The Association between ANXA2 mutations and Clinical Pathological Features in Patients with Breast Cancer

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Abstract

Background

Genetic mutations have been reported in many tumors. In this study, we aimed to examine whether ANXA2 mutations occur in breast cancer and to investigate their association with clinicopathological characteristics in patients.

Materials and Methods

We collected breast cancer and adjacent normal tissue samples from 112 patients, extracted total RNA, and performed PCR-SSCP and bidirectional Sanger sequencing. ANXA2 mutations were identified by NCBI BLAST (blastn and blastx), and their correlation with clinical data were analyzed.

Results

ANXA2 mutations were detected in breast cancer tissues (missense mutations 38.39%) at a higher incidence than in adjacent normal tissues (missense mutations 8.04%) and mainly located in domain and repeats 1. Moreover, mutations in breast cancer tissues were associated with clinical stages, molecular subtype, ER, PR, and lymph-node metastasis of patients. Within a mean follow-up time of 52.5 months, the 5-year OS of patients with missense mutations was lower than those without. Among those mutations, c.(350AG > GA), c.(375G > A), c.(487T > A) and c.(693G > A) were associated with younger patients. c.(350AG > GA) was related to higher clinical stage and lymph-node metastasis. c.(375G > A) was linked to HER2(-) cases, while c.(693G > A) tended to be TNBC cases.

Conclusion

ANXA2 missense mutations mainly occurred on domain and repeats 1 in breast cancer, which may be associated with pathogenesis, clinical stage, molecular subtyping, lymph node metastasis and 5-year OS of breast cancer. These mutations may contribute to the early screening, diagnosis, and targeted treatment of breast cancer.

Background

In China, the incidence of breast cancer is increasing every year, and represents a serious threat to women's health[1–3]. Breast cancer is considered to be a polygenic heterogenous disease [4, 5], and identifying the mutations closely associated with the pathogenesis of breast cancer is of considerable significance for the screening, early diagnosis, treatment, and prevention of this disease.

Mutations can be categorized as somatic and germline mutations, according to whether the cells have reproductive capacity. Most cancer mutations are somatic. Somatic mutations are more complex and diverse than embryonic mutations[6–8]. Single nucleotide polymorphism (SNP) refers to the variation of a single nucleotide in the genome, also called a point mutation and is one of the most common types of mutations in humans (about 1 in every 1000 genes) [9].

ANXA2, a member of the Annexin family, comprises four domains and repeats (Fig. 1). Therefore, it can bind anionic membranes in response to Ca^{2+} activation and induce membrane folding and blebbing [10], and is also involved in the transduction of numerous signaling pathways, including the AKT [11, 12], JNK-p53 [13], and NF-kappa B [14] pathways. Increased expression of ANXA2 is associated with the occurrence and distant metastasis of a number of tumor types, including esophageal [15–17], gastric [18–20], prostate [21–23], ovarian [24], and breast cancers [25–27]. ANXA2 is associated with poor pathological response and inhibits apoptosis [28]; It is also associated with worsened prognosis in triple negative breast cancer patients [29]. ANXA2 overexpression in TNBC contributes to an aggressive pathology [30]. Owing to its association with tumor-derived exosomes, ANXA2 plays an important role in angiogenesis and metastasis in breast cancer[27, 31].

To date, a few ANXA2 mutations have been reported to be associated with osteonecrosis in children with sickle cell anemia and low-density lipoprotein cholesterol [32–34]. However, although ANXA2 function is critical to cell growth and closely associated with the occurrence and metastasis of breast cancer, it remains to be determined whether ANXA2 mutations occur in breast cancer and, if so, to identify the relationship between ANXA2 mutations and breast cancer patients' clinical characteristics.

In this study, we used PCR-SSCP to identify ANXA2 mutations occurrence, and bidirectional Sanger sequencing to determine the sequences of ANXA2 PCR products. These sequences were then blasted against the NCBI nucleic acid and protein databases, and thereafter, we assessed the relationship between the mutations and patients' clinicopathological features, in order to identify potentially novel targets for characterizing the pathogenesis of breast cancer and facilitating its diagnosis.

Materials And Methods

Tissue samples

This study was approved by the ethics committee of North Sichuan Medical College and informed consent was obtained from each patient. From April 2018 to December 2019, samples of fresh breast cancer tissue and adjacent normal tissue were collected from 112 female patients with invasive breast ductal carcinoma in the Affiliated Hospital of North Sichuan Medical College. All tissues were flash-frozen in liquid nitrogen immediately after collection and stored at a temperature of -80°C until analysis. The identity of both tumor and adjacent normal tissue samples was confirmed through pathological examination. None of the patients had received chemotherapy or radiotherapy prior to surgery.

Main Reagents

The major reagents used in this study included the total RNA extraction reagent TRIzol, Golden viewer I type nucleic acid dyes, 30% Acr-Bis, 1 M Tris pH8.8, 10% Ap and TEMED (Beyotime Biotechnology, China), a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA), DNA markers (Tiangen, China), and Taq Master Mix (Novo Protein, China).

Extraction of total RNA

After obtaining the fresh human breast specimens (breast cancer tissue and adjacent normal tissue), they were immediately placed in a thermal insulation bucket containing liquid nitrogen, and approximately 50–80 mg tissue samples were powdered in liquid nitrogen. Total RNA was extracted using TRIzol reagent as per the manufacturer's instructions. The total RNA thus obtained was assessed by 1.2% agarose gel electrophoresis (80–100 V for 10–15 min), with detection under a BIO-MED ultraviolet detector.

Synthesis of cDNA

cDNA was synthesized according to the procedure of the Revert Aid first strand cDNA Synthesis Kit (Thermo Science), using a 20-μL reaction system.

Primers

There are four transcriptional versions of ANXA2: transcriptional version 1, which encodes a 357-amino acid protein (isoform 1); versions 2, 3 and 4, which encode 339-amino acid proteins (isoform 2). Version 4 has an alternate 5' UTR, as compared to variant 1. It uses a downstream AUG start codon and encodes isoform 2 which has a shorter N-terminus, as compared to isoform 1. Versions 2, 3 and 4 encode the same isoform 2. All versions, however, contain the same domain and repeats.

