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Original Article

A novel PTEN mutant caused by polymorphism in cis-regulatory elements is involved in chemosensitivity in breast cancer

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Abstract: Phosphatase and tensin homolog (*PTEN*) is one of the most important tumor suppressor genes. Although studies have shown the association between cancer and genetic polymorphisms of *PTEN*, the underlying molecular mechanisms of breast cancer (BC) chemosensitivity that results from *PTEN* polymorphism is still unclear. This study aims to investigate potential links between *PTEN* polymorphisms in cis-regulatory elements and BC chemosensitivity in the Chinese population. A total of 172 BC patients who received neoadjuvant chemotherapy were included in the study, including 104 chemosensitive cases and 68 chemoresistant cases. The results showed a significant association between the rs786204926 polymorphism and BC chemosensitivity. Logistic multivariate regression analysis showed that age, lymph node metastasis, and the rs786204926 genotype were risk factors for BC chemoresistance. The G allele of rs786204926 is more prone to increasing the risk of chemosensitivity in BC. Additionally, analysis using Alamut Visual showed a preference of the G allele of rs786204926 to produce a novel PTEN mutant with an insertion of 18 bases from intron 4. While the transcriptional level of PTEN remained similar in chemosensitivity and chemoresistant samples, its protein level changed significantly. Interestingly, there were significant differences in both transcription and protein levels of the novel PTEN mutant between the two groups. Furthermore, we found that the mutant was more susceptible to dephosphorylation compared with wildtype PTEN, leading to chemosensitivity through the PI3K-AKT signaling pathway. These findings indicate that novel PTEN mutants caused by polymorphisms in cis-regulatory elements may be involved in BC chemosensitivity.

Keywords: *PTEN*, single nucleotide polymorphism, chemosensitivity, alternative splicing, breast cancer

Introduction

Breast cancer (BC) occurs in breast epithelial tissues and is the most common malignant tumor in women [1]. BC has become a serious health problem threatening women all over the world [2]. Chemotherapy is one of the main treatments for BC, but chemoresistance remains the main reason for treatment failures. However, a subset of patients are sensitive to chemotherapy [3-6]. Therefore, the identification of genetic mechanisms involved in chemosensitivity is crucial to predicting drug response [4, 7]. Studies have reported that key mutations in tumor suppressors gene may be a major factor involved in chemosensitivity and the treatment of cancer patients [8-10].

Many tumor suppressor genes have been found to be involved in BC chemosensitivity [11-13]. Phosphatase and tensin homolog (*PTEN*) is a major tumor suppressor genes [14]. It is a dual-specificity phosphatase and the most important negative regulator of the PI3K-AKT signaling pathway [15]. PTEN can reverse the action of PI3K, thereby regulating many cellular pro-

cesses, such as cell cycle, translation, apoptosis, self-renewal, and epithelial-to-mesenchymal-transition [16-18]. Germline mutations in the *PTEN* gene have been found in subsets of seemingly disparate syndromes [19]. Studies have identified germline *PTEN* mutations in ~85% of Cowden syndrome cases and elucidated how clinical phenotypes result from these underlying germline mutations [20-22]. A growing body of research on transcriptomes and cancer genomes has revealed that many tumor-specific genetic mutations are located in cis-regulatory elements. Single nucleotide polymorphisms (SNPs) often emerge in cis-regulatory elements, which can lead to pathogenic promoter-enhancer interactions, ultimately resulting in abnormal gene expression [23]. The *PTEN* gene is a highly polymorphic with multiple SNPs [9, 24]. Although studies have linked BC to genetic polymorphisms in the cis-regulatory elements of *PTEN*, the underlying molecular mechanism leading to BC chemosensitivity is still elusive. In recent years, there have been reports of polymorphism affecting the process of alternative splicing (AS), which leads to cancer development [25-27]. A small number of studies have found that PTEN can produce new isoforms, but how abnormal AS of PTEN leads to chemosensitivity was yet not reported [28, 29].

Despite numerous studies on the association between genetic polymorphisms of *PTEN* and drug resistance [30], the underlying molecular mechanism of *PTEN* polymorphism leading to BC chemosensitivity is still uncertain. As far as we know, SNPs located in cis-regulatory elements can affect the AS process and cause diseases, but the link between SNP and AS of PTEN in the Chinese populations at risk of BC chemosensitivity has not been reported. This study was designed to evaluate the relationship between *PTEN* polymorphisms in cis-regulatory elements and BC chemosensitivity. Our work should provide theoretical guidance for individualized BC chemotherapy treatment in the Chinese population.

Materials and methods

Study population

In this study, peripheral blood and fresh breast cancer tissue samples of BC patients who had received neoadjuvant chemotherapy were used. Of the 234 patients originally screened, 172 were enrolled for blood sample collection, including 104 chemosensitive and 68 chemoresistant samples. Inclusion criteria were: (1) pathological confirmation of BC; (2) recipients of anthracycline-based neoadjuvant chemotherapy; (3) at least 2 courses of chemotherapy; (4) tumor size was recorded before and after chemotherapy; (5) the presence of complete clinical data. Exclusion criteria were: (1) incomplete data; (2) recipients of postoperative chemotherapy; (3) without anthracycline drugs; (4) fewer than 2 courses of chemotherapy; (5) chemotherapy efficacy not evaluated. For fresh BC tissue samples, we initially collected 32 samples (18, chemosensitive; 14, chemoresistant). According to the genotyping results, we randomly selected 9 tissue samples whose genotypes and clinical date were consistent with defined chemosensitivity and chemoresistance, including 3 chemosensitive samples with the GG genotype, 3 chemosensitive samples with the AG genotype, and 3 chemoresistant samples with the AA genotype. Chemotherapy efficacy was evaluated and scored according to the Response Evaluation Criteria in Solid Tumour (RECIST) criteria [31-34], divided into a complete response, partial response, stable disease, or progressive disease, and classified as chemoresistant or chemosensitive [35-37]. Patients were susceptible to chemotherapy if all tumor masses or pathological lymph nodes disappeared (complete response), and the tumor was at least 30% smaller than the longest diameter of the tumor (partial response). Patients were chemoresistance if the tumor increased by at least 20% of the longest diameter (progressive disease), and the tumor size reduction was insufficient to produce any partial response but tumor size did not increase or become a progressive disease (stable disease) [31, 32, 38, 39]. All samples were collected from the former Lanzhou General Hospital of Lanzhou Military Region between September 2013 and April 2020. The ethics committee of Lanzhou University School of Basic Medicine approved the study.

Extraction of genomic DNA and genotyping

All peripheral blood samples were collected in sterile tubes with EDTA and were stored at -80°C. The extracted peripheral blood DNA was stored at -80°C until used. 4 SNPs (rs786204926, rs701848, rs12402181, rs35-

770269) were genotyped using the Sequenom MassArray typing technology for samples with a DNA concentration of more than 20 ng/μl (Beijing Bomiao Biotechnology Co., Ltd., China). Assay Design 3.1 software was used to design the primers required for Sequenom MassArray (Supplementary Table 1). After PCR amplification, SAP-PCR, purification, and spotting of the purified extension product, Spectro CHIP was prepared. After spotting, a MALDI-TOF mass spectrometer was employed to detect the Spectro CHIP and TYPER 4.0 software was used to analyze the results.

Sequencing verification of genotyping

Partial samples were randomly selected for direct sequencing verification of genotyping. The primers were designed and sent to the company (General Biosystems (Anhui) Co., Ltd., China) for synthesis. The primers are shown in Supplementary Table 2. PCR amplification consisted of 31 cycles with an annealing temperature of 58°C. The synthesized products were electrophoresed on 1% agarose gels. The PCR amplified products were verified by sequencing (General Biosystems (Anhui) Co., Ltd., China).

