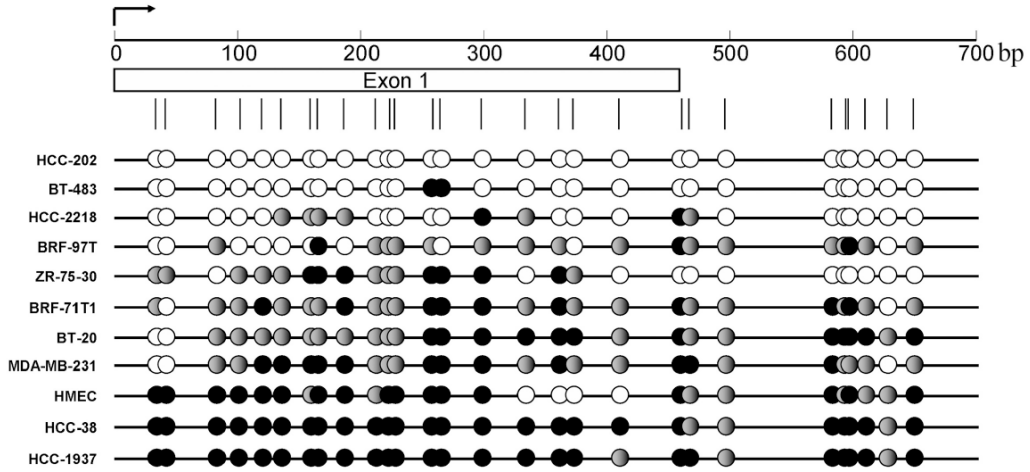

Figure 3.

Promoter methylation of LZTS1 in breast cancer cell lines. Size of the fragment containing the CpG island is shown at the top, with the location of exon 1 marked. Location of each CpG is marked by a vertical line. For each CpG dinucleotide, its methylation status in a cell line is indicated by an open circle (unmethylated), grey circle (partially methylated), or solid circle (methylated).

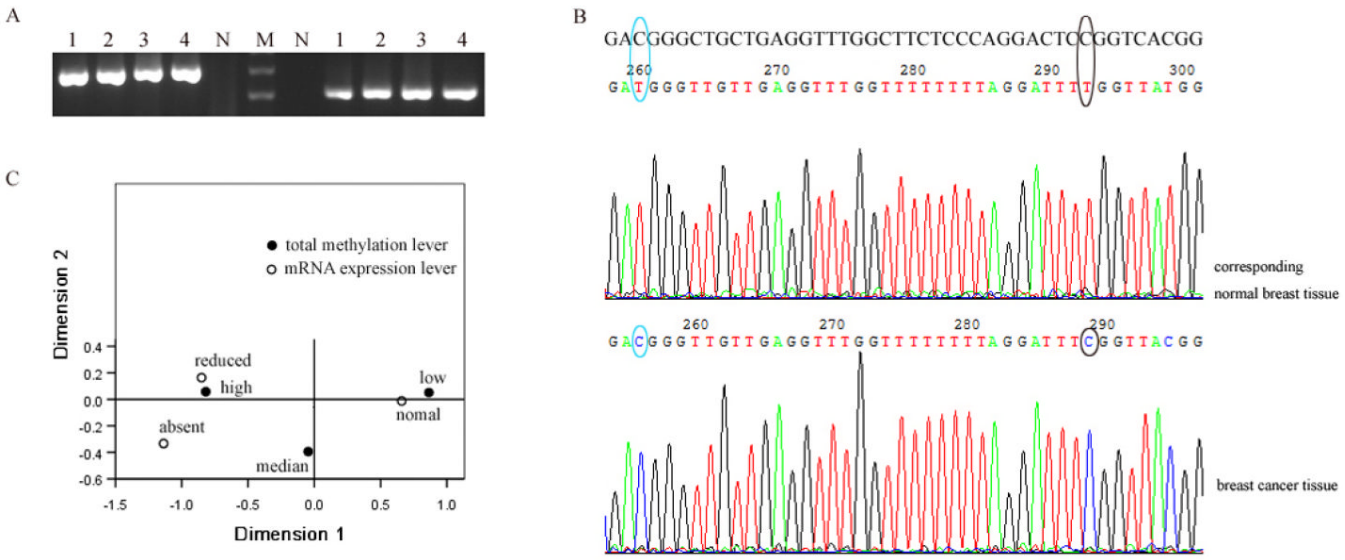

Figure 4.