For better description, we selected transcriptional version 4 (NM_001136015.3, 1659 bp) to design the primers used for gene amplification. To maximum cover the coding sequence (CDS:161–1180) of ANXA2, we designed five primers using primer3.0 input based on reference to NCBI databases (Table 1). The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. GAPDH was used as the reference gene.

Table 1
Primers of ANXA2 based on transcript 4.

Name	Primer	Start	length	Sequence	Size
Primer 1	F	173	20	cacgaaatcctgtgcaagct	204
	R	376	20	ggcaatatcctgtctctgtgc	
Primer 2	F	250	21	tactaactttgatgctgagcg	291
	R	540	20	gagtctcgtcggttcccag	
Primer 3	F	510	18	ccatgaaggggctgggaa	269
	R	778	20	tttctcttcactccagcgt	
Primer 4	F	746	22	cgggatctctatgacgctggag	247
	R	992	22	tggagtcatacagccgatcagc	
Primer 5	F	945	21	gcattcagaacaagcccctgt	222
	R	1166	19	cacacaggtacagcagcgc	
Note: F: Forward primer; R: Reverse primer.					

Gene amplification

PCR amplification used Taq Master Mix (Novo Protein Company) in a 50-μL reaction system following manufacturer instructions. The denaturation temperature was 95 °C, the annealing temperature of different primers was different (primer 1 was 53 °C, primer 2 was 56 °C, primer 3 was 56 °C, primer 4 was 55 °C, primer 5 was 54 °C), and the extension temperature was 72 °C, the refolding time was 30 s, and the cycle number was 30 times.

PCR product assessment

PCR products were examined by running samples on 1.2% agarose gels, and the electrophoresis was performed at 80–100 V for 10–15 min in 1× TAE buffer. Agarose gels were viewed under Molecular imager® ChemiDoc™ XRS + and analyzed by image Lab™ Software (BIO-RAD, USA) to determine whether the bands were concentrated, clear, bright, and of the expected size.

PCR-SSCP

10 μl PCR products were denatured at 98 °C for 10 min, frozen rapidly for 10 min, then added to the 12% non-denatured acrylamide gel (ddH₂O 39ml, 30% Acr-Bis 51ml, 1 M Tris pH8.8 57ml, 10% Ap 1.5ml, TEMED 0.06ml) to perform electrophoresis in 0.5x TBE solution at 4°C (pre-electrophoresis for 10min under 400 V, then electrophoresis for 16 h under 200 V). Put the gel into Goldenviwer I nucleic acid dye liquid (Beyourtime, diluted with ultrapure water 1:10000) for 30min. Gels were viewed under a Molecular imager® ChemiDoc™ XRS + and analyzed through image Lab™ Software (BIO-RAD, USA).

PCR product sequencing

The amplified products were sent to Sangon Biotech (Shanghai) Company for bidirectional Sanger sequencing using an ABI 3730XL sequencer. The sequences thus obtained were spliced using DNASTAR-Seqman. Overlapping peaks were labeled in the sequence peak map, and then the sequences were blasted in NCBI Nucleotide BLAST blastn[35]. Select results were blasted with transcript 4 of ANXA2 to confirm mutations and exact loci. By comparing the nucleotide sequences with the translated protein database, we can find out whether the mutated gene will lead to a change in the corresponding protein. Next, these sequences were blasted in NCBI Nucleotide BLAST blastx[36]. to identify changes in the proteins according to results blasted with transcript 4 of ANXA2. On the basis of the sequence results obtained, we determined the relationships between mutations and clinicopathological characteristics.

Statistical analysis

SPSS 26 software was used for statistical analysis. The expression of ANXA2 in breast cancer tissues and the adjacent normal tissues was analyzed using a paired *t*-test. The relationships between mutations and the clinicopathological features of patients were analyzed using one-way analysis of variance (ANOVA) and a Chi-square test. Kaplan–Meier and multivariate Cox proportional risk regression model analyses were used to evaluate the prognosis of patients. Mean and standard deviations were used to summarize the data. Differences were deemed to be statistically significant at $P < 0.05$.

Results

ANXA2 mutations were identified in breast cancer patients

112 paired total RNA and PCR products electrophoresis of patients' breast cancer and adjacent normal tissues revealed that bands were suitable for PCR-SSCP and bidirectional Sanger sequencing (Fig. 2A-B). PCR-SSCP showed that mutations existed among samples. Bidirectional Sanger sequencing and nucleotide blast results also identified the presence of ANXA2 mutations in both breast cancer and normal adjacent tissues (Fig. 2C, Fig. 3, Tables 2–3, Additional file1). Among those mutations, the top-four mutations were as follows:

Table 2
ANXA2 mutations in adjacent normal tissues based on transcript 4.

Loci	CBS	Gene	Codon change	AA. change	Occur.	DR
338	r.338a > c	c.(338T > G)	60TTG > GTG	p. (Leu60Val)	1/112	DR 1
350-1	r.350tc > ct	c.(350AG > GA)	64AGC > GAC	p. (Ser64Asp)	1/112	DR 1
375	r.375g > a	c.(375C > T)	72GCC > GTC	p. (Ala72Val)	3/112	DR 1
380	r.380c > a	c.(380G > T)	74GCC > TCC	p. (Ala74Ser)	2/112	DR 1
382	r.382c > t	c.(382G > A)	74GCC > GCA	p. (Ala74=)	1/112	DR 1
487	r.487a > t	c.(487T > A)	109TAT > TAA	p. (Tyr 109Ter)	1/112	DR 2
952	r.952c > g	c.(952G > C)	264CAG > CAC	p. (Gln264His)	1/112	DR 4
					10/112	
CBS: Complementary base by sequencing; AA: amino acid; Occur: occurrence; DR: domain and repeats; ">": changed into; "=": synonymous mutation; Amino acid abbreviation: Ala-Alanine, Asp-Aspartic acid, Gln-Glutamine, His-Histidine, Leu-Leucine, Ser-Serine, Tyr-Tyrosine, Val-Valine. Ter: Stop codon.						