Semi-quantitative RT-PCR and sequencing verification of novel mutant type of PTEN

We performed RNA extraction on 9 fresh BC tissue samples that were finally enrolled. While 3 samples were rejected due to poor RNA quality, 6 samples were finally analyzed, including 2 chemosensitive samples each of the GG and AG genotypes and 2 chemoresistant samples (AA genotype). Total RNA was extracted from BC tissues using TRIzol (Invitrogen, Carlsbad, CA, USA), and cDNA was reverse transcribed using the RT MasterMix (Cwbiotech, Beijing, China). Based on software prediction, polymorphisms located in cis-regulatory elements affects the production of two transcripts (PTEN and PTEN mutant). Primers were designed for the two transcripts (PTEN-F: AATTGCAGAGTTGCACAATATCC; PTEN mutant-F: GTTATCTTTTTACCACGGTTGC; PTEN-R: GTCTCTGGTCCTTACTTCCCC; β-actin-F: AGGATTCCTATGTGGGCGAC; β-actin-R: ATAGCACAGCCTGGATAGCAA) and PCR amplification was performed. For PCR, template was initially heated to 94°C for 3 min, followed by 32 cycles of 30 s at 94°C, 30 s at 60°C, and 25 s at 72°C, and finally 72°C for 5 min. The product was analyzed on a 1% agarose gel and evaluated by Quantity One software. PCR amplified products were verified by sequencing (General Biosystems (Anhui) Co., Ltd.).

Western blot analysis

We extracted total protein from the 9 BC tissues from above, and the final 9 samples were used to analyze protein expression, including 3 each of chemosensitive samples of the GG and AG genotypes and 3 chemoresistant samples (AA genotype). Total protein was extracted using the radioimmunoprecipitation assay lysis buffer (Beyotime Biotechnology, Shanghai, China), and the protein concentration was determined with a protein concentration determination kit (Solarbio, Beijing, China). The proteins were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes were incubated overnight at 4°C with primary antibodies against human phosphorylated PI3K (1:1000; 20584-1-AP), ASF/SF2 (1:1000; 12929-2-AP), PTEN (1:1000; 22034-1-AP), SFRS5 (1:1000; 16237-1-AP), and GAPDH (1:2000; 60004-1-Ig). Then the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000; S0001) and visualized using a chemiluminescence kit (NCM Biotech Co., Ltd., Suzhou China). The results were analyzed by the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Silicon analysis

Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/index.html) was used to analyze transcript levels of PTEN in 1,085 BC tissues and 291 normal tissues with boxplots. The overall survival status of patients with PTEN and PTEN mutation was assessed with cBioPortal for Cancer Genomics version 3.7.17 (https://www.cbioportal.org/), and included in 24 studies querying 10,550 patients/11,253 samples. To study the correlation between *PTEN* mutation genome and epirubicin drug sensitivity, Genomics of Drug Sensitivity in Cancer version 8.3 (GDSC, https://www.cancerrxgene.org/) was used [40]. In order to predict whether rs786204926 affects splicing, Alamut Visual v.2.15 and Human Splicing Finder (HSF, https://www.genomnis.com/access-hsf) were used for in silico analysis. We searched for PTEN (PDB ID: 5BUG) and INS (1, 3, 4, 5) P4 (PDB ID: 1BWN)

[41] protein structures in the PDB (https://www.rcsb.org/) database. Pymol-2.3.0 and Swiss-Model were performed for single point mutations on protein structures and introduced in Zdock for docking.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was evaluated in two groups of subjects to detect genotype distribution of the rs786204926, rs701848, rs12402181, and rs35770269 polymorphisms using online software (http://shesisplus.bio-x.cn/SHEsis.html). Haplotype estimation was performed with the SHEsis software (http://shesisplus.bio-x.cn/SHEsis.html) [42, 43]. All values with two-tailed $P<0.05$ were considered statistically significant. Clinical data and genotyping results were analyzed using the SPSS 22.0 software (IBM Corp., Armonk, NY, USA), independent sample t-test, chi-square test, and Fisher's exact test (sample size <5). $P<0.05$ was considered statistically significant. Logistic regression analysis was applied to analyze correlation risk factors associated with chemosensitivity in BC. Data were presented as the mean ± SD and analyzed with SPSS 22.0 and GraphPad Prism 5.0. Differences between the two groups were analyzed with Student's t-test.

Results

In silico analysis of the association of PTEN mutations with drug sensitivity in BC

According to the GEPIA database, the expression of PTEN was compared by boxplot. The BC tumor group included 1,085 samples, and the normal group included 291 samples. The results showed that PTEN was slightly expressed in the normal group (**Figure 1A**). According to the cBioPortal for Cancer Genomics database, we analyzed samples with *PTEN* mutation data in BC [44, 45]. Approximately 9% patients had *PTEN* mutations (**Figure 1B**), and *PTEN* mutations were associated with overall survival (**Figure 1C**). To study the correlation between the *PTEN* mutation genome and drug sensitivity in BC, we used the Genomics of Drug Sensitivity in Cancer version 8.3 online software. The results showed that having *PTEN* mutation in the genome would increase the sensitivity of epirubicin drugs in BC (**Figure 1D**).

Distributions of the clinical characteristics in BC patients

The study included 172 peripheral blood BC samples with 104 (60.5%) being chemosensitive and 68 (39.5%) chemoresistant. **Figure 2A** shows the imagological examination data of chemosensitivity and chemoresistance. **Table 1** shows the clinical characteristics and statistical information of these 172 patients. All participants are female, with a median age of 48 years. Based on American Joint Committee on Cancer (AJCC) Clinical Stage, most patients were diagnosed with stage II or stage III BC. There were statistical differences in PR and molecular subtypes ($P<0.05$) but not in other variables ($P>0.05$) between the sensitive and resistant groups.

Statistical analysis of 4 SNP genotyping

MassArray genotyping was performed on the rs786204926, rs701848, rs12402181, and rs35770269 polymorphisms in 172 peripheral blood BC samples. Our analysis showed that the genotype results of rs786204926, rs701848, and rs12402181 conformed to HWE ($P>0.05$) (**Table 2**), which indicates that the genotype genetic equilibrium of rs786204926, rs701848, rs35770269, and the data were derived from the same Mendel population, with the results of this test being representative. We further statistically calculated genotypes and compared the distribution of genotypes under different genetic models. The results are listed in **Table 3**. In the heterozygote model, the AG genotype was significantly different from the AA genotype (OR=0.227, 95% CI=0.049-1.050, $P=0.048$). Under the dominant model, the AG+GG genotype was significantly altered (OR=0.195, 95% CI=0.043-0.886, $P=0.029$). In the Additive model, the AA+GG genotype was significantly different from the AG genotype (OR=4.304, 95% CI=0.932-19.878, $P=0.049$). In the allele analysis, there was a statistical difference between alleles G and A (OR=0.179, 95% CI=0.041-0.792, $P=0.012$), therefore carrying the G allele or AG genotype might increase the risk of chemosensitivity in BC. There was no correlation between BC chemosensitivity and rs701848, rs12402181, or rs35770269. We randomly selected several samples to verify the genotyping results by Sanger sequencing. PCR products following agarose gel electrophoresis are shown in **Figure 2B**. Wildtype homozygous, heterozygous, and mutant homozygous