Promoter methylation of LZTS1 in primary tumors of breast cancer and its inverse association with RNA expression. (A) PCR products from genomic DNA treated with bisulfite. Four tumor samples numbered 1, 2, 3 and 4 are shown for both pairs of primers. The negative control (N) was amplified from untreated DNA of a normal sample. M, molecular weight markers. (B) Sequencing results of methylation PCR products from a pair of normal (upper) and cancer (lower) tissues. Wildtype sequence is shown at the top. Each of the three cytosines (C) in a CpG dinucleotide was converted to thymine (T) in the normal tissue but not in the cancer tissue. (C) Inverse correlation between methylation and expression of LZTS1 in breast cancer samples, as detected by correspondence analysis. The spot for low level of AML for the promoter region and that for normal level of LZTS1 expression are located closely on one area of the chart, whereas the spot for a high level of AML is located closely to that for reduced or absent expression of LZTS1.

Table 1

Association between reduced LZTS1 expression and lymph node metastasis in breast cancer.

Expression		Cases with lymph node metastasis		*P* value*
		Negative	Positive	
mRNA	Reduced	12	13	0.041
	Not reduced	19	6	
Protein	+	21	7	0.022
	+/-	22	7	
	-/+	12	16	
	-	7	7	

Note: Chi square statistic was used to compare cases with reduced expression and those without reduction for difference in lymph node metastasis. For protein expression, samples with reduced expression included those with +/-, -/+ and -.

Table 2

Expression of LZTS1 protein in different types of breast cancer.

Tissue Type	Cases	Expression of LZTS1 protein			
		+	+/−	−/+	−
IDC	88	23	25	26	14
DCIS	3	2	0	1	0
ILC	3	1	1	1	0
IMPC	3	2	1	0	0
IPC	2	0	1	1	0
MC	1	1	0	0	0

Note: IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; ILC, invasive lobular carcinoma; IMPC, invasive micropapillary carcinoma; IPC, invasive papillary carcinoma; MC, medullary carcinoma.

Breast Cancer Res Treat. Author manuscript; available in PMC 2010 August 24.

Table 3

Promoter methylation of LZTS1 in six breast cancer cell lines.

Methylation	HCC-202	BT-483	HCC-2218	BRF-97T	ZR-75-30	BRF-71T1
No. of sites methylated	0	2	8	18	16	26
Rate	0%	7.1%	28.6%	64.3%	57.1%	92.9%

Table 4

Methylation levels of LZTS1 promoter in primary breast cancer and normal breast tissue.

CpG site	39	47	87	110	127	145	173	180	194	224	232	235	268	274	304	341	366	379	Average	*P* value
Normal (n = 9)	0.44	0.39	0.61	0.61	0.33	0.50	0.44	0.44	0.44	0.39	0.39	0.33	0.50	0.33	0.44	0.44	0.61	0.61	0.46	< 0.001
Tumor (n =49)	0.55	0.51	0.55	0.60	0.71	0.69	0.73	0.81	0.84	0.64	0.77	0.67	0.80	0.73	0.82	0.78	0.69	0.69	0.70	

Note: CpG sites within the PCR product, along with their methylation levels in matched normal and tumors tissues, are listed. Student's *t* test was used to compare normal and tumor cells for the difference in methylation levels.

Breast Cancer Res Treat. Author manuscript; available in PMC 2010 August 24.

Table 5

Relationship between the expression of LZTS1 RNA and promoter methylation as well as LZTS1 protein expression.

LZTS1		mRNA			*P* value
		Absent	Reduced	Normal	
Methylation	Low	0	2	23	< 0.005
	Median	1	2	4	
	High	6	13	7	
Protein	+	0	2	12	< 0.005
	+/-	1	8	8	
	-/+	1	6	5	
	-	5	2	0	