Table 3
ANXA2 mutations in breast cancer tissues based on transcript 4.

Loci	RNA	Gene	Codon change	AA change	Occur.	DR
338	r.338a > c	c.(338T > G)	60TTG > GTG	P. (Leu60Val)	3/112	DR 1
350	r.350tc > ct	c.(350AG > GA)	64AGC > GAC	p. (Ser64Asp)	13/112	DR 1
373	r.373a > t	c.(373T > A)	71ATT > ATA	p. (Ile71=)	2/112	DR 1
375	r.375g > a	c.(375C > T)	72GCC > GTC	p. (Ala72Val)	15/112	DR 1
380	r.380c > a	c.(380G > T)	74GCC > TCC	p. (Ala74Ser)	4/112	DR 1
487	r.487a > t	c.(487T > A)	109TAT > TAA	p. (Tyr109Ter)	16/112	DR 2
504	r.504t > a	c.(504A > T)	115AAA > ATA	p. (Lys115Ile)	3/112	DR 2
	r.504t > g	c.(504A > C)	115AAA > ACA	p. (Lys115Thr)	1/112	
530	r.530c > a	c.(530G > T)	124GAC > GTC	p. (Asp124Try)	4/112	DR 2
531	r.531t > c	c.(531A > G)	124GAC > GGC	p. (Asp124Gly)	2/112	DR 2
693	r.693c > t	c.(693G > A)	178AGA > AAA	p. (Arg178Lys)	14/112	DR 2–3
1071	r.1071c > g	c.(1071G > C)	304AGG > ACG	p. (Arg304Thr)	2/112	DR 4
1083	r.1083t > c	c.(1083A > G)	308AAG > AGG	p. (Lys308Arg)	1/112	DR 4
					80/112	
CBS: Complementary base by sequencing; AA: amino acid; Occur: occurrence; DR: domain and repeats; ">": changed into; "Syn.": synonymous mutation; Amino acid abbreviation: Ala- alanine, Asp-Aspartic acid, Arg-Arginine, Gln-Glutamine, Gly-Glycine, His-Histidine, Ile-Isoleucine, Leu-Leucine, Lys-Lysine, Ser-Serine, Tyr-Tyrosine, Val-Valine. DR 2–3: located between DR2 and DR3.						

c.(693G > A) (r.693c > t, p. (Arg178Lys), also reported as rs1165464246 in NCBI SNP database) appeared 14 times in breast cancer tissues but was not detected in the adjacent normal tissues ($P \leq 0.001$) (Fig. 3A); **c.(487T > A)** (r.487a > t, p. (Tyr109Ter)) mutation is a terminator codon mutation, which was detected 16 times in breast cancer tissues and 1 time in adjacent normal tissues ($\chi^2 = 12.476$, $P \leq 0.001$) (Fig. 3B); **c.(375C > T)** (r.375g > a, p. (Ala72Val)) mutation appeared 15 times in breast cancer tissues and 3 times in adjacent normal tissues ($\chi^2 = 7.310$, $P = 0.007$) (Fig. 3C); **c.(350AG > GA)** (r.350tc > ct, p. (Ser64Asp)) mutation appeared 13 times in breast cancer tissues and 1 time in adjacent normal tissues ($\chi^2 = 9.219$, $P = 0.002$) (Fig. 3D). Based on above data, we inferred that mutations occurred in breast cancer and may be related to the germination of breast cancer.

ANXA2 mutations were different in breast cancer tissues and adjacent normal tissues

Among the 112 patients, 47 patients's breast cancer tissues (45 missense mutations and 2 synonymous mutations) were found to harbor ANXA2 mutations, whereas mutations in adjacent normal tissues were found in 9 patients (8 missense mutations and 1 synonymous mutation) (missense mutation: $\chi^2 = 33.368$, $P < 0.001$) (Fig. 4A, Table 4). Moreover, ANXA2 missense mutations number in breast cancer tissues (78/112) was 8.67 times higher than adjacent normal tissues (9/112). Paired t -test results showed that missense mutations average number in each breast cancer tissue (0.70 ± 0.101) was also higher than corresponding adjacent normal tissues (0.08 ± 0.026) ($t = 7.187$, $P \leq 0.001$) (Fig. 4B). These data implied that ANXA2 missense mutations may be related to the occurrence of breast cancer.

Table 4

The relationship between ANXA2 missense mutations and clinicopathological features.

Clinical features	N	missense mutations in breast cancer tissues		χ ² -value	P-value
		With	Without		
Ages					
≤ 45	76	31(40.79%)	45(59.21%)	0.037	1.000
> 45	36	14(38.88%)	22(61.12%)		
Clinical stage					
I, II	35	9(25.71%)	26(74.29%)	4.431	0.040
III,IV	77	36(46.75%)	41(53.25%)		
Molecular subtype					
Luminal A	57	15(26.32%)	42(73.68%)	14.271	0.002
Luminal B	26	12(46.15%)	14(53.85%)		
HER-2+	12	5(41.67%)	7(58.33%)		
TNBC	17	13(72.73%)	4(27.27%)		
ER					
+	83	27(32.53%)	56(67.47%)	7.802	0.008
-	29	18(62.07%)	11(37.93%)		
PR					
+	57	15(26.32%)	42(73.68%)	9.281	0.004
-	55	30(54.55%)	25(45.45%)		
HER2					
+	28	12(42.86%)	16(57.14%)	0.111	0.825
-	84	33(39.29%)	51(60.71%)		
Ki-67					
≤ 30%	68	23(33.82%)	45(66.18%)	2.908	0.115
> 30%	44	22(50.00%)	22(50.00%)		
Lymph-node metastasis					
Yes	45	27(60.00%)	18(40.00%)		
P-value < 0.05, statistically significant. BC: breast cancer.					

