

# rs621554 single nucleotide polymorphism of DLC1 is associated with breast cancer susceptibility and prognosis

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**Abstract.** Deleted in liver cancer 1 (DLC1) on chromosome 8p22, is an important tumor suppressor gene originally identified to be deleted in hepatocellular carcinoma. It can regulate the structure of the actin cytoskeleton and inhibit cell proliferation, motility and angiogenesis, which predominantly depends on its homology to rat RhoGAP. There are many genetic variants in DLC1, which may influence its antitumor efficacy. The rs621554 (IVS19+108C>T) polymorphism is a synonymous single nucleotide polymorphism (SNP) previously found to be associated with hepatocellular carcinoma. In the present study, 453 patients with breast cancer and 330 healthy females were analyzed using a cycling probe method. It was determined that the rs621554 polymorphism of DLC1 was associated with breast cancer susceptibility, with the CC and CT genotypes resulting in a higher risk of developing breast cancer. In regard to clinicopathological variables, it was demonstrated that the CT and CC genotype were associated with tumor size, lymph node metastasis and progesterone receptor status. Patients with the CT and CC genotype had shorter disease-free survival and overall survival rates compared with those with the TT genotype. Additionally, it was demonstrated that the rs621554 polymorphism was correlated with DLC1 expression at the mRNA level. These results suggested that the rs621554 polymorphism is associated with breast cancer susceptibility and prognosis, and may serve as a biomarker for breast cancer development and progression.

## Introduction

Breast cancer is one of the most common types of malignancies among females worldwide and has become a serious health burden for women with 1,380,000 new cases and 458,000 breast cancer-related fatalities annually (1,2). However, mechanisms underlying the onset and development of breast cancer remain to be fully elucidated. Like other malignancies, breast cancer development is the result of a combination of genetic and environmental factors (3-6). The most frequent type of genetic alteration, single nucleotide polymorphisms (SNPs), are known to be important carcinogenesis and cancer development (7,8). It is well known that breast cancer is associated with SNPs in a number of genes, such as BCL-2 and XRCC3 (9,10).

Deleted in liver cancer 1 (DLC1), mapped to chromosome 8p22, is an important tumor suppressor gene originally identified to be deleted in hepatocellular carcinoma (HCC). Recently, increased attention has been paid to its actions in other types of malignancies, including breast, prostate, cervical and lung (11) cancer. DLC1 can regulate the structure of the actin cytoskeleton and inhibit cell proliferation, migration, invasion and angiogenesis, which predominantly depends on its homology to rat RhoGAP, which acts as a catalyzing enzyme in the conversion of the active GTP-bound rho complex to the inactive GDP-bound form (12-14). Mutation screening of DLC1 was performed in 159 patients with prostate cancer indicated 8 exonic and 23 intronic SNPs of DLC1; however, no association with cancer was identified (15). Among these mutations, rs621554 (IVS19+108C>T) polymorphisms, a synonymous SNP located 108 bp downstream of exon 19, was found to be correlated with HCC susceptibility in patients with hepatitis B virus (16). However, little is known regarding the influence of the DLC1 rs621554 polymorphism in breast cancer. Thus, the present study aimed to determine the association between the rs621554 polymorphism and breast cancer susceptibility, clinicopathological variables and prognosis.

## Materials and methods

**Patients and samples.** This study involved 453 patients who were diagnosed with breast cancer and 330 healthy females who were recruited from annual physical examinations between 2008 and 2012 at the Qilu hospital of Shandong

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**Abbreviations:** DLC1, deleted in liver cancer 1; SNPs, single nucleotide polymorphisms; HWE, Hardy-Weinburg equilibrium

