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RESEARCH ARTICLE

The diplotype *Fas* −1377A/−670G as a genetic marker to predict a lower risk of breast cancer in Chinese women

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Abstract This study was designed to reveal the effects of *Fas* and *FasL* polymorphisms of interest on breast cancer risk. A total of 439 patients with breast cancer and 439 controls were enrolled in this study. The genotypes *Fas* −1377G/A, *Fas* −670A/G, and *FasL* −844 T/C were detected by MassARRAY. The protein expressions of estrogen receptor, progesterone receptor, and CerbB-2 were determined by immunohistochemistry. Among the 439 patients, *Fas* mRNA levels in 22 samples of breast cancer and adjacent normal tissues were detected by real-time polymerase chain reaction, and the soluble Fas and Fas ligand concentrations of 180 patients were measured by enzyme-linked immunosorbent assay. The *Fas* −1377GA, *Fas* −1377AA, *Fas* −670AG, *Fas* −670GG, and *FasL* −844TC genotypes were associated with a reduced risk of breast cancer. Haplotype analysis indicated that *Fas* −1377G/−670A was associated with an increased risk of breast cancer, whereas *Fas* −1377A/−670A was associated with the opposite effect. Furthermore, gene–gene interaction analysis revealed that the *Fas* −1377GA/AA (−670AG/GG) and *FasL* −844CC or TC/TT genotypes were associated with a decreased risk of breast cancer. Meanwhile, −1377GG and −670AA genotypes were associated with higher soluble Fas concentrations than other genotypes. We conclude that *Fas* and *FasL* polymorphisms can affect breast cancer risk and that *Fas* polymorphisms are likely to affect breast cancer risk by regulating the soluble Fas concentration.

Keywords *Fas* · *FasL* · Polymorphism · Breast cancer

Introduction

Breast cancer is one of the most common cancers among women, accounting for 23 % of the total cancer burden and 14 % of cancer deaths worldwide [1]. It has become a great threat to public health and a major burden on the global economy. Therefore, new diagnostic markers are urgently needed for the early detection and prevention of breast cancer. A large number of studies have demonstrated that the pathogenesis of various tumors, including breast cancer, is associated not only with unlimited proliferation but also with the suppression of apoptosis [2]. Therefore, an in-depth and complete exploration of the abnormal proliferation and apoptosis associated with the pathogenesis of cancer may be of interest.

Apoptosis, a complex process in which cells neatly commit suicide, plays a critical role in the development and maintenance of homeostasis, and the elimination of malignant cells [3]. Accumulating evidence suggests that aberrant regulation of apoptosis can result in unchecked cell growth and proliferation during carcinogenesis and that malignant cells possess the ability to resist apoptotic stimuli [4, 5]. Fas and Fas ligand (FasL) are critical components of apoptosis and have special status in the apoptotic process. Moreover, they are involved in

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immune escape, by which malignant cells can protect themselves from attack by immune cells [6]. Fas is an apoptosis-signaling transmembrane receptor that belongs to the tumor necrosis factor receptor superfamily [5]. Upon ligation of its natural ligand, FasL, Fas triggers molecular interactions, resulting in initiation of a proteolytic cascade and eventually apoptosis [7]. Previous studies have suggested that decreased Fas or increased FasL expression may facilitate the development and progression of tumors by reducing tumor cell apoptosis or inducing immune cell apoptosis [8, 9].