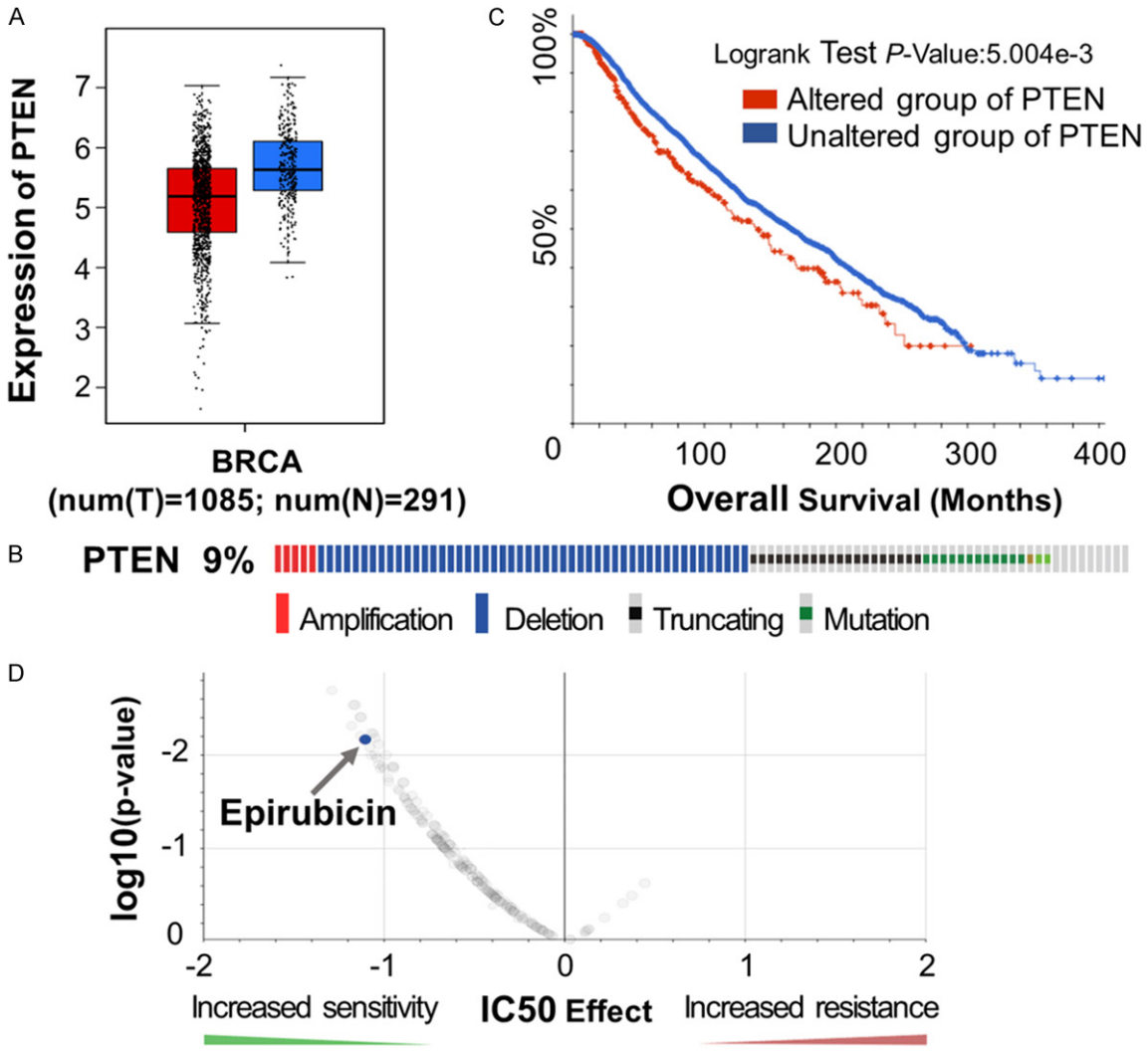


Figure 1. Association of *PTEN* mutations with drug sensitivity in breast cancer. A. The GEPIA database analyses the expression of the *PTEN* gene in breast cancer by boxplot. B. The cBioPortal for Cancer Genomics database analysis of samples with mutation data in breast cancer, *PTEN* had mutations in 9% of the patients. C. The relationship between genomic *PTEN* mutation and overall survival in breast cancer patients. D. The association of genomic *PTEN* mutations and chemotherapeutic drug sensitivity in breast cancer. Each circle represents an association between a genomic marker and drug sensitivity analyzed using ANOVA. The blue circle represents the association between epirubicin sensitivity and the genomic *PTEN* mutation.

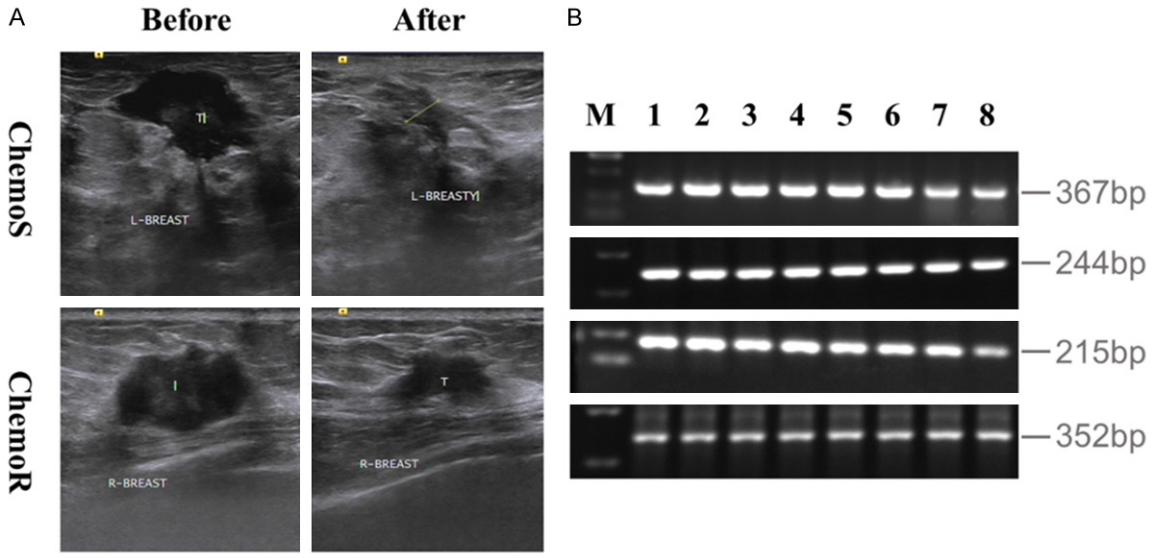
sequences of the rs786204926 polymorphism are shown in **Figure 2C**.

Logistic regression analysis

Logistic regression analysis was used to determine correlative risk factors associated with chemosensitivity in BC. Chemotherapy response was set as the dependent variable, while related factors (age, lymph node metastasis, rs786204926 genotype, tumor size, AJCC clinical stage, and molecular subtypes.) were set as independent variables. Logistic regression analysis showed that the rs786204926 GG/AG genotype (P=0.015), age (P=0.000), and lymph node staging (P=0.027) were clearly related to BC chemoresistance (**Figure 2D**), while no association between other associated factors and chemoresistance in BC were found.

Linkage disequilibrium (LD) and haplotype analysis

The SHEsis software was employed to analyze the LD of the two polymorphisms of *PTEN*. LD was detected in rs701848 and rs786204926



D

	B	S.E.	Wald	*P*	Exp(B)
Genotype	-2.173	0.892	5.940	**0.015**	0.114
Age	0.245	0.061	16.028	**0.000**	1.278
Menopausal state	-0.371	0.614	0.365	0.546	0.690
Tumor size	-0.549	0.482	1.301	0.254	0.577
AJCC clinical stage	-0.140	0.344	0.165	0.684	0.870
Molecular subtype	0.601	0.319	3.557	0.059	1.824
Lymph node staging	-0.844	0.382	4.871	**0.027**	0.430

Figure 2. The relationship between *PTEN* polymorphism and chemosensitivity. A. The imagological examination data of breast cancer patients. According to the tumor size before and after chemotherapy, samples are divided into chemosensitivity and chemoresistance groups. B. PCR electropherograms of four SNPs in partial DNA samples, followed by rs786204926, rs701848, rs12402181, and rs35770269. C. Sequence verification of the rs786204926 polymorphism: GG-wild type homozygous, AG-heterozygous, AA-SNP homozygous. The red box is the position of the rs786204926 polymorphism base mutation. D. Multiple logistic regression analysis was used to stepwise analyze the relationship between chemosensitivity and its related factors. Chemotherapy response (chemosensitivity/chemoresistance) served as a dependent variable. Age (<48/≥48), lymph node stage (N0/1/2/3), rs786204926 (AA/AG/GG), tumor size (T1/2/3), AJCC clinical stage (II/IIIA/IIIB/IIIC+IV), molecular subtype (lumina A/lumina B/HER2 overexpression/triple negative) served as independent variables.