**Key words:** DLC1, breast cancer, susceptibility, prognosis, polymorphism

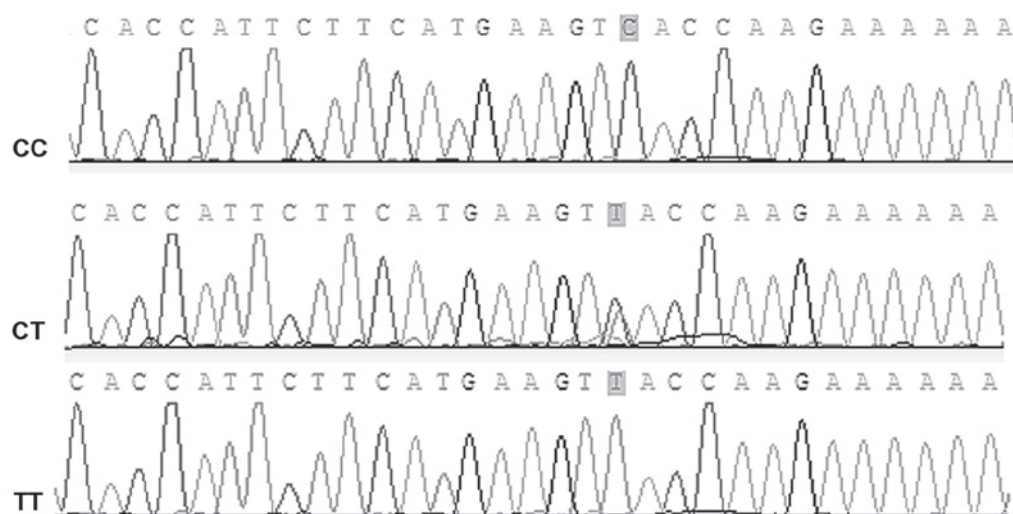


Figure 1. Genotyping results of rs621554 polymorphism were shown to be consistent using DNA sequencing and cycling probe methods. Genotype shown on the left indicated the results of the cycling probe and those highlighted in the figure show the results of the DNA sequencing based on different peaks.

University (Jinan, China). The average age of the case and control group was  $50.00 \pm 11.40$  and  $48.23 \pm 7.93$  years, respectively, and there was significant difference in the age of the two groups as determined by Student's t-test ( $P=0.367$ ). In order to investigate the correlation between the DLC1 rs621554 polymorphism and changes in DLC1 mRNA levels, 20 tumor tissue samples were obtained from the patient group during tumor resection. To detect whether the rs621554 polymorphism is associated with breast cancer prognosis, 245 patients with long-term clinical follow-up were enrolled. The patients were followed up every three months, and disease free survival and overall survival were analyzed. Whole blood samples ( $200 \mu\text{l}$ ) were obtained from each individual in the case and control groups and were stored at  $-80^\circ\text{C}$  until use. The following clinicopathological characteristics were determined from patient notes: Age at diagnosis, pathological type, tumor size, histological grade, lymph node status, as well as biological markers, such as the presence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). This study was approved by ethical committee of Shandong University. Written informed consent was signed by each subject involved in the study.

**DNA extraction.** DNA was extracted from whole blood cells using the TIANamp Genomic DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. DNA concentration and purity were measured using an ultraviolet spectrophotometer (GE Healthcare, Pittsburgh, PA, USA). The extracted DNA samples were routinely stored at  $-20^\circ\text{C}$ .

**SNP genotyping analysis.** Cycling probes were synthesized by Takara Biotechnologies (Dalian), Co. Ltd. (Dalian, China), and an allelic discrimination assay [Cycleave PCR Core kit, Takara Biotechnologies (Dalian), Co. Ltd.] was performed using an ABI 7900HT thermal cycler. The PCR amplification conditions were conducted according to the manufacturer's instructions:  $95^\circ\text{C}$  for 30 sec, 45 cycles of  $95^\circ\text{C}$  for 5 sec,  $55^\circ\text{C}$  for 10 sec, and  $72^\circ\text{C}$  for 25 sec. Several DNA samples

were randomly selected for DNA sequencing to confirm the genotyping.

**Reverse transcription-quantitative polymerase chain reaction analysis (RT-qPCR).** Total RNA was extracted using TRIzol reagent [Takara Biotechnologies (Dalian), Co. Ltd.]. The cDNA was reverse transcribed from  $1 \mu\text{g}$  total RNA using the PrimerScript RT Reagent kit [Takara Biotechnologies (Dalian), Co. Ltd.]. mRNA quantification was performed using SYBR PCR mix [Takara Biotechnologies (Dalian), Co. Ltd.] on an ABI 7900HT platform (Applied Biosystems, Foster City, CA, USA). Primer sequences were as follows: Forward: 5'-GGGCTGCTTTTAACTCTGGTAAAG-3' and reverse: 5'-CCATGGGTGGAATCATATTGG-3' for glyceraldehyde 3-phosphate dehydrogenase; and forward: 5'-CCAAACCCAAGACTACGGCTA-3' and reverse: 5'-GCTGTGCATACTGGGGGAA-3' for DLC1. Primers were obtained from Sangon Biological Engineering, Co., Ltd. (Shanghai, China). A  $20\text{-}\mu\text{l}$  reaction system was used and contained 1X Cycleave PCR reaction mixture,  $0.4 \mu\text{M}$  cycling probe,  $0.2 \mu\text{M}$  PCR forward primer,  $0.2 \mu\text{M}$  PCR reverse primer and 50 ng DNA template. The cycling probe method was used, the CC genotype presented strong fluorescence, TT genotype presented no fluorescence and CT genotype present weak fluorescence (17). Sequence detection system software 1.2.1 (SDS1.2.1, Applied Biosystems) was used for analysis. The experiment was repeated in triplicate to confirm the results.