Clinical features	N	missense mutations in breast cancer tissues		χ^2 -value	P-value
		With	Without		
No	67	18(26.87%)	49(73.13%)	12.296	0.001
Tumor size					
≤ 2cm	56	23(41.07%)	33(58.93%)	0.0860	1.000
> 2cm, ≤ 5cm	45	18(40.00%)	27(60.00%)		
> 5cm	11	4(36.36%)	7(63.64%)		
Tissues	112	45(40.18%)	67(59.82%)	33.368	< 0.001
BC tissues	112	9(8.04%)	103(91.96%)		
Adjacent tissues					
P-value < 0.05, statistically significant. BC: breast cancer.					

ANXA2 protein contains four domain and repeats (DRs), and among the four variable transcripts, transcript 1 differs in terms of size from that of the other three transcripts (Fig. 1). In adjacent normal tissues, missense mutations were primarily (7/9) concentrated on DR1 ($\chi^2 = 12.279$, $P = 0.004$). In breast cancer tissues, missense mutations were distributed not only on DR1 (35/78) but also on DR2 (26/78) ($\chi^2 = 12.514$, $P = 0.002$) and others, but the DRs difference between adjacent normal tissues and breast cancer tissues was not significant ($\chi^2 = 3.746$, $P = 0.236$) (Fig. 4C).

However, taking all the missense mutations (including adjacent normal tissues and breast cancer tissues) into consideration, DR1 was with the highest mutation frequency among these 4 groups DRs (42/87) ($\chi^2 = 49.824$, $P < 0.001$), which implied that ANXA2 mutations are more likely to occur in DR1.

The relationship between ANXA2 mutations and clinicopathological features

We found that the 45 patients with distant metastasis had 58 missense mutations, whereas the other 67 patients without metastasis had 20 missense mutations. The observation that each metastatic patient had a greater number of missense mutations (1.289 ± 0.186) than patients without metastasis (0.299 ± 0.080) ($t = 4.428$, $P \leq 0.001$) (Fig. 4D) indicates that the number of mutations in individuals may be associated with breast cancer lymph node metastasis.

The average number of missense mutations in breast cancer tissues at different clinical stage were as follow: stage I ~ II (0.3143 ± 0.1067 , $N = 35$), stage III ~ IV (0.8571 ± 0.1290 , $N = 77$), analysis revealed that the difference between groups was significant ($t = 2.651$, $P = 0.009$) (Fig. 4E), which indicated that missense mutations may be associated with a higher clinical stage.

The numbers of missense mutations in breast cancer tissues at each molecular subtype were also significantly different ($F = 14.271$, $P = 0.002$) (Fig. 4F, Table 4), which implied that patients with TNBC were more likely to occur ANXA2 mutations. The difference of missense mutations also appeared in ER and PR ($P < 0.05$). However, we were unable to detect any obvious correlation between missense mutations and tumor grade and tumor size ($P > 0.05$).

5-year OS and related factors

By the end of 5-years of observation, 77 of 112 patients in this study survived, and the overall survival rate was 68.75% (Fig. 5A). To find the relationship between factors and the 5-year OS of patients, Kaplan-Meier univariate survival analysis was conducted and the results revealed 6 potential influencing factors including clinical stage, molecular subtyping, ER, PR, lymph-node metastasis and missense mutations in breast cancer tissues ($P < 0.05$, Fig. 5B, Table 5). Subsequently, multivariate Cox proportional risk regression model analysis was carried out and results showed that lymph-node metastasis ($P < 0.001$), PR ($P = 0.036$) and missense mutations ($P = 0.009$) in breast cancer tissues may be associated with 5-year OS (Table 6).

Table 5
Kaplan-Meier analysis for 5-year overall survival rate.

Clinical features	N	5-year OS		X ² -value	P-value
		Censored subjects	Events		
Ages					
≤ 45	76	54(71.1%)	22(28.9%)	0.2197	0.6392
> 45	36	23(63.9%)	13(26.1%)		
Clinical stage					
I, II	35	33(94.3%)	2(5.7%)	14.92	0.0001
III,IV	77	44(57.1%)	33(42.9%)		
Molecular subtype					
Luminal A	57	47(82.5%)	10(17.5%)	36.91	< 0.0001
Luminal B	26	16(61.5%)	10(38.5%)		
HER-2+	12	9(75.0%)	3(25.0%)		
TNBC	17	5(29.4%)	12(70.6%)		
ER					
+	83	63(75.9%)	20(24.1%)	14.25	0.0002
-	29	14(48.3%)	15(51.7%)		
PR					
+	57	47(82.5%)	10(17.5%)	12.21	0.0005
-	55	30(54.5%)	25(45.5%)		
HER2					
+	28	20(71.4%)	8(28.6%)	0.1494	0.6991
-	84	57(67.9%)	27(32.1%)		
Lymph-node metastasis					
Yes	45	17(37.8%)	28(62.2%)	40.91	< 0.0001
No	67	60(89.6%)	7(10.4%)		
Tumor size					
≤ 2cm	56	42(75.0%)	14(25.0%)		
BC: breast cancer; P-value < 0.05, statistically significant.					

Clinical features	N	5-year OS		χ^2 -value	P-value
		Censored subjects	Events		
≤2cm, ≤5cm	45	28(62.2%)	17(37.8%)	1.979	0.3718
≤5cm	11	7(63.6%)	4(36.4%)		
Mutations in breast cancer tissues	67	56(83.6%)	11(16.4%)	19.19	< 0.0001
Without	45	21(46.7%)	24(53.3%)		
With					
BC: breast cancer; P-value < 0.05, statistically significant.					

Table 6

Multivariable Cox proportional risk regression model analysis for 5-year overall survival rate.

Factors	B	SE	Wald	df	P-value	Exp(B)	95.0% CI
Lymph node Metastasis	1.932	0.431	20.057	1	< 0.001	6.904	2.964 ~ 16.802
PR	0.795	0.379	4.400	1	0.036	2.215	1.054 ~ 4.658
Missense mutations	-0.974	.373	6.836	1	0.009	0.378	0.182 ~ 0.784
P-value < 0.05, statistically significant.							

45 patients with missense mutations and clinical features.