The *Fas* and *FasL* genes are localized at chromosome 10q24.1 and 1q23, consisting of nine and four exons, respectively. Polymorphisms in the *Fas* and *FasL* genes have been linked to their differential expression. Thus, we hypothesized that polymorphisms in *Fas* and *FasL* could contribute to the variability in individual susceptibility to breast cancer. The most extensively investigated polymorphisms are −670A>G (rs1800682) and −1377G>A (rs2234767) in the promoter region of *Fas*, and −844C>T (rs763110) in the promoter region of *FasL* [10–12] (Fig. 1). Previous studies have investigated these three polymorphisms in the etiology of breast cancer [12, 2, 13, 11, 10, 14]. However, the association between *Fas* and *FasL* polymorphisms and breast cancer risk has not been conclusively established. Moreover, circulating soluble Fas (sFas), another form of Fas that has five variants because of alternative messenger RNA (mRNA) splicing, can inhibit Fas-mediated apoptosis by neutralizing FasL or anti-Fas antibodies [15–17]. Furthermore, increased concentrations of serum sFas have been observed in patients with breast cancer [18, 19]. On the other hand, sFasL, released from the cell surface by cleavage of matrix metalloproteinase-like enzyme, has been reported to have the opposite function to membrane-bound FasL and is involved in anti-inflammatory processes [20].

In order to investigate the effects of *Fas* and *FasL* polymorphisms on breast cancer risk among the Chinese population, we conducted a population-based case-control study with 439 breast cancer patients and 439 healthy, age-matched controls. In addition, to assess the risk of potential functional polymorphisms in *Fas* for breast cancer, 22 tumor tissues and adjacent normal tissues were collected to measure differences in *Fas* expression. Furthermore, sFas and sFasL concentrations were measured in 180 breast cancer patients to elucidate the associations between sFas and sFasL expression levels and breast cancer risk. Finally, the clinical characteristics of patients' tumors, such as estrogen receptor (ER), progesterone receptor (PR), and CerbB-2 status, were detected by immunohistochemistry (IHC).

Materials and methods

Study population

A total of 439 female patients with histologically confirmed and classified breast cancer (according to the World Health Organization classification) were recruited from January 2010 to December 2012 at Nanjing First hospital, Nanjing Medical University, China, after exclusion of their history of cancer,

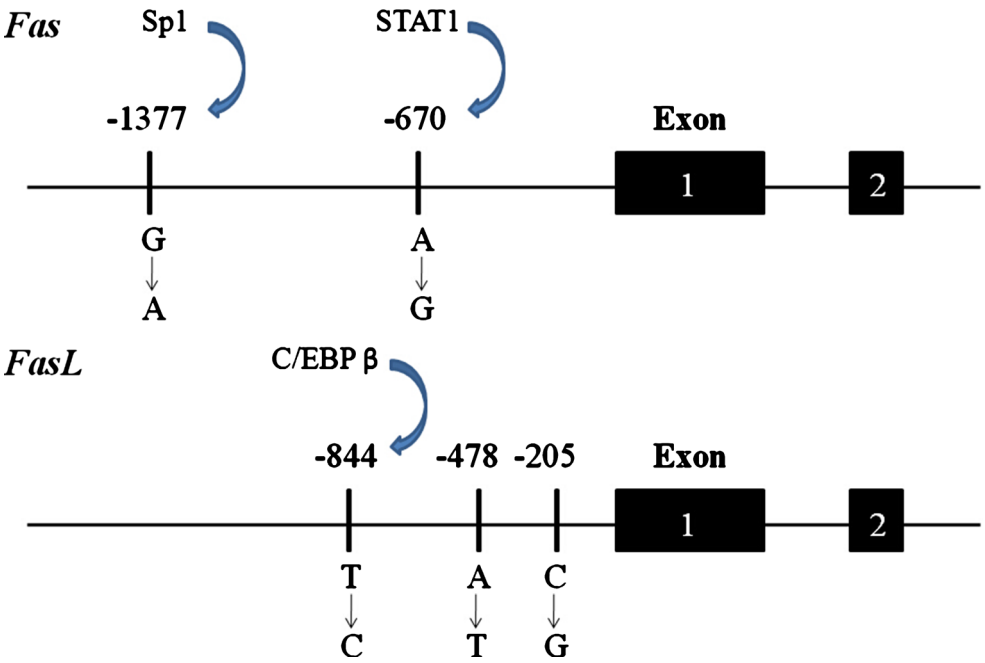

Fig. 1 The mode chart of the human *Fas* and *FasL* polymorphism positions indicated

chemotherapy, or radiotherapy. A cohort of 439 healthy, age-matched women who visited the same hospital for a routine physical examination were enrolled as controls. All subjects were unrelated, of Han ethnicity, and residents of Nanjing and its suburbs. Upon receipt of written informed consent (before the initiation of any study-related procedure), each subject was interviewed to collect additional information about breast cancer risk factors. Meanwhile, three indexes (ER, PR, and CerbB-2) were detected using IHC. The protocol was approved by the ethics committees of Nanjing First Hospital, Nanjing Medical University, China.