**Statistical analysis.** SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze data in this study. The genotype and allele frequencies were tested using the public statistical web-tool <http://www.oege.org/software/hwe-mr-calc.shtml> for Hardy-Weinberg equilibrium (HWE).  $P>0.05$  was considered to indicate non-deviation from HWE. Logistic regression models were used to analyze the association between the rs621554 polymorphism and breast cancer susceptibility, as well as the clinicopathological characteristics. Differences in patient survival rates were analyzed using the Kaplan-Meier

Table I. Genotype distribution in cancer cases and controls.

Genotype	No. of patients		P-value	OR (95% CI)
	Control (n=330)	Cancer (n=453)		
TT	54	44		
CT	166	220	0.032	1.627 (1.041, 2.541)
CC	110	189	0.001	2.109 (1.328, 3.348)

OR, odds ratio; CI, confidence interval.

Table II. Association between rs621554 and clinicopathological variables.

Variable	Genotype		P-value
	TT	CC+CT	
Age (years)			0.561
≤45	20	148	
>45	24	214	
Pathological type			0.759
Ductal	37	296	
Other	7	64	
Tumor size (cm)			0.035
≤2	24	136	
>2	15	175	
Histological grade			0.242
Low	23	165	
High	6	75	
Lymph node metastasis			0.047
No	28	170	
Yes	14	167	
ER			0.359
Negative	26	178	
Positive	16	149	
PR			0.031
Negative	12	151	
Positive	30	176	
HER2			0.476
Negative	38	283	
Positive	4	44	

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

method. The Cox hazard regression model was used to analyze the independent prognostic value of the rs621554 polymorphism.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*DNA sequencing shows complete reliability of genotyping using cycling probe method.* Ten DNA samples randomly

selected from the patient group were sent to Sangon Biological Engineering Co., Ltd. for DNA sequencing. As shown in Fig. 1, DNA sequencing showed consistent results, which suggest reliability of the genotyping method.

*rs621554 polymorphisms are associated with breast cancer susceptibility.* In total, 453 patients with breast cancer and 330 healthy females were involved in this study. Subjects from the two groups were confirmed to meet HWE using the  $\chi^2$