Kaplan-Meier analysis was performed on 45 patients with different types of mutations in breast cancer tissues (Fig. 5C-F, Table 7), and found that age, clinical stage, molecular subtyping, lymph node metastasis, ER, mutation number, c.(350AG > GA), c.(375G > A) and c.(693G > A), may be linked to 5-year OS ($P < 0.05$). However, statistical significance was not found in the furtherly multivariate Cox proportional risk regression model analysis results ($P > 0.05$) (Table 8).

Table 7
Kaplan-Meier analysis for 5-year OS of 45 patients with missense mutations in breast cancer tissues.

Types of SNPs	N	5-year OS		X ² -value	P-value
		Censored subjects	Events		
Age					
≤ 45	16	5(31.25%)	11(68.75%)	4.797	0.0285
> 45	29	16(55.17%)	13(44.83%)		
Tumor size					
≤ 2cm	23	11(47.83%)	12(52.17%)	0.5459	0.7611
> 2cm, ≤ 5cm	18	9(50.00%)	9(50.00%)		
> 5cm	4	1(25.00%)	3(75.00%)		
Clinical stage					
I, II	9	7(77.78%)	2(22.22%)	4.465	0.0346
III,IV	36	14(38.89%)	22(61.11%)		
Molecular subtype					
Luminal A	15	9(60.00%)	6(40.00%)	12.65	0.0055
Luminal B	12	6(50.00%)	6(50.00%)		
HER-2+	5	3(60.00%)	2(40.00%)		
TNBC	13	3(23.08%)	10(76.92%)		
Lymph-node metastasis					
Yes	27	7(25.93%)	20(74.07%)	14.23	0.0002
No	18	14(77.78%)	4(22.22%)		
ER					
+	27	15(55.56%)	12(44.44%)	6.469	0.0110
-	18	6(33.33%)	12(63.67%)		
PR	15	9(60.00%)	6(40.00%)	2.720	0.0991
+	30	12(40.00%)	18(60.00%)		
-					

P-value < 0.05, statistically significant.

Types of SNPs	N	5-year OS		X ² -value	P-value
		Censored subjects	Events		
HER2	12	7(58.33%)	5(41.67%)		
+					
-	33	14(42.42%)	19(57.58%)	1.002	0.3168
Ki-67					
≤ 30%	23	12(52.17%)	11(47.83%)		
> 30%	22	9(40.91%)	13(59.09%)	0.5679	0.4511
Mutation number					
≤ 2	35	20(57.14%)	15(42.86%)		
> 3	10	1(10.00%)	9(90.00%)	12.87	0.0003
c.(338T > G)	3	3(100.00%)	0(0.00%)	2.574	0.1086
With	42	18(42.86%)	14(57.14%)		
Without					
c.(350TC > CT)					
With	13	2(15.38%)	11(84.62%)		
Without	32	19(76.8%)	13(23.2%)	10.63	0.0011
c.(375G > A)					
With	15	3(20.0%)	12(80.0%)		
Without	30	18(60.0%)	12(40.0%)	8.526	0.0035
c.(380G > T)					
With	4	3(75.00%)	1(25.00%)	1.045	0.3066
Without	41	18(43.90%)	23(56.10%)		
c.(487T > A)					
With	16	6(37.50%)	10(62.50%)		
Without	29	15(51.72%)	14(48.28%)	0.5419	0.4616
c.(504A > T/C)					
With	4	2(50.00%)	2(50.00%)		

P-value < 0.05, statistically significant.

Types of SNPs	N	5-year OS		χ^2 -value	P-value
		Censored subjects	Events		
Without	41	19(46.34%)	22(53.66%)	0.008919	0.9248
c.(530GA > TT/CG)	6	3(50.00%)	3(50.00%)	0.1550	0.6938
With	39	18(46.15%)	21(53.85%)		
Without					
c.(693G > A)	14	3(21.43%)	11(78.57%)	9.658	0.0019
With	31	18(58.06%)	13(41.94%)		
Without					
c.(1071G > C)					
With	2	1(50.00%)	1(50.00%)	0.01249	0.9110
Without	43	20(46.51%)	23(53.49%)		
c.(1083A > G)					
With	1	1(100.0%)	0(0.00%)		
Without	44	20(45.45%)	24(54.55%)	0.8021	0.3705
P-value < 0.05, statistically significant.					

Table 8

Multivariable Cox proportional risk regression model analysis for 5-year overall survival rate of 45 patients with missense mutations in breast cancer tissues.

Factors	B	SE	Wald	df	P-value	Exp(B)	95.0% CI
Lymph node Metastasis	7.151	4.283	2.788	1	0.095	1275.591	0.289 ~ 5637123.637
P-value < 0.05, statistically significant.							

We then analyzed the relationship between the top four high occurrence mutations and clinical features (Table 9–12). Results showed that c.(350AG > GA), c.(375G > A), c.(487T > A) and c.(693G > A) were all likely to occur in younger patients (≤ 45 years) ($P < 0.05$, Table 10–13). Patients with c.(350AG > GA) have higher clinical stage ($P = 0.035$) and lymph-node metastasis ($P = 0.045$) (Table 9). Patients with c.(375G > A) were inclined to be HER2(-) cases ($P < 0.05$) (Table 10), while c.(693G > A) tended to be TNBC cases ($P < 0.05$, Table 12). The characteristics of different mutations may provide potential reference indexes for our future clinical application.

Table 9
c.(350TC > CT) and clinical features in 45 patients with missense mutations.