(r^2=0.05, D'=1.000) (**Figure 3A** and **3B**). There was a discordance between D' and r^2 due to the low genotype frequency of the rs786204926 polymorphism. Therefore, this result should be further validated with larger sample size. Haplotype analysis was also performed in the

Table 1. Characteristics of study subjects

Characteristics	Total	Chemosensitive	Chemoresistant	*P*
	172	104 (0.605)	68 (0.395)	
Age (years)				0.106
<48	89 (0.517)	59 (0.343)	30 (0.174)	
≥48	83 (0.483)	45 (0.262)	38 (0.221)	
Menopausal state				0.062
Menopause	59 (0.343)	30 (0.174)	29 (0.169)	
Premenopausal	113 (0.657)	74 (0.430)	39 (0.227)	
AJCC Clinical Stage				0.190
II	77 (0.448)	40 (0.233)	37 (0.215)	
IIIA	41 (0.238)	29 (0.169)	12 (0.069)	
IIIB	50 (0.291)	32 (0.186)	18 (0.105)	
IIIC+IV	4 (0.023)	3 (0.017)	1 (0.006)	
Tumor size				0.564
T1 (size ≤2)	43 (0.250)	24 (0.140)	19 (0.110)	
T2 (2< size ≤5)	84 (0.488)	50 (0.291)	34 (0.198)	
T3 (size >5)	45 (0.262)	30 (0.174)	15 (0.087)	
Lymph node staging				0.874
N0	57 (0.331)	33 (0.192)	24 (0.140)	
N1	59 (0.343)	35 (0.203)	24 (0.140)	
N2	46 (0.267)	29 (0.169)	17 (0.099)	
N3	10 (0.058)	7 (0.041)	3 (0.017)	
ER				0.263
Negative	90 (0.523)	58 (0.337)	32 (0.186)	
Positive	82 (0.477)	46 (0.267)	36 (0.209)	
PR				0.001
Negative	57 (0.331)	45 (0.262)	12 (0.070)	
Positive	115 (0.669)	59 (0.343)	56 (0.326)	
C-erbB-2[a]				0.503
Negative	24 (0.140)	16 (0.093)	8 (0.047)	
Positive	148 (0.860)	88 (0.512)	60 (0.349)	
Molecular Subtype				0.028
Luminal A	40 (0.233)	18 (0.105)	22 (0.128)	
Luminal B	98 (0.570)	68 (0.395)	30 (0.174)	
HER2 overexpression	14 (0.081)	6 (0.035)	8 (0.047)	
Triple-negative	20 (0.116)	12 (0.070)	8 (0.047)	

[a]C-erbB-2-positive: HER2 (+++) or Fish (+); ER, Estrogen Receptor; PR, Progesterone Receptor; C-erbB-2, human epidermal growth factor receptor 2.

two groups, with haplotype frequencies of <3% excluded from further analysis. We did not find any relationships between the CA, TA and TG haplotypes (rs701848, rs786204926) with chemosensitivity (**Figure 3C**).

rs786204926 affects AS of a novel PTEN mutant

SNP located in cis-regulatory elements may affect AS and possible effects can be detected by Alamut Visual v.2.15 and HSF. A previous study has reported that Alamut Visual had the greatest accuracy in predicting splicing abnormalities (approximately 85%), and the accuracy of HSF prediction was about 58% [46]. HSF prediction results showed that rs786204926 is a splice acceptor site located in cis-regulatory elements. With the G allele, it will likely inhibit the function of the wild-type acceptor site thus affect splicing (Supplementary Table 3). Further analysis using Alamut Visual

Table 2. The test of Hardy-Weinberg Equilibrium of four SNPs

SNP	Group	Genotype			*P*
		11	12	22	
rs786204926	Chemosensitive	90 (86.54%)	12 (11.54%)	2 (1.92%)	0.161
	Chemoresistant	66 (97.06%)	2 (2.94%)	0 (0.00%)	0.992
rs701848	Chemosensitive	47 (45.19%)	37 (35.58%)	20 (19.23%)	0.054
	Chemoresistant	33 (48.53%)	20 (29.41%)	15 (22.06%)	0.010
rs12402181	Chemosensitive	59 (56.73%)	40 (38.46%)	5 (4.81%)	0.863
	Chemoresistant	33 (48.53%)	30 (44.12%)	5 (7.35%)	0.876
rs35770269	Chemosensitive	34 (32.69%)	53 (50.96%)	17 (16.35%)	0.891
	Chemoresistant	24 (35.29%)	34 (50.00%)	10 (14.71%)	0.936

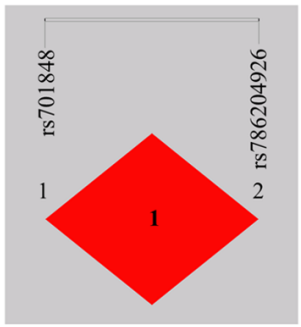
Table 3. The association between polymorphisms and the risk of breast cancer chemoresistance

SNP	Model	Allele/ Genotype	Chemosensitive	Chemoresistant	OR (95% CI)	*P*
rs786204926	Heterozygote	AA	90 (0.523)	66 (0.384)		
		AG	12 (0.070)	2 (0.012)	0.227 (0.049-1.050)	0.048*
	Homozygote	AA	90 (0.523)	66 (0.384)		
		GG	2 (0.012)	0 (0.000)	/	/
	Dominant	AA	90 (0.523)	66 (0.384)		
		AG+GG	14 (0.084)	2 (0.012)	0.195 (0.043-0.886)	0.029*
	Recessive	GG	2 (0.012)	0 (0.000)		
		AG+AA	102 (0.593)	68 (0.395)	/	/
	Additive	AG	12 (0.070)	2 (0.012)		
		AA+GG	92 (0.535)	66 (0.384)	4.304 (0.932-19.878)	0.049*
	Allele	A	192 (0.558)	134 (0.389)		
		G	16 (0.046)	2 (0.005)	0.179 (0.041-0.792)	0.012*
rs701848	Heterozygote	TT	47 (0.273)	33 (0.192)		
		TC	37 (0.215)	20 (0.116)	0.770 (0.381-1.555)	0.465
	Homozygote	TT	47 (0.273)	33 (0.192)		
		CC	20 (0.116)	15 (0.087)	1.068 (0.478-2.387)	0.872
	Dominant	TT	47 (0.273)	33 (0.192)		
		TC+CC	57 (0.331)	35 (0.203)	0.875 (0.474-1.614)	0.668
	Recessive	CC	20 (0.116)	15 (0.087)		
		TC+TT	84 (0.488)	53 (0.308)	0.841 (0.396-1.786)	0.652
	Additive	TC	37 (0.215)	20 (0.116)		
		TT+CC	67 (0.390)	48 (0.279)	1.325 (0.686-2.560)	0.401
	Allele	T	131 (0.381)	86 (0.250)		
		C	77 (0.224)	50 (0.145)	0.989 (0.632-1.548)	0.962
rs12402181	Heterozygote	GG	59 (0.343)	33 (0.192)		
		GA	40 (0.233)	30 (0.174)	1.341 (0.709-2.535)	0.366
	Homozygote	GG	59 (0.343)	33 (0.192)		
		AA	5 (0.029)	5 (0.029)	1.788 (0.482-6.631)	0.494*
	Dominant	GG	59 (0.343)	33 (0.192)		
		GA+AA	45 (0.262)	35 (0.203)	1.391 (0.753-2.569)	0.292
	Recessive	AA	5 (0.029)	5 (0.029)		
		GA+GG	99 (0.576)	63 (0.366)	0.636 (0.177-2.287)	0.519*
	Additive	GA	40 (0.233)	30 (0.174)		

		GG+AA	64 (0.372)	38 (0.221)	0.792 (0.426-1.473)	0.460
	Allele	G	158 (0.459)	96 (0.279)		
		A	50 (0.145)	40 (0.116)	1.317 (0.809-2.143)	0.268
rs35770269	Heterozygote	AA	34 (0.198)	24 (0.140)		
		AT	53 (0.308)	34 (0.198)	0.909 (0.462-1.789)	0.782
	Homozygote	AA	34 (0.198)	24 (0.140)		
		TT	17 (0.099)	10 (0.058)	0.833 (0.326-2.133)	0.704
	Dominant	AA	34 (0.198)	24 (0.140)		
		AT+TT	70 (0.407)	44 (0.256)	0.890 (0.467-1.696)	0.724
	Recessive	TT	17 (0.099)	10 (0.058)		
		AT+AA	87 (0.506)	58 (0.337)	1.133 (0.485-2.648)	0.772
	Additive	AT	53 (0.308)	34 (0.198)		
		AA+TT	51 (0.297)	34 (0.198)	1.039 (0.564-1.915)	0.902
	Allele	A	121 (0.352)	82 (0.238)		
		T	87 (0.253)	54 (0.157)	0.916 (0.590-1.423)	0.696

OR, Odds Ratio; CI, Confidence Interval. *: Pass Fisher's exact test calculation.