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood samples using the GoldMag-Mini Whole Blood Genomic DNA Purification Kit (GoldMag, Xi'an, China) according to the manufacture's protocol, and DNA concentrations were measured using spectrometry (DU 530 UV/Vis spectrophotometer, Beckman Instruments, Fullerton, CA, USA). Genotyping was determined using the Sequenom MassARRAY RS-1000 (Sequenom, San Diego, CA, USA) according to the standard protocol. Sequenom Typer 4.0 software was used for data management and analysis [21, 22].

Quantitative real-time PCR assay

Fas mRNA levels were measured in 22 breast cancer and adjacent normal tissue sample pairs. The total RNA was extracted from the tissues using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol, followed by RNA quantification with spectrophotometry. Complementary DNA (cDNA) synthesis from total RNA was carried out in a final volume of 20 μL using a cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer's protocol. Expression of *Fas* and the housekeeping *GAPDH* gene was measured using quantitative real-time PCR (qRT-PCR). Samples were assayed in a 20-μL reaction mixture including 2 μL cDNA, 10 μL SYBR Premix Ex Taq II (TaKaRa, Dalian, China), 0.8 μL of each specific primer (10 μM), 0.4 μL ROX reference dye, and 6 μL RNase-free water. The qRT-PCR reactions were performed using the ABI 7500 System (Applied Biosystems, Foster, CA, USA) with an initial incubation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. The primers for qRT-PCR of *Fas* (forward: 5′-TGA AGG ACA TGG CTT AGA AGT G-3′; reverse: 5′-GGT GCA AGG GTC ACA GTG TT-3′) and *GAPDH* (forward: 5′-GTC AAC GGA TTT GGT CTG TAT T-3′; reverse: 5′-AGT CTT CTG GGT GGC AGT GAT-3′) were used. To

Table 1 Clinical features of breast cancer patients and healthy controls

Characteristic	Patients	Controls	*P* value
Sample size, *n*	439	439	–
Age (years)			
Range	29–87	29–87	–
Age at diagnosis (mean±SD)	52.89±10.78	52.95±10.89	0.933
Number of births			
0	2 (0.5)	–	–
1	228 (51.9)	–	–
2–5	209 (47.6)	–	–
Menopausal status			
Premenopausal	198 (45.1)	–	–
Postmenopausal	241 (54.9)	–	–
Tumor size			
T1	85 (19.4)	–	–
T2	232 (52.8)	–	–
T3	114 (26.0)	–	–
T4	8 (1.8)	–	–
Stage			
0	2 (0.5)	–	–
I	36 (8.2)	–	–
II	268 (61.0)	–	–
III	80 (18.2)	–	–
IV	53 (12.1)	–	–
Lymph node involvement			
Negative	211 (48.1)	–	–
Positive	228 (51.9)	–	–
Estrogen receptor			
Negative	166 (37.8)	–	–
Positive	273 (62.2)	–	–
Progesterone receptor			
Negative	205 (46.7)	–	–
Positive	234 (53.3)	–	–
CerbB-2			
Negative	92 (21.0)	–	–
Positive	347 (79.0)	–	–
Family history of cancer			
No	424 (96.6)	–	–
Yes	15 (3.4)	–	–
Age at menarche			
Mean (year)	14.60	–	–
SD	1.12	–	–
BMI			
Mean (kg/m^2)	24.52	–	–
SD	4.50	–	–

Data are *n* (%)

SD standard deviation, *BMI* body mass index

Table 2 Frequency distribution of *Fas* and *FasL* polymorphisms and their association with breast cancer risk in breast cancer patients and healthy controls