Clinical features	N	c.(350TC > CT)		χ²	P
		With	Without		
Age					
≤ 45	16	9(56.25%)	7(43.75%)	1.456	0.005
> 45	29	4(13.79%)	25(86.21%)		
Tumor size					
≤ 2cm	23	7(30.43%)	16(69.57%)	1.456	0.567
> 2cm, ≤ 5cm	18	4(22.22%)	14(77.78%)		
> 5cm	4	2(50.00%)	2(50.00%)		
Clinical stage					
I, II	9	0(0.00%)	9(100.00%)	4.465	0.035
III,IV	36	13(36.11%)	23(63.89%)		
Molecular subtype					
Luminal A	15	2(13.33%)	13(86.67%)	4.105	0.248
Luminal B	12	3(25.00%)	9(75.00%)		
HER-2+	5	2(40.00%)	3(60.00%)		
TNBC	13	6(46.15%)	7(53.85%)		
Lymph-node metastasis					
Yes	27	11(40.74%)	16(59.26%)	1.456	0.045
No	18	2(11.11%)	16(88.89%)		
ER					
+	27	5(18.52%)	22(81.48%)	3.534	0.940
-	18	8(44.44%)	10(55.56%)		
PR					
+	15	2(13.33%)	13(86.67%)	1.456	0.165
-	30	11(36.67%)	19(63.33%)		

Only the first four types are shown. P-value < 0.05, statistically significant.

Clinical features	N	c.(350TC > CT)		χ^2	P
		With	Without		
HER2	12	4(33.33%)	8(66.67%)		
+					
-	33	9(27.27%)	24(72.73%)		0.721
Ki-67					
$\leq 30\%$	23	6(26.09%)	17(73.91%)		
$> 30\%$	22	7(31.82%)	15(68.18%)	0.180	0.749
Only the first four types are shown. P-value < 0.05, statistically significant.					

Table 10
c.(375G > A) and clinical features in 45 patients with missense mutations.

Clinical features	N	c.(375G > A)		χ ²	P
		With	Without		
Age					
≤ 45	16	11(68.75%)	5(31.25%)		0.001
> 45	29	4(13.79%)	25(86.21%)		
Tumor size					
≤ 2cm	23	7(30.43%)	16(69.57%)		0.532 0.894
> 2cm, ≤ 5cm	18	7(38.89%)	11(61.11%)		
> 5cm	4	1(25.00%)	3(75.00%)		
Clinical stage					
I, II	9	2(22.22%)	7(77.78%)		0.695
III,IV	36	13(36.11%)	23(63.89%)		
Molecular subtype					
Luminal A	15	3(20.00%)	12(80.00%)		0.104
Luminal B	12	3(25.00%)	9(75.00%)		
HER-2+	5	1(20.00%)	4(80.00%)		
TNBC	13	8(61.54%)	5(38.46%)		
Lymph-node metastasis					
Yes	27	11(40.74%)	16(59.26%)		0.333
No	18	4(22.44%)	14(77.56%)		
ER					
+	27	6(22.22%)	21(77.78%)		0.105
-	18	9(50.00%)	9(50.00%)		
PR	15	3(20.00%)	12(80.00%)		0.315
+	30	12(40.00%)	18(60.00%)		
-					

P-value < 0.05, statistically significant.

Clinical features	N	c.(375G > A)		χ^2	P
		With	Without		
HER2	12	1(8.33%)	11(91.67%)		
+					
-	33	14(42.42%)	19(57.58%)		0.038
Ki-67					
$\leq 30\%$	23	7(30.43%)	16(69.57%)		
$> 30\%$	22	8(36.36%)	14(63.64%)	0.178	0.673
P-value < 0.05, statistically significant.					

Table 11
c.(487T > A) and clinical features in 45 patients with missense mutations.

Clinical features	N	c.(487T > A)		χ²	P
		With	Without		
Age					
≤ 45	16	15(93.75%)	1(6.25%)	2.498	P < 0.001
> 45	29	1(3.45%)	28(96.55%)		
Tumor size					
≤ 2cm	23	10(43.48%)	13(56.52%)	2.498	0.346
> 2cm, ≤ 5cm	18	6(33.33%)	12(66.67%)		
> 5cm	4	0(0.00%)	4(100.00%)		
Clinical stage					
I, II	9	2(22.22%)	7(77.78%)	0.456	
III,IV	36	14(38.89%)	22(61.11%)		
Molecular subtype					
Luminal A	15	4(26.67%)	11(73.33%)	1.891	0.608
Luminal B	12	6(50.00%)	6(50.00%)		
HER-2+	5	2(40.00%)	3(60.00%)		
TNBC	13	4(30.77%)	9(69.23%)		
Lymph-node metastasis					
Yes	27	13(48.15%)	14(51.85%)	0.055	
No	18	3(16.67%)	15(83.33%)		
ER					
+	27	10(37.04%)	17(62.96%)	0.065	1.000
-	18	6(33.33%)	12(66.67%)		
PR	15	4(26.67%)	11(73.33%)	0.514	
+	30	12(40.00%)	18(60.00%)		
-					

P-value < 0.05, statistically significant.

Clinical features	N	c.(487T > A)		χ ²	P
		With	Without		
HER2	12	6(50.00%)	6(50.00%)	1.490	0.296
+					
-	33	10(30.30%)	23(69.70%)	0.538	0.542
Ki-67					
≤ 30%	23	7(30.43%)	16(69.57%)	0.538	0.542
> 30%	22	9(40.91%)	13(59.09%)		
P-value < 0.05, statistically significant.					

Table 12
c.(693G > A) and clinical features in 45 patients with missense mutations.

Clinical features	N	c.(693G > A)		χ²	P
		With	Without		
Age					
≤ 45	16	11(68.75%)	5(31.25%)	1.639	P < 0.001
> 45	29	3(10.34%)	26(89.66%)		
Tumor size					
≤ 2cm	23	8(34.78%)	15(65.22%)	1.639	0.456
> 2cm, ≤ 5cm	18	4(22.22%)	14(77.78%)		
> 5cm	4	2(50.00%)	2(50.00%)		
Clinical stage					
I, II	9	1(11.11%)	8(88.89%)	0.236	
III,IV	36	13(36.11%)	23(63.89%)		
Molecular subtype					
Luminal A	15	2(13.33%)	13(86.67%)	8.615	0.028
Luminal B	12	2(16.67%)	10(83.33%)		
HER-2+	5	2(40.00%)	3(60.00%)		
TNBC	13	8(61.54%)	5(38.46%)		
Lymph-node metastasis					
Yes	27	11(40.74%)	16(59.26%)	0.111	
No	18	3(16.67%)	15(83.33%)		
ER					
+	27	10(37.04%)	17(62.96%)	0.343	
-	18	4(22.22%)	14(77.78%)		
PR					
+	15	2(13.33%)	13(86.67%)	0.094	
-	30	12(40.00%)	18(60.00%)		

P-value < 0.05, statistically significant.