A



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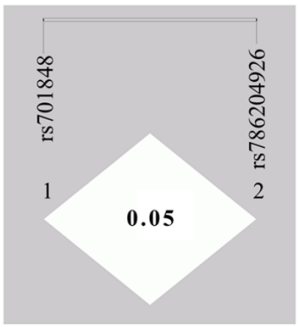

C

Haplotype	Frequency		OR (95%CI)	*P*
	ChemoR	ChemoS		
CA	51 (0.375)	77 (0.370)	1.020 (0.652~1.595)	0.928
TA	72 (0.529)	115 (0.552)	0.909 (0.589~1.403)	0.669
TG	16 (0.095)	16 (0.076)	1.268 (0.589~2.728)	0.542

Figure 3. Linkage disequilibrium and haplotype analysis for two polymorphisms of the *PTEN* gene. A, B. SHEsis software analyses the LD relationship of two polymorphisms of *PTEN*, and uses D' and r^2 to measure the LD relationship between the two SNPs. C. Results of haplotype analysis of two *PTEN* polymorphisms in chemosensitive and chemoresistant groups.

v.2.14 showed that the rs786204926A>G mutation attenuated the function of the original acceptor site while increasing the function of a new acceptor site, which likely produced a novel PTEN mutant with an insertion of 18 bases from intron 4 (GTTATCTTTTTACCACGG). In addition, it showed changes between the exon splicing enhancers (ESE), so the ESE binding ability (SC35, SRP40, ASF/SF2) may be weakened to varying degrees with the mutation (**Figure 4A**). **Figure 4B** is a schematic diagram of PTEN AS based on the above predictions and clinical genotyping results. With the A allele, PTEN produced from normal splicing is associated with chemoresistance; with the G allele, the PTEN mutant is more likely produced from abnormal splicing (intron retention) and associated with chemosensitivity. Pymol-2.3.0 and Swiss-Model were used to generate the model of mutant PTEN protein structure. The regions that differ between the two protein structures are shown in boxes.

Higher levels of novel PTEN mutant in chemosensitive samples of BC

To determine the expression of the two transcripts (PTEN vs. PTEN mutant) in chemosensitivity and chemoresistance patients, we analyzed 6 fresh BC tissue samples. cDNAs were then amplified with primers flanking the spliced exons and at the intron junction. The PCR products (PTEN: 243 bp, PTEN mutant: 251 bp) were verified by sequencing. There was no

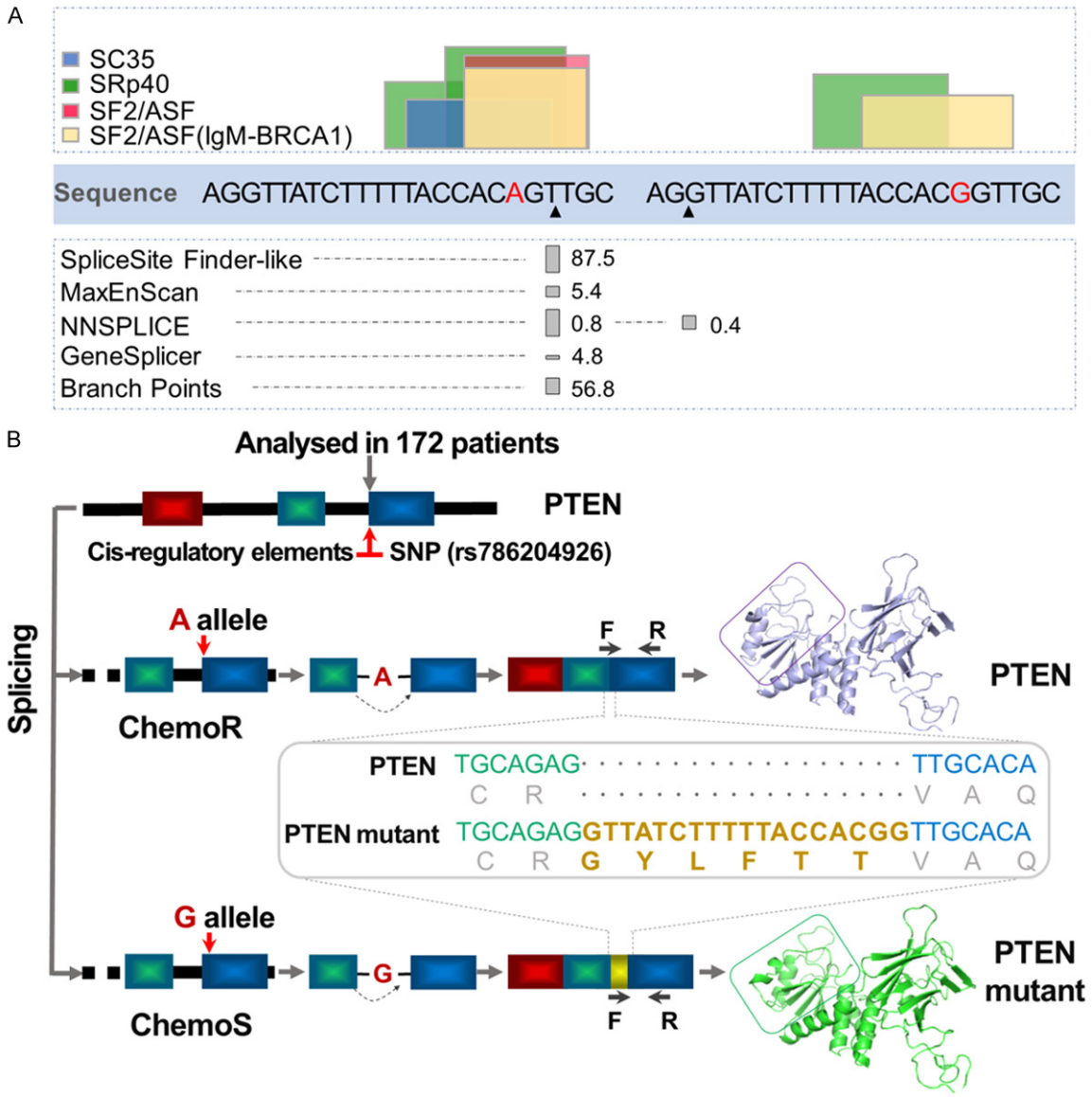

Figure 4. rs786204926 located in cis-regulatory elements affects alternative splicing of a novel PTEN mutant. A. Alamut Visual was used to analyze the effect of rs786204926 on alternative splicing of the *PTEN* gene. rs786204926A>G destroys the original 3' splice site and creates a new 3' splice site. The triangle represents the new splice site. The figure also shows the changes of splicing factor after rs786204926A>G mutation. B. Schematic illustration of the *PTEN* rs786204926 polymorphism generating two isoforms (PTEN and PTEN mutant) by alternative splicing. Boxes showed how the two proteins differ in structure. Yellow is the retained intron fragments in the PTEN mutant.

change in PTEN transcription levels in chemosensitive vs. chemoresistant samples, but a significant difference in the PTEN mutant could be detected ($P<0.05$). While rs786204926 preferentially produced two transcripts, the A allele was more likely to produce PTEN (**Figure 5A** and **5B**). We sequenced the PTEN mutant and confirmed that it indeed contained an 18-bases retention from intron 4 (GTTATCTTTTTACCACGG) (**Figure 5C**), while the rest of sequences were identical to PTEN.

We collected 9 fresh BC tissue samples to analyze the expression of PTEN and PTEN mutant proteins. The molecular weight of PTEN is 55 KDa. The PTEN mutant has an additional 6 amino acids (GYLFTT). Therefore, we think the lower band is PTEN and the upper band is pos-