Genotype	Patients, *n* (%)	Controls, *n* (%)	OR (95 % CI)[a]	*P* value
Fas				
−1377G>A				
GG	168 (38.27)	99 (22.55)	1.00	<0.0001
GA	202 (46.01)	227 (51.71)	**0.52 (0.38–0.72)**	<0.0001
AA	69 (15.72)	113 (25.74)	**0.36 (0.24–0.53)**	<0.0001
GA/AA	271 (84.28)	370 (74.49)	**0.47 (0.35–0.63)**	<0.0001
Fas				
−670A>G				
AA	183 (41.69)	127 (28.93)	1.00	<0.0001
AG	205 (46.70)	210 (47.84)	**0.68 (0.50–0.91)**	0.0102
GG	51 (11.62)	102 (23.23)	**0.34 (0.23–0.52)**	<0.0001
AG/GG	256 (58.31)	321 (73.12)	**0.57 (0.43–0.75)**	<0.0001
FasL				
−844 T>C				
CC	258 (58.77)	206 (46.92)	1.00	0.0180
TC	153 (34.85)	201 (45.79)	**0.61 (0.46–0.81)**	0.0005
TT	28 (6.38)	32 (7.29)	0.70 (0.41–1.21)	0.1920
TC/TT	181 (41.23)	225 (51.25)	**0.62 (0.47–0.81)**	0.0004

Significant results are in bold

OR odds ratio, *CI* confidence interval

[a] Adjusted by age

ensure the reproducibility of the results, all measurements were conducted in triplicate.

Enzyme-linked immunosorbent assay

Serum samples were extracted from whole blood by centrifuging at 3,000 rpm for 10 min and stored at −20 °C until use. The concentrations of sFas and sFasL in serum were determined by ELISA using the sFas and sFasL kits (USCN, Wuhan, China). In brief, each serum sample was diluted and added to the microtiter wells, which were precoated with monoclonal antibodies specific for Fas and FasL, respectively, followed by additional incubation with enzyme-linked polyclonal antibodies specific for Fas and FasL. Color development was then produced by adding tetramethylbenzidine substrate solutions and stopped using sulfuric acid. Finally, absorbance was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The concentration analysis of sFas and sFasL was conducted using standard curves. The sensitivities of the sFas and sFasL assays were 12 pg/mL and 0.038 ng/mL, respectively.

Immunohistochemistry assay

An IHC assay was used to assess ER, PR, and CerbB-2 expression in paraffin-embedded tumor tissue. The IHC procedure has been described elsewhere [23]. In brief, 3-μm tissue sections taken from paraffin blocks were placed on adhesive-coated slides and incubated overnight at 37 °C. After deparaffinization by xylene, the slides were passed through graded alcohols for 15 min and rinsed in distilled water. The slides were then placed in citrate buffer (pH 6.0) and in a microwave oven for 20 min to retrieve antigen. Incubation with 3 % hydrogen peroxide for 10 min can block endogenous peroxidase activity. The three primary antibodies used in this procedure were polyclonal rabbit antihuman CerbB-2 oncoprotein (Dako, Copenhagen, Denmark; 1:10 dilution, 90 min), monoclonal rabbit ER antibody (Spring Bioscience, Pleasanton, CA, USA; 1:100 dilution, 90 min), and monoclonal mouse antihuman PR antibody (Spring Bioscience; 1:100 dilution, 90 min). The secondary antibodies were applied for 30 min with primary antibody enhancer (Spring Bioscience) for 30 min. The slides were then incubated for 30 min with polyvalent HRP polymer (Spring Bioscience). AEC chromogen system was used to slides for 15 min (Spring Bioscience). The results were recorded as the percentage of positively stained target cells, with positivity defined as samples with more than 10 % stained neoplastic cell nuclei.