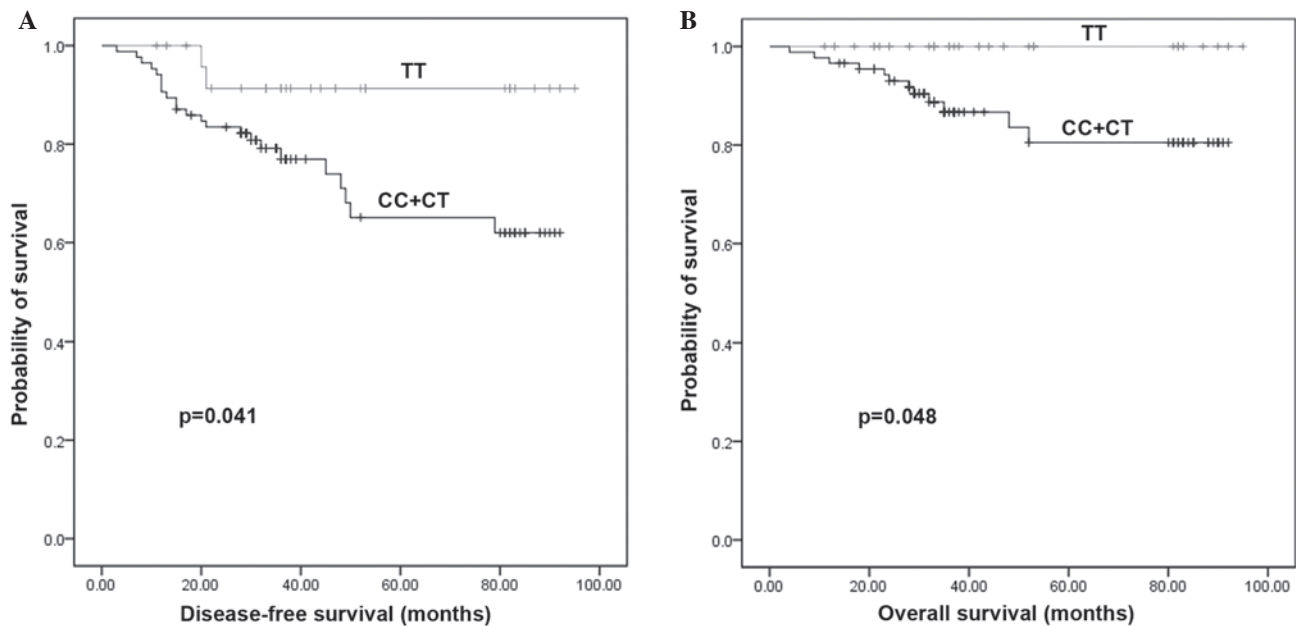


Figure 2. rs621554 polymorphisms were correlated with breast cancer prognosis. (A) The CC+CT genotypes of rs621554 had shorter relapse-free survival ( $P=0.041$ ). (B) The CC+CT genotypes of rs621554 were associated with shorter overall survival ( $P=0.048$ ).

test. The genotype and allele frequencies were tested using Hardy-Weinberg equilibrium (HWE).  $\chi^2$  test was performed to confirm the representativeness of each group.  $P>0.05$  was considered as non-deviation from HWE. The  $\chi^2$  result was 3.06 for the patient group and 0.43 for control group, respectively ( $P>0.05$ ).

Table I shows a significantly different distribution of rs621554 polymorphism in the patient and control groups. A higher percentage of CT or CC genotypes were observed in the patient group compared with the control group (CT: 83.33 vs. 75.45%; CC: 81.12 vs. 67.07%, respectively). Logistic regression analysis showed significant differences between the two groups ( $P=0.032$  and  $P=0.001$ , respectively). Moreover, the CT and CC genotypes were associated with a higher risk of breast cancer [Odds ratio (OR), 1.627; 95% confidence interval (CI), 1.041-2.541; and OR, 2.109; 95% CI, 1.328-3.348, respectively]. Based on the above, our study indicated that the C allele may be a risk factor for breast cancer susceptibility. Accordingly, the CT and CC genotypes were taken together and compared with the TT group.

**Association between rs621554 polymorphisms and breast cancer clinicopathological variables.** Table II shows the association between the TT and CT+CC genotype and clinicopathological characteristics of breast cancer patients, including age at diagnosis, pathological type, tumor size, histological grade, lymph node status, and biological markers, such as ER, PR and HER2. It was demonstrated that the CT and CC genotype were strongly associated with tumor size ( $P=0.035$ ), lymph node metastasis ( $P=0.047$ ) and PR status ( $P=0.031$ ). No significant association was found between genotype and age at diagnosis, pathological type, histological grade, and status of ER and HER2.

**rs621554 polymorphisms are correlated with breast cancer prognosis.** The association between rs621554 polymorphisms

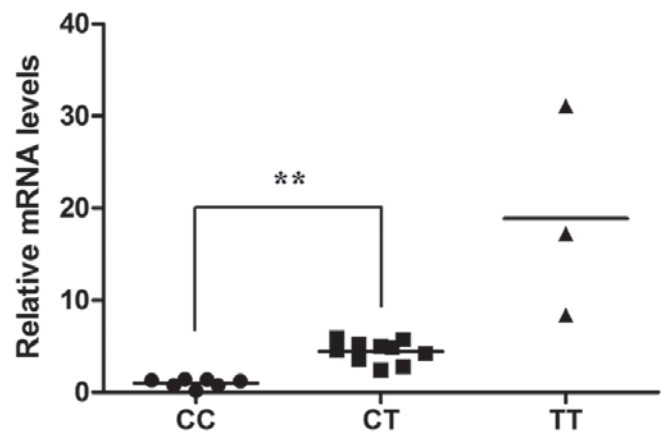


Figure 3. rs621554 polymorphisms are associated with the mRNA level of DLC1 *in vivo*. The CC genotype resulted in a significantly lower mRNA level of DLC1. No significant differences were identified between CC and TT genotypes, or CT and TT genotypes. \*\* $P<0.01$ .

and prognosis was also analyzed using the Kaplan-Meier method. As shown in Fig. 2, compared with patients with the TT genotype, patients with CT and CC genotypes had shorter disease-free survival ( $P=0.041$ ) and overall survival ( $P=0.048$ ) rates. Additionally, COX hazard logistic regression analysis was performed to demonstrate that rs621554 polymorphisms are not an independent prognostic factor in breast cancer, with  $P=0.192$  for disease-free survival and  $P=0.131$  for overall survival (data not shown).