Clinical features	N	c.(693G > A)		χ^2	P
		With	Without		
HER2	12	2(16.67%)	10(83.33%)		
+					
-	33	12(36.36%)	21(63.64%)		0.287
Ki-67					
≤ 30%	23	5(21.74%)	18(78.26%)		
> 30%	22	9(40.91%)	13(59.09%)		0.208
P-value < 0.05, statistically significant.					

Discussion

The ANXA2 gene is located on the long arm of chromosome 15 (15q21-q22), is approximately 40 kb in length, and contains 13 exons and 12 introns. The length of its mRNA is approximately 1665 bp, and the coding region spans base pairs 161 to 1180. The ANXA2 protein comprises 339 amino acids with a molecular weight of 36 kDa, and is accordingly referred to as p36. It is also known by several other names, including Annexin II, P39, calpactin α heavy chain, and Lipocortin α [37]. ANXA2 is a calcium-dependent membrane phospholipid-binding protein, and is one of the most important members of the Annexin family; it is closely associated with the occurrence, development, invasion, and metastasis of many tumors, including breast cancer.

Because of the reduced evolutionary constraints on somatic cells, somatic cell mutations continue to accumulate and eventually lead to tumorigenesis. At the same time, persistent somatic cell evolution promotes genetic heterogeneity in the development of tumors [38–40]. It has been reported that many gene mutations are associated with breast cancer, including the polymorphisms of rs2046210 and rs3803662, single-nucleotide polymorphisms (SNPs) of the FGFR1 gene, the SNP of rs10800708 within the KIF14 miRNA, and SNPs of the CD44 gene [41–44]. However, whether ANXA2 mutation is associated with breast cancer has not been reported up to now.

In the present study, we identified ANXA2 mutations in breast cancer patients.

Breast cancer tissues exhibited more mutations (80/112) than adjacent normal tissues (10/112) ($P < 0.05$). Moreover, most mutations (8/10) in adjacent normal tissues were found in breast cancer tissues, few mutations (2/10) in adjacent normal tissues were different from the breast cancer tissues. The presence of mutations in normal tissues may be due to sequencing artifacts, presence of tumor cells in the normal bulk or preneoplastic evolution of the tumor-adjacent tissue. However, all the mutations of ANXA2 in adjacent normal tissues still need further study and confirmation.

We detected a high incidence of missense mutation in the tumor samples (40.18%) and 8.04% of normal tissues carrying a mutation, which was higher than known data: a combined analysis of 6995 breast cancer patients selected in 19 studies in cbiportal hub[45] (<https://www.cbiportal.org/>) showed that ANXA2 mutation incidence was only 0.7% (less than 5%). The difference may lie in: Firstly, the limitation of samples in this paper involved; Secondly, data in the cbiportal hub were based on DNA level, while our results were based on mRNA level. The function of mRNA is active, which is related to the on / off of promoter, RNA degradation and post transcriptional silencing, etc. Because of this, it is more likely and easier to be changed than DNA in the state of breast cancer. At the same time, mRNA can also better reflect the molecular changes caused by breast cancer.

Previous reports mainly focused on the relationship between expression of ANXA2 and cancer genesis and prognosis of patients, and found that the high expression of ANXA2 was associated with the occurrence and development of tumors, distant metastasis, higher clinical stages, and poor outcomes and prognosis [4–16]. While, our results showed that ANXA2 mutations may be associated with the occurrence, molecular subtype, clinical stage, ER, PR and lymph node metastasis of breast cancer patients ($P < 0.05$) (Tables 4–5). The consistency of the results suggested that the high expression of ANXA2 may be associated with dysfunction or abnormality caused by mutations. However, this still needs further verification.

Because of the high heterogeneity and lack of typical receptors (ER, PR, HER-2+) and effective targeted therapies, TNBC is often considered to be synonymous with an increased risk of relapse and shorter survival time[46]. Pearson Chi-Square results showed that TNBC patients with higher rate of ANXA2 mutations (16/22: 16 patients occurred ANXA2 mutations in 22 TNBC patients) than Luminal A (7/32), Luminal B (10/34) and HER-2+ (10/22), and the difference was significant ($\chi^2 = 16.149$, $P = 0.001$). Results of LSD-t showed that ANXA2 mutations frequency in TNBC (37/22: 37 mutations in 22 patients) was significantly different from three other groups ($P < 0.05$). These data implied that patients with TNBC molecular subtype were more likely to occur ANXA2 mutations, ANXA2 mutations may be potential diagnostic and therapeutic targets for TNBC.

In this study, Kaplan-Meier univariate survival analysis for 5-year OS results showed that c.(350TC > CT), c.(375G > A) and c.(693C > T) were meaningful ($\chi^2 = 7.499$, $P = 0.006$) (Fig. 5C-F, Table 8), though significant mutation and factor was not identified in multivariate Cox proportional risk regression model analysis for 5-year OS, (Tables 5–7), it still provided a reference for future early diagnoses and prognoses evaluation of breast cancer, and may be potential target for gene therapy of breast cancer.

Owing to an insufficient number of samples, we were unable to fully elucidate other mutations, and further investigations are necessary to determine whether there are false-positive and currently unidentified mutations. In this study, we only examined the mutation of ANXA2 coding area, but not in its non-coding area. Secondly, we only chose invasive ductal carcinoma as the research object, which does not reflect other types of breast cancer. Finally, the follow-up time was short, which can only reflect the

survival rate within 5 years. Whether there is a difference between ANXA2 mutation and non-mutation patients after 5 years cannot be predicted.