Statistical analysis

The allele frequencies of cases and controls were analyzed using the χ^2 test. Odds ratios (ORs) and 95 % confidence intervals (CIs) were calculated using logistic regression

Table 3 Genotype frequencies of *Fas* (−1377G>A, −670A>G) and *FasL* (−844 T>C) polymorphisms among patients and their associations with breast cancer risk in relation to patient characteristics and clinical features of breast cancer severity

Genotype	Patients, *n*		OR (95 % CI)[a]	*P* value
Birth status	≤1	≤2		
Fas −1377G>A				
GG	92 (40)	51 (35.42)	1.00	0.5818
GA	101 (43.91)	71 (49.31)	0.73 (0.43–1.25)	0.3088
AA	37 (16.09)	22 (15.28)	0.89 (0.43–1.85)	0.8271
GA/AA	138 (60.00)	93 (64.59)	0.77 (0.47–1.27)	0.3750
Fas −670A>G				
AA	93 (40.93)	61 (42.36)	1.00	0.8785
AG	110 (47.83)	65 (45.14)	1.16 (0.70–1.94)	0.6460
GG	27 (11.74)	18 (12.50)	0.80 (0.36–1.78)	0.9625
AG/GG	137 (59.57)	83 (57.64)	1.09 (0.67–1.77)	0.7126
FasL −844 T>C				
CC	140 (60.87)	89 (61.87)	1.00	0.9757
TC	76 (33.04)	46 (31.94)	0.94 (0.56–1.59)	0.8319
TT	14 (6.09)	9 (6.25)	0.90 (0.31–2.55)	0.9801
TC/TT	90 (39.13)	55 (38.19)	0.93 (0.57–1.54)	0.8567
Birth status	≤1	>2		
Fas −1377G>A				
GG	92 (40.00)	25 (38.46)	1.00	0.9497
GA	101 (43.91)	30 (46.15)	0.99 (0.35–2.77)	0.7717
AA	37 (16.09)	10 (15.38)	1.16 (0.33–4.11)	0.9898
GA/AA	138 (60.00)	40 (61.54)	1.03 (0.42–2.55)	0.8290
Fas −670A>G				
AA	93 (40.93)	29 (44.62)	1.00	0.7676
AG	110 (47.83)	30 (46.15)	1.24 (0.50–3.06)	0.6509
GG	27 (11.74)	6 (9.23)	1.42 (0.21–9.82)	0.4973
AG/GG	137 (59.57	36 (55.38)	1.27 (0.52–3.13)	0.5459
FasL −844 T>C				
CC	140 (60.87)	29 (44.62)	1.00	0.0627
TC	76 (33.04)	31 (47.69)	0.61 (0.24–1.54)	**0.0216**
TT	14 (6.09)	5 (7.69)	0.29 (0.05–1.65)	0.3303
TC/TT	90 (39.13)	36 (55.38)	0.54 (0.22–1.33)	**0.0204**
Menopausal status	Premenopausal	Postmenopausal		
Fas −1377G>A				
GG	76 (38.38)	92 (38.17)	1.00	0.2503
GA	85 (42.93)	117 (48.55)	0.75 (0.33–1.69)	0.5418
AA	37 (18.69)	32 (13.28)	1.65 (0.56–4.87)	0.2412
GA/AA	122 (61.62)	149 (61.83)	0.93 (0.44–1.96)	0.9641
Fas −670A>G				
AA	82 (41.41)	101 (41.91)	1.00	0.1788
AG	87 (43.94)	118 (48.96)	0.54 (0.23–1.24)	0.6384
GG	29 (14.65)	22 (9.13)	2.22 (0.72–6.85)	0.1292
AG/GG	116 (58.59)	140 (58.09)	0.79 (0.37–1.67)	0.9167
FasL −844 T>C				
CC	112 (56.57)	146 (60.58)	1.00	0.6911

Table 3 (continued)