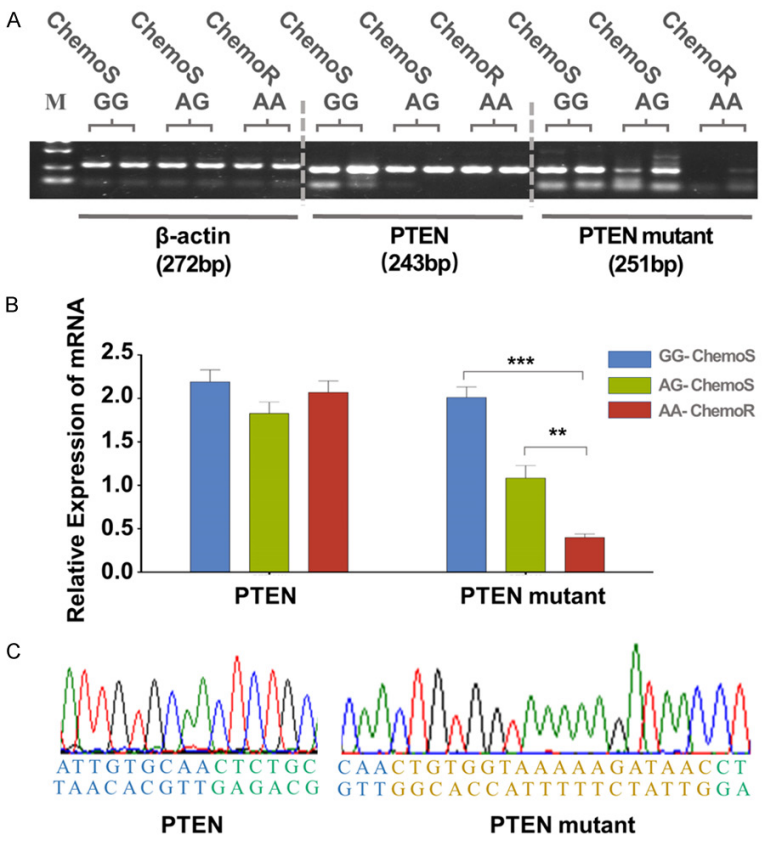

Figure 5. The relative transcript levels of PTEN and the PTEN mutant in breast cancer samples. A. After the sample was amplified by RT-PCR, it was subjected to 1% agarose gel electrophoresis, and the electrophoresis bands of PTEN and PTEN mutant. According to the different genotypes (GG, GA, AA) of the 6 samples, they were divided into chemosensitive and chemoresistant groups. B. Statistical analysis chart of the gray value of PTEN and the PTEN mutant. C. Sequencing verification for PCR production. Yellow is the retained intron fragments in the PTEN mutant.

sibly PTEN mutant. Proteins results showed that PTEN and PTEN mutant differ significantly in protein level in chemosensitive (GG genotype) and chemoresistant (AA genotype) samples ($P<0.05$). The difference was not significant for the chemosensitive AG genotype group. In addition, the genotype of rs78620-4926 tends to produce the two proteins, while the A allele will preferentially produce the 55 KDa PTEN protein. Splicing factors SRP40 and ASF/SF2 were statistically significant in the three groups ($P<0.05$) (**Figure 6A** and **6B**).

The response of PTEN and PTEN mutant to PI3K drug-resistance pathway

We then used Pymol-2.3.0 and Swiss-Model for the homology model based on the predicted amino acid sequence, and predicted changes in the PTEN protein structure with the mutation (**Figure 7A**). The results showed spatial structure changes of the protein with the mutation. In the PTEN structure, amino acids D324, Y177, Y176, Q149, K183, and R189 of INS form hydrogen bonds with residues R46, E45, R48, E7, and Q91 of PTEN respectively; amino acids I92, F44, I95, Y42, K41, D43, M89, I94, I9, L29, and R49 have hydrophobic interactions with amino acids L318, F278, I280, P281, F279, P283, Y180, D187, E284, L186, L181, Y188, N184, K147 of PTEN respectively, and constitute a hydrophobic surface. The above residues might constitute the active interaction interface. In the PTEN mutant structure, amino acids N24, K26, K53, R28, R48, E45, and Q91 of INS form hydrogen bonds with mutant K295, V296, E294, E291, Y182, Y183, and D193 respectively; amino acids L11, E76, F98, E96, P75 of INS, K27, F44, Y42, D43, G47, I9, R49, S21, I92, P22, L29, L23 and mutant C310, I312, F285, I286, G288, L324, Q313, Y186, P289, P287, E290, N190, F284, S293, K153, T292, L192, R195, D307, G299, Q297, Y182 have a hydrophobic effect and constitute a hydrophobic surface. The above residues may constitute an active interaction interface. The results of protein docking showed that hydrogen bonding and hydrophobic interactions increased in the PTEN mutant, mediating the interaction between PTEN and 3,4,5-phosphatidylinositol triphosphate (PIP3), making the dephosphorylation of PIP3 to PIP2 easier. Compared with PTEN, the PTEN mutant is more prone to dephosphorylation, leading to chemosensitivity (**Figure 7B**). We further confirmed the relationship of PTEN and the PTEN mutant with PI3K-AKT pathway signaling in chemosensitivity. The results showed that increased expression of the PTEN mutant produced by the G allele could

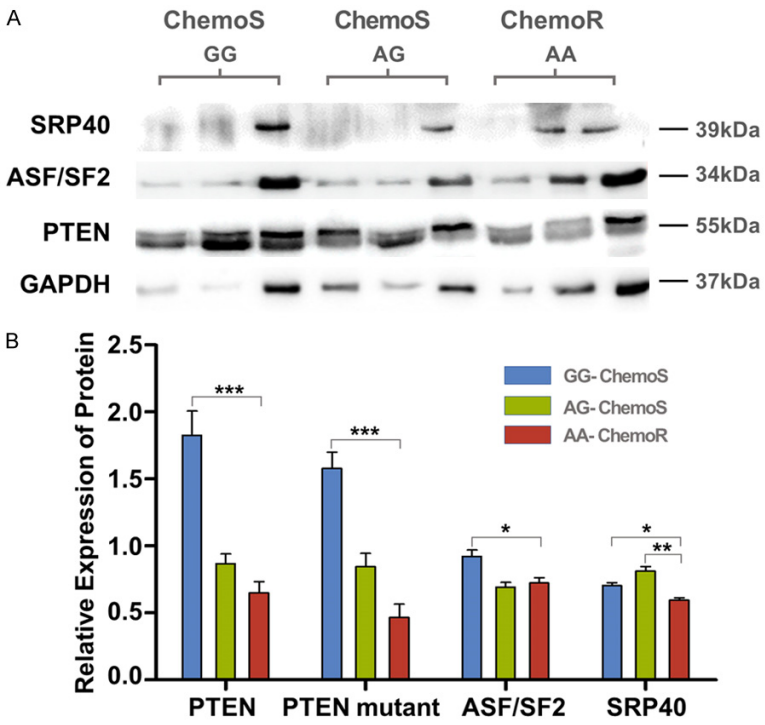

Figure 6. The relative protein level of PTEN and the PTEN mutant in breast cancer samples. A. Protein expression of PTEN, ASF/SF2, SRP40; PTEN is the lower band (55 KDa), the PTEN mutant is the upper band. According to the different genotypes (GG, GA, AA) of the 9 samples, they were divided into chemosensitive and chemoresistant groups. B. A gray value analysis of protein expression of PTEN and the PTEN mutant, ASF/SF2, SRP40.

inhibit P-PI3K, promoting the dephosphorylation of PIP3 to PIP2, resulting in chemosensitivity (**Figure 7C** and **7D**).

Discussion

PTEN is the first reported tumor suppressor gene with phosphatase activity and plays a vital role in cell apoptosis, cell cycle arrest, and migration [47]. It is closely linked to the occurrence and development of a variety of human malignant tumors [14, 48]. Mutations or deletions of *PTEN* in a variety of malignant tumors result in weakened or lost tumor suppressor function [16]. *PTEN* has a high rate of deletions and mutations in BC, and its loss of function is closely linked to the malignant transformation of BC. *Liang H et al.* have found that the PTEN can produce the new isoforms PTENβ, which specifically localizes in nucleolus, binds to nucleolar and regulates its phosphorylation level, thereby regulating rDNA transcription and ribosome production, and inhibiting cell proliferation [28]. *Agrawal S et al.* found new PTEN isoforms in sporadic BC tissues. They produced a short-lived structure of PTEN by different splicing methods of PTEN introns 3 and 5, which also limited its phosphatase activity. The short-lived structure PTEN plays an important role in the occurrence and development of sporadic BC [49]. In addition, many studies have reported that the PTEN plays an important role in multidrug resistance in cancers. It can specifically dephosphorylate PIP3 in the cell membrane, thus antagonizing the PI3K-AKT pathway and leading to the development of drug resistance [50]. It has been widely believed that high expression of PTEN is associated with chemosensitivity, although the underlying molecular mechanisms of BC chemosensitivity resulting from PTEN mutants is still unclear.