**rs621554 polymorphisms are associated with the mRNA level of DLC1 *in vivo*.** The possible correlation between the rs621554 polymorphism and the mRNA level of DLC1 was also investigated. In total, 20 samples of breast cancer tissue were taken for analysis and the result is shown in Fig. 3. The



relative mRNA level of DLC1 was  $1.000 \pm 0.451$  for the CC genotype,  $4.452 \pm 1.18$  for the CT genotype, and  $18.886 \pm 11.433$  for the TT genotype. Significant differences were identified in the mRNA level between patients with the CC and CT genotype ( $P < 0.001$ ), and no significance was found between the CC and TT genotypes ( $P = 0.113$ ) or the CT and TT genotypes ( $P = 0.160$ ).

## Discussion

As with other types of malignancies, genetic and environmental factors are involved in the development of breast cancer. SNPs are the most frequent gene mutation, and certain SNPs have been shown to result in a higher risk of cancer (18). Since the 6q25.1 rs2046210 was first reported by Zheng *et al* (19) as a novel SNP associated with breast cancer risk, increased attention has been paid to cancer-associated SNPs with the aim of identifying sensitive genetic markers to assess cancer risk and to monitor therapeutic response.

The human DLC1 gene is a tumor suppressor that acts in a Rho-dependent manner, since the protein it encodes is highly homologous to the rat p122-RhoGAP (20,21). It is found to be crucial in regulating the structure of the actin cytoskeleton, and inhibit cell proliferation, migration, invasion and angiogenesis (12-14). It has been shown to be downregulated or absent in a number of different types of cancer (22). According to previous studies, genetic variants widely exist in DLC1 and may influence the antitumor effect of DLC1 (16,23,24). rs621554, located in intron 19, is a synonymous SNP in DLC1, which is associated with HCC (16). Nevertheless, whether the rs621554 polymorphism is associated with breast cancer remains to be determined.

In the present study, the CT and CC genotypes (CT+CC genotype) of rs621554 DLC1 were shown to be associated with a significantly higher risk of breast cancer compared with that of the TT genotype. It appears that the T allele of rs621554 DLC1 is protective against breast cancer. Since clinicopathological characteristics are vital in evaluating cancer prognosis their correlation with the rs621554 polymorphism was investigated, and it was demonstrated that the CT+CC genotype was associated with larger tumor size, lymph node metastasis and negative PR status, but was not associated with ER or HER2 status. This may due to the limited number of samples, which suggests that a larger number of cases is required in a further study. In addition, it was demonstrated that the CT+CC genotype was a prognostic indicator for disease-free survival and overall survival in breast cancer, but a COX hazard regression analysis indicated that, with the limited sample size, CT+CC genotype was not shown to be an independent indicator. Despite this, however, a conclusion can be made that the CT+CC genotype is associated with breast cancer susceptibility, development and prognosis.

Next, in order to determine the possible correlation between the rs621554 polymorphism and DLC1 expression, DLC1 mRNA expression was detected. It was demonstrated that it is higher in patients with the CT genotype compared with the CC genotype, suggesting the protective effect of the T allele. However, the DLC1 expression level in patients with the TT genotype is not significantly different compared with that in CT and CC group. This phenomenon may due to small

number patients with the TT genotype (only 3 samples) and large standard error. It was hypothesized that the change in the mRNA level of DLC1 due to the SNP may be responsible for the development and progression of breast cancer. The specific mechanism underlying this effect may involve alterations in the binding site of transcriptional factors (25), since rs621554 polymorphism is located in the intron of DLC1, where a variety of cis elements are involved. However, further investigation is required to confirm this.

In conclusion, the results demonstrated that individuals with the CT and CC genotype of the DLC1 rs621554 polymorphism are more susceptible to breast cancer compared with those with the TT genotype and the two genotypes together are associated with larger tumor size, lymph node metastasis and negative PR. In addition, the CT or CC genotype may be associated with a poor prognosis. Further research is required to determine the underlying molecular mechanisms with larger patient cohorts to further confirm rs621554 polymorphism as a clinical biomarker for early diagnosis and prognosis of breast cancer.

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