Genotype	Patients, *n*		OR (95 % CI)[a]	*P* value
Birth status	≤1	≤2		
TC	73 (36.87)	80 (33.20)	2.07 (0.90–4.78)	0.3970
TT	13 (6.57)	15 (6.22)	1.47 (0.26–8.27)	0.7599
TC/TT	86 (43.43)	95 (39.42)	1.97 (0.89–4.35)	0.3952
Tumor size	T1–T2	T3–T4		
Fas −1377G>A				
GG	117 (36.91)	51 (41.87)	1.00	0.1170
GA	155 (48.90)	47 (38.52)	1.44 (0.91–2.29)	0.1247
AA	45 (14.20)	24 (19.67)	0.82 (0.45–1.49)	0.5061
GA/AA	200 (63.09)	71 (58.20)	1.23 (0.80–1.89)	0.3449
Fas −670A>G				
AA	127 (40.06)	56 (45.90)	1.00	0.1283
AG	157 (49.53)	48 (39.34)	1.44 (0.92–2.26)	0.1115
GG	33 (10.41)	18 (14.75)	0.80 (0.42–1.55)	0.5243
AG/GG	190 (59.94)	66 (54.10)	1.26 (0.83–1.93)	0.2668
FasL −844 T>C				
CC	190 (59.94)	68 (55.74)	1.00	0.5984
TC	106 (33.44)	47 (38.52)	0.82 (0.53–1.28)	0.3413
TT	21 (6.62)	7 (5.74)	1.08 (0.44–2.64)	0.8768
TC/TT	127 (40.06)	54 (44.26)	0.85 (0.56–1.30)	0.4235
Stage	0~I	II		
Fas −1377G>A				
GG	24 (63.16)	162 (60.45)	1.00	0.4415
GA	10 (26.32)	90 (33.58)	0.73 (0.33–1.61)	0.4707
AA	4 (10.53)	16 (5.97)	1.74 (0.54–5.69)	0.3834
GA/AA	14 (36.84)	106 (39.55)	0.87 (0.43–1.77)	0.7489
Fas −670A>G				
AA	17 (44.74)	102 (38.06)	1.00	0.1167
AG	14 (36.84)	140 (52.24)	0.60 (0.28–1.27)	0.1830
GG	7 (18.42)	26 (9.70)	1.61 (0.60–4.29)	0.3374
AG/GG	21 (55.26)	166 (61.94)	0.76 (0.38–1.51)	0.4304
FasL −844 T>C				
CC	13 (34.21)	101 (37.69)	1.00	0.3865
TC	16 (42.11)	127 (47.39)	0.98 (0.45–2.13)	0.9569
TT	9 (23.68)	40 (14.93)	1.75 (0.69–4.41)	0.2369
TC/TT	25 (65.79)	167 (62.31)	1.16 (0.57–2.36)	0.6785
Stage	0~I	III~IV		
Fas −1377G>A				
GG	24 (63.16)	72 (54.14)	1.00	0.2522
GA	10 (26.31)	53 (39.85)	0.53 (0.23–1.23)	0.1730
AA	4 (10.53)	8 (6.01)	1.49 (0.41–5.41)	0.5365
GA/AA	14 (36.84)	61 (45.86)	0.66 (0.31–1.40)	0.3244
Fas −670A>G				
AA	17 (44.74)	64 (48.12)	1.00	0.7513
AG	14 (36.84)	51 (38.35)	1.04 (0.47–2.30)	0.9355
GG	7 (18.42)	18 (13.53)	1.48 (0.53–4.12)	0.4654

Table 3 (continued)