Conclusions

ANXA2 missense mutations (mainly on DR1) occurred in breast cancer, which may be associated with pathogenesis, higher clinical stage, molecular subtyping, lymph node metastasis and 5-year OS of breast cancer. c.(350AG > GA), c.(375G > A), c.(487T > A) and c.(693G > A) were associated with younger patients. c.(350AG > GA) was related to higher clinical stage and lymph-node metastasis. c.(375G > A) was linked to HER2(-) cases, while c.(693G > A) tended to be TNBC cases. These mutations may contribute to the early screening, diagnosis, and targeted treatment of breast cancer.

Abbreviations

Single-nucleotide polymorphism (SNP)

Domain and repeats (DRs)

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of North Sichuan Medical College and informed consent was obtained from each patient.

Consent for publication

All authors consent for the publication of the manuscript.

Availability of data and materials

The data and materials used to support the findings of this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Author's contributions

Shishan Deng and Lingmi Hou conceived of and designed this paper; Xiaoming Zhang and Mei Zeng provided a lot of administrative support; Jinsui Li, Lulan Pu, Mingfei Xu and Fangfang Zhou collected samples and managed the clinical data of patients; Jiayou Liu, Shaoli Xie, Linlong Mo, Jingjia Li, and Yi Yang performed the RT-PCR and SSCP-PCR; Jiayou Liu, Shaoli Xie, Linlong Mo, Qian Yi, Xinxin Rong conducted the data analysis and interpretation. All authors participated in the manuscript writing and final approval.

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Figures

Figure 1

Domain and repeats of the different transcripts of ANXA2. A. Domain and repeats of transcript 1 of ANXA2. DR1: domain and repeats 1, from amino acid 55 to 120; DR2: domain and repeats 2, from amino acid 127 to 192; DR3: domain and repeats 3, from amino acid 211 to 277; DR4: domain and repeats 4, from amino acid 287 to 352. B. Domain and repeats of transcript 2, 3, and 4 of ANXA2. DR1: domain and repeats 1, from amino acid 37 to 102; DR2: domain and repeats 2, from amino acid 109 to 174; DR3: domain and repeats 3, from amino acid 193 to 259; DR4: domain and repeats 4, from amino acid 269 to 334.

Figure 2

Electrophoresis results (partial). A. Electrophoresis results for total RNA. 1N: adjacent tissue of patient 1; 1T: breast cancer tissue of patient 1; 2N: adjacent tissue of patient 2; 2T: breast cancer tissue of patient 2; 3N: adjacent tissue of patient 3; 3T: breast cancer tissue of patient 3. B. Electrophoresis results for PCR products. M: marker; 1TA: ANXA2 expressed in breast cancer tissue of patient 1; 1TG: GAPDH expressed in breast cancer tissue of patient 1; 2TA: ANXA2 expressed in breast cancer tissue of patient 2; 2TG: GAPDH expressed in breast cancer tissue of patient 2; 3TA: ANXA2 expressed in breast cancer tissue of patient 3; 3TG: GAPDH expressed in breast cancer tissue of patient 3; 4TA: ANXA2 expressed in breast cancer tissue of patient 4; 4TG: GAPDH expressed in breast cancer tissue of patient 4. C. PCR-SSCP results. M: DNA marker. 1-13 represents the serial number of the specimen. T: breast cancer tissues, N: adjacent normal tissues. 1-3 were the products of primer 1, 4-8 were the products of primer 2, 9-13 were the products of primer 3.

Figure 3

Peak maps of bidirectional Sanger sequencing results (main mutations). The vertical arrows indicate the sites of mutation, whereas the horizontal arrows indicate the reverse and forward sequence: The horizontal red arrows pointing to the left indicate reverse sequencing results, whereas the horizontal green arrows pointing to the right indicate forward sequencing results. A. The 693rd base C in the mRNA changed to T, according to base-pair complementation, in DNA, the 693rd base was G and changed to A,

and the 178th amino acid Arg changed to Lys. B. The 487th base A in the mRNA changed to T, in DNA, the 487th base was T and changed to A, leading to the 109th amino acid Try changing to a terminator codon (Stop). C. The 380th base C in mRNA changed to A, according to base-pair complementation, in DNA, the 380th base was G and changed to T, and the 74th amino acid Ala changed to Ser. The 375th base G in mRNA changed to A, in DNA, the 375th base was C and changed to T, and the 72nd amino acid Ala changed to Val. D. The 350th–351st bases TC in mRNA changed to CT, in DNA, the 350th–351st bases were AG and mutated to GA, and the 64th amino acid Ser changed to Asp.

Figure 4

ANXA2 mutations and their relationship with clinical characters. N: Adjacent normal tissues; T: Breast cancer tissues. A. Histogram of mutations in patients' breast cancer tissues and adjacent normal tissues. B. Histogram of missense mutations and synonymous mutations in adjacent normal tissues and breast cancer tissues. C. Scatter plot of mutations in domain and repeats of ANXA2. D. Histogram and scatter plot of missense mutations and metastasis. E. Histogram and scatter plot of missense mutations and clinical stages. Stage IV was significantly different from other stages. F. Histogram and scatter plot of missense mutations and molecular subtypes.

Figure 5

Independent association of ANXA2 mutations with patients' 5-year overall survival. A. Total survival curve of 112 patients. B. Kaplan–Meier curves (log-rank) for patients with ANXA2 mutations versus without from breast cancer tissues for overall survival. C. Kaplan–Meier curves (log-rank) of overall survival rate between patients with ANXA2 c. (350AG>GA) mutation and patients with ANXA2 non- c. (350AG>GA) mutation in 45 mutated breast cancer tissues. D. Kaplan–Meier curves (log-rank) of overall survival rate between patients with ANXA2 c.(375C>T) mutation and patients with ANXA2 non- c.(375C>T) mutation in 45 mutated breast cancer tissues. E. Kaplan–Meier curves (log-rank) of overall survival rate between patients with ANXA2 c.(487T>A) mutation and patients with ANXA2 non- c.(487T>A) mutation in 45 mutated breast cancer tissues. F. Kaplan–Meier curves (log-rank) of overall survival rate between patients with ANXA2 c.(693G>A) mutation and patients with ANXA2 non- c.(693G>A) mutation in 45 mutated breast cancer tissues.

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