High frequencies of *PTEN* mutation have been found in cancers, especially mutations in cis-regulatory elements that also affect PTEN function [22]. SNP is the most common type of mutations. Studies have shown that *PTEN* SNPs may be a candidate pharmacogenomic factors for assessing the susceptibility of BC and the response and prognosis of chemotherapy [51, 52]. Although studies have shown the association between genetic polymorphisms of *PTEN* and BC, the underlying molecular mechanism of *PTEN* polymorphism leading to BC chemosensitivity is still unclear. Therefore, we investigated SNPs in the binding sites of trans-regulatory factors (rs701848, rs12402181, rs35770269) and cis-regulatory elements (rs786204926) of *PTEN* in BC chemoresistance. Our laboratory is committed to studying the role of AS in cancer and has published many high-quality articles on the subject and its clinical application [25, 53-55]. Since mutations at the canonical splicing site (GU-AG) in cis-regulatory elements can affect the AS of the gene, we selected the SNP (rs786204926) that has a greater impact on the AS through previous analysis, and investigated the potential association

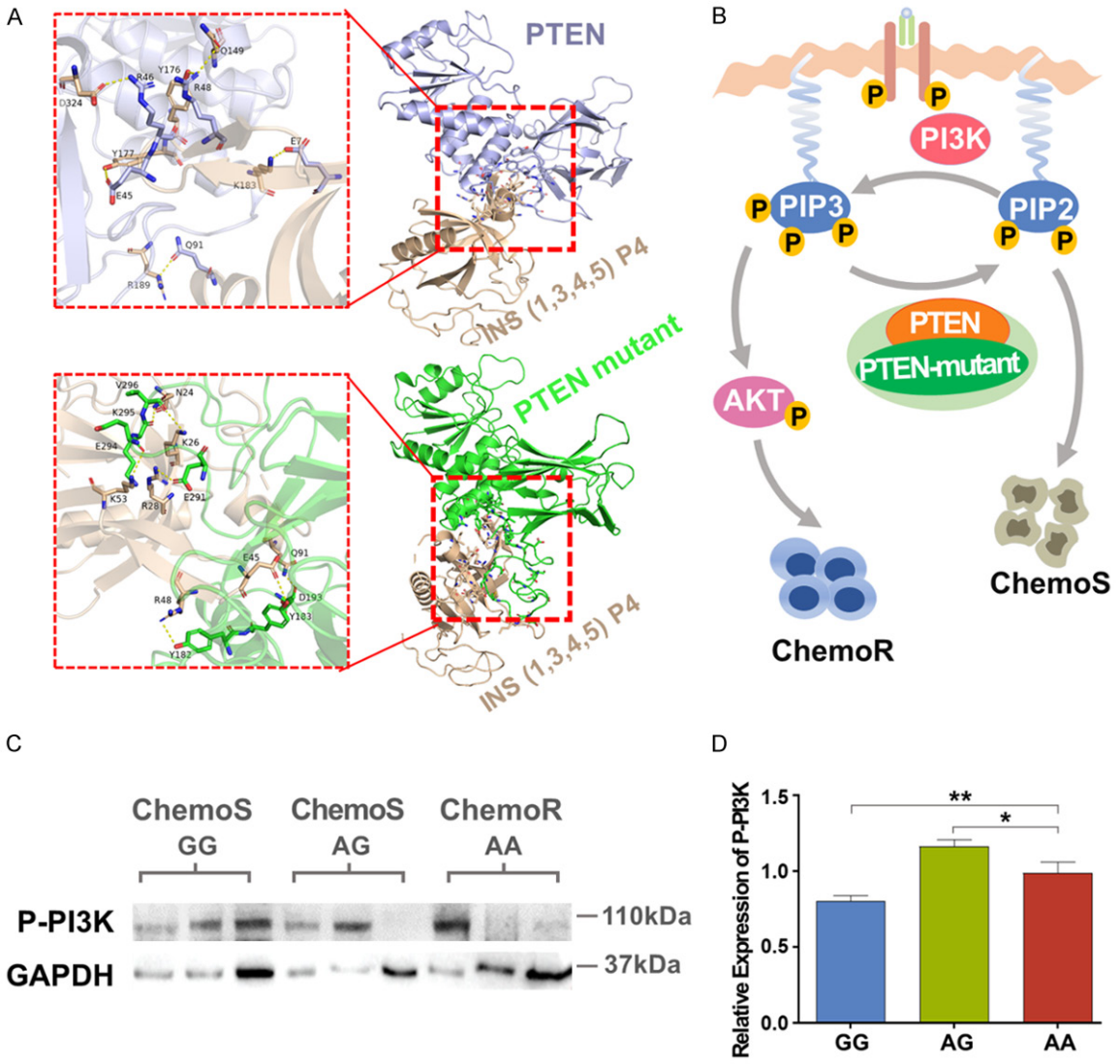

Figure 7. The response of PTEN and the PTEN mutant to PI3K drug-resistance pathway. A. Model of INS (1, 3, 4, 5) P4 binding to PTEN and the PTEN mutant, INS (1, 3, 4, 5) P4 and PIP3 have the same domain. B. Model diagram of PTEN and the PTEN mutant and PI3K resistance pathways. C. Protein expression of P-PI3K. According to the different genotypes (GG, AG, AA) of the 9 samples, they were divided into chemosensitive and chemoresistant groups. D. The gray value of protein expression of P-PI3K.

between *PTEN* mutations locating in cis-regulatory elements and BC chemosensitivity in the Chinese population. Interestingly, our research showed that rs786204926 of *PTEN* was closely linked to BC chemosensitivity, and carrying the G allele can increase chemosensitivity risks in BC patients. Previous studies have confirmed that rs701848, rs12402181, and rs35770269 are related to the susceptibility of cancers and response and prognosis prediction for chemotherapy [51, 56-58]. Song et al. suggested that the rs701848 polymorphism was associated with increased cancer risks in the Asian population, especially Chinese, but not in non-Asian populations (e.g., American, Mexico American, and Polish) [59]. In addition, the rs701848 polymorphism has been shown to be a candidate pharmacogenomic factor for assessing the susceptibility of BC and response of individualized CE(A)F chemotherapy in BC patients [51]. Mao et al. demonstrated that rs35770269 was significantly associated with the occurrence of side effects of capecitabine-based chemotherapy in advanced colon cancer patients [56]. However, we have found no association of rs701848, rs12402181, or

rs35770269 with BC chemotherapy response. It may be the association between genetic polymorphisms and BC varies greatly among different populations, signaling that different genetic backgrounds may have different mutation frequencies [60]. In addition, studies have shown that genetic SNPs may influence drug response, and there are significant differences between different populations [61]. This signifies that SNPs have different therapeutic effects on people with different genetic backgrounds. With its vast size in territory and population, China exhibits vast differences in eating habits, cultural customs, living environments, and genetic backgrounds in different regions. Our samples were mainly collected from patients from Northwest China. All samples came from patients in the hospital, so we analyzed patients who did not meet the HW equilibrium state. This study is the first to detect and analyze the *PTEN* polymorphism of rs786204926 in Northwest China. It provides a framework for establishing a database of *PTEN* gene polymorphisms in Northwest China and should facilitate pharmacogenomics research in personalized medicine.

Compared with previous studies related to chemosensitivity, our research not only finds a novel SNP related to chemosensitivity, but also used Alamut Visual to analyze the correlation between rs786204926 polymorphism and AS. Up to 50% of mutations that lead to genetic diseases result in abnormal splicing [62]. It has been found that *PTEN* mutants found at the junction of the splicing sites might cause abnormal splicing [63]. Cis-regulatory elements can "signal" the snRNAs and auxiliary splicing factors to recognize the "staging area" to initiate the assembly of the spliceosome, eventually leading to intron excision and exons joining to yield a mature mRNA [64]. This is one of the important mechanisms of AS involved in the occurrence and development of various diseases. Studies have found that the *PTEN* gene can produce novel isoforms by AS, such as retained intron 3 (3a, 3b, 3c) and intron 5 (5a, 5b, 5c) regions; excluding part of exon 5 (DelE5) or all of exon 6 (DelE6); PTENα and PTENβ. But the involvements of abnormal AS of the *PTEN* gene in BC chemosensitivity was not reported [28, 49]. In this study, we identified a novel PTEN mutant that might be caused by rs786204926 polymorphism in cis-regulatory elements. When an A>G mutation occurs at the cis-regulatory elements, it will likely reduce the function of the wild-type receptor site and affect splicing. With the mutation, 18 bases from pre-mRNA intron 4 will be retained in the mature mRNA, and the ESE binding ability will also change, which might eventually cause the mutant protein to have 6 additional amino acids. *PTEN* is a tumor suppressor gene with phosphatase activity [47]. On the cell membrane, PTEN mediates the conversion of PIP3 to PIP2 and inhibits the PI3K-Akt pathway to arrest the cell cycle in the G1 phase, leading to the development of drug resistance [67-69]. In the PTEN protein, the phosphatase domain is composed of its N-terminal amino acids 15-185 [65, 66]. The 6 extra amino acids produced by the mutant are also located in this domain, which will induce structural changes in the protein and likely functional changes as well. With the mutation, we found varying degrees of protein structural changes. In the rs786204926 polymorphism, AS might cause a change in mRNA and protein, resulting in a novel PTEN mutant, which will eventually lead to the reduction or loss of the function of PTEN phosphatase. Loss of PTEN function will mean the loss of its negative regulatory effect on PI3K-Akt, Akt will be constitutively activated, which will dysregulate the cell cycle, leading to unchecked cell growth and proliferation and increasing the risk of BC chemosensitivity. Previously, it was thought that PTEN was related to chemosensitivity, but the expression of PTEN mutants was not examined. In our research, we discovered a novel PTEN mutant in BC. In past studies of *PTEN*, the sum of PTEN and PTEN mutant expression was generally taken as PTEN expression. In our more detailed study, our results suggested that close attention should be paid to the respective expression levels of both PTEN and PTEN mutants. We analyzed the collected clinical samples and found a correlation between rs786204926 polymorphism and chemosensitivity in BC, and that the G allele was associated with chemosensitivity in BC. We also performed transcript and protein level verification. We showed that the G allele was more prone to producing the PTEN mutant in chemosensitive patients. There was no alteration in PTEN transcript levels in either chemosensitive or chemoresistant samples, but the protein level was significantly different. Interestingly, there were significant

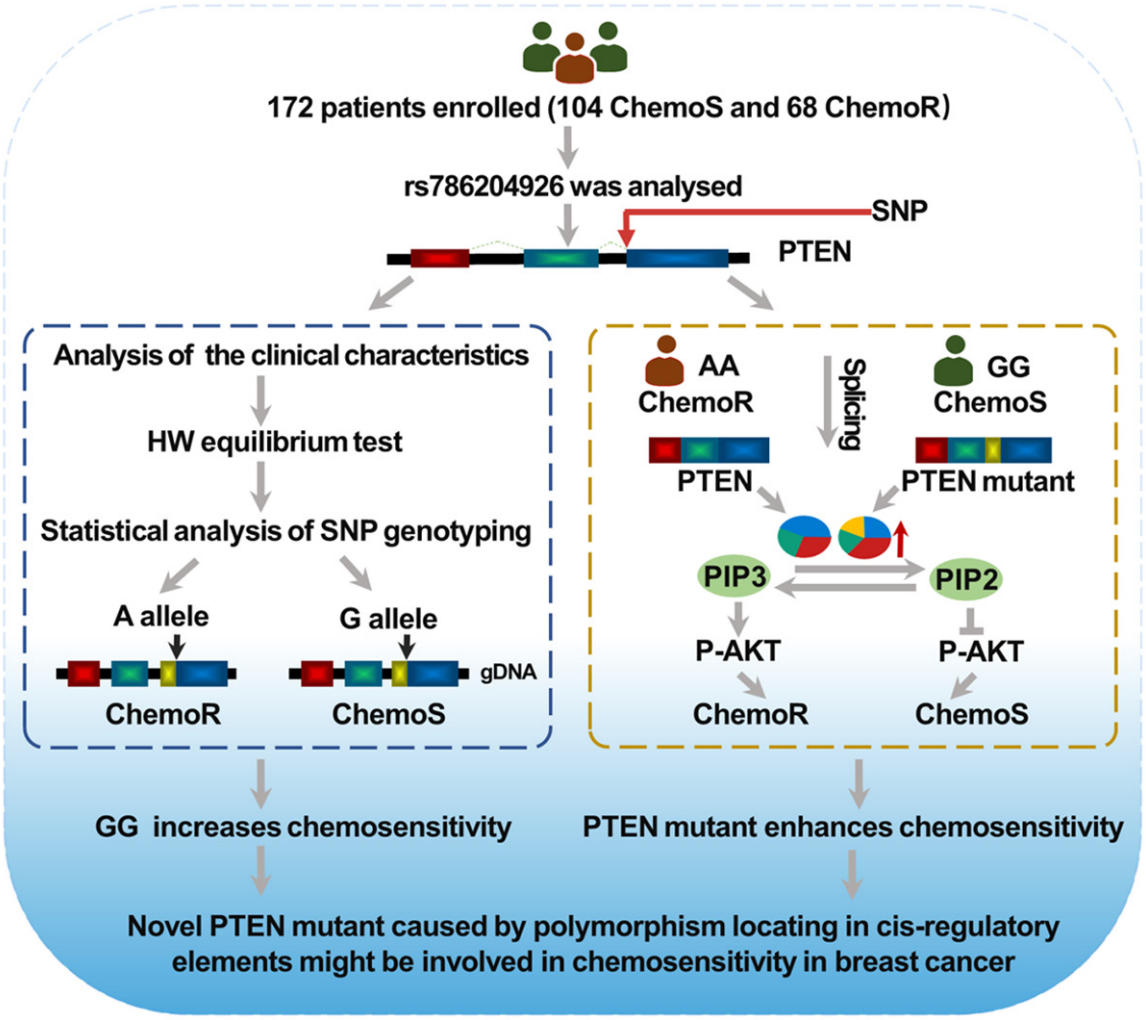

Figure 8. Schematic representation of the mechanism of chemosensitivity in breast cancer.

differences in both transcription and protein levels of the novel PTEN mutant between the two groups. Concurrent computational analysis also revealed that the PTEN mutant has a stronger binding ability with PIP3 and more likely to exert its dephosphorylation effect, thus inhibiting the PI3K-AKT pathway and leading to chemosensitivity. These findings indicate that the novel PTEN mutant caused by polymorphism in cis-regulatory elements is involved in chemosensitivity in BC. Our study should provide theoretical guidance for the individualized treatment of clinical BC patients (**Figure 8**).

In conclusion, we hypothesize that a novel PTEN mutant caused by polymorphism in cis-regulatory elements may be involved in chemosensitivity in BC. Our work not only provides theoretical guidance for the individualized treatment of clinical BC patients in the Chinese population, but also signifies new avenues of research into the mechanisms of BC chemosensitivity.

Acknowledgements

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Primer sequences used for genotyping of gene SNP with the sequenom platform

SNP-ID	1st-PCR Primer Sequences	2nd-PCR Primer Sequences	UEP Sequences
rs786204926	ACGTTGGATGCTCTGGAATCCAGTGTTTCT	ACGTTGGATGTCTAGCTGTGGTGGGTTATG	ATGTTCTTCAAAAGGATATTGTGCAAC
rs35770269	ACGTTGGATGGGTTTATGCCACTTCTCCAC	ACGTTGGATGCAGCAAAGGATAAAGCTGGG	AGCGGCTGTTAATGATTTTAACAGT
rs701848	ACGTTGGATGCCGCTTAAAATCGTATGCAG	ACGTTGGATGATTGAAAGAATAGGGTTTTCCTT	ACCGAGTTGGGACTAGGGC
rs12402181	ACGTTGGATGCTAGATGGAATCGAATTTCCC	ACGTTGGATGTGTCTTCATTCAGAAATGG	GGCCCCTGGCACTATATGAGTC

Supplementary Table 2. Primers for polymerase chain reaction

SNP	Forward primer	Reverse primer	Products size
rs786204926	GGGGAAAATAATACCTGGCTTCC	AGTTCGTCCCTTTCCAGCTT	367
rs35770269	TGAGCCTAGAGAGAAGCATGG	GGATGTGTCAGGTAGGCAGT	352
rs701848	GTAAACTTTCAATGCTGCACA	AAATCGTATGCAGTCTGGGC	244
rs12402181	AGAAGACCTGGGCTGTAGTC	GTCCCACAAAACACCTGGC	215

Supplementary Table 3. HSF software predicted the rs786204926A>G mutation affected alternative splicing

Type	Interpretation		
Broken WT Acceptor Site	Alteration of the WT Acceptor site, most probably affecting splicing		
Algorithm/Matix	position	sequences	variation
MaxEnt Acceptor site	chr10:87932993	-REF: AGGTTATCTTTTTACCACAGTTG -ALT: AGGTTATCTTTTTACCACGGTTG	5.35>-2.6 =>-148.6%
HSF Acceptor site (matrix AG)	chr10:87933001	-REF: TTTTTACCACAGTT -ALT: TTTTTACCACGGTT	89.48>61.61 =>-31.15

Red fonts represent the mutation position of rs786204926.