Genotype	Patients, *n*		OR (95 % CI)[a]	*P* value
Birth status	≤1	≤2		
AG/GG	21 (55.26)	69 (51.88)	1.14 (0.55–2.36)	0.7127
FasL −844 T>C				
CC	13 (34.21)	54 (40.60)	1.00	0.4393
TC	16 (42.11)	59 (44.36)	1.12 (0.49–2.15)	0.7758
TT	9 (23.68)	20 (15.04)	1.92 (0.71–5.21)	0.2168
TC/TT	25 (65.79)	79 (59.40)	1.29 (0.61–2.76)	0.4774
Lymph node involvement	Negative	Positive		
Fas −1377G>A				
GG	80 (37.91)	88 (38.60)	1.00	0.6383
GA	101 (47.87)	101 (44.30)	1.10 (0.73–1.66)	0.6484
AA	30 (14.22)	39 (17.10)	0.85 (0.48–1.49)	0.5616
GA/AA	131 (62.09)	140 (61.40)	1.03 (0.70–1.51)	0.8833
Fas −670A>G				
AA	87 (41.23)	96 (42.11)	1.00	0.6869
AG	102 (48.34)	103 (45.17)	1.09 (0.73–1.62)	0.6631
GG	22 (10.43)	29 (12.72)	0.84 (0.45–1.56)	0.5774
AG/GG	124 (58.77)	132 (57.89)	1.03 (0.71–1.51)	0.8530
FasL −844 T>C				
CC	119 (56.40)	139 (60.96)	1.00	0.1877
TC	74 (35.07)	79 (34.65)	1.11 (0.74–1.65)	0.6596
TT	18 (8.53)	10 (4.39)	2.10 (0.93–4.73)	0.0725
TC/TT	92 (43.60)	89 (39.04)	1.22 (0.83–1.78)	0.3317
Estrogen receptor	Negative	Positive		
Fas −1377G>A				
GG	96 (57.83)	162 (59.34)	1.00	0.1996
GA	55 (33.13)	98 (35.90)	0.94 (0.62–1.42)	0.7980
AA	15 (9.04)	13 (4.76)	1.94 (0.88–4.26)	0.0959
GA/AA	70 (42.17)	111 (40.66)	1.06 (0.71–1.56)	0.7551
Fas −670A>G				
AA	71 (42.77)	112 (41.03)	1.00	0.4202
AG	80 (48.19)	125 (45.79)	1.01 (0.67–1.53)	0.9626
GG	15 (9.04)	36 (13.19)	0.66 (0.34–1.29)	0.2208
AG/GG	95 (57.23)	161 (58.97)	0.93 (0.63–1.38)	0.7187
FasL −844 T>C				
CC	68 (40.96)	100 (36.63)	1.00	0.5667
TC	75 (45.18)	127 (46.52)	0.87 (0.57–1.32)	0.5104
TT	23 (13.86)	46 (16.85)	0.74 (0.41–1.32)	0.3052
TC/TT	98 (59.04)	173 (63.37)	0.83 (0.56–1.24)	0.3652
Progesterone receptor	Negative	Positive		
Fas −1377G>A				
GG	81 (39.51)	87 (37.18)	1.00	0.8706
GA	93 (45.37)	109 (46.58)	0.92 (0.61–1.38)	0.6765
AA	31 (15.12)	38 (16.24)	0.88 (0.50–1.54)	0.6453
GA/AA	124 (60.49)	147 (62.82)	0.91 (0.62–1.33)	0.6157
Fas −670A>G				

Table 3 (continued)

Genotype	Patients, *n*		OR (95 % CI)[a]	*P* value
Birth status	≤1	≤2		
AA	89 (43.41)	94 (40.17)	1.00	0.7592
AG	92 (44.88)	113 (48.29)	0.86 (0.58–1.29)	0.4592
GG	24 (11.71)	27 (11.54)	0.94 (0.50–1.75)	0.8423
AG/GG	116 (56.59)	140 (59.83)	0.88 (0.60–1.28)	0.4916
FasL −844 T>C				
CC	118 (57.56)	140 (59.83)	1.00	0.3071
TC	70 (34.15)	83 (35.47)	1.00 (0.67–1.49)	0.9976
TT	17 (8.29)	11 (4.70)	1.83 (0.82–4.06)	0.1360
TC/TT	87 (42.44)	94 (40.17)	1.09 (0.75–1.60)	0.6300
CerbB-2	Negative	Positive		
Fas −1377G>A				
GG	31 (33.70)	137 (39.48)	1.00	0.2948
GA	42 (45.65)	160 (46.11)	1.16 (0.69–1.94)	0.5736
AA	19 (20.65)	50 (14.41)	1.68 (0.87–3.24)	0.1215
GA/AA	61 (66.30)	210 (60.52)	1.28 (0.79–2.08)	0.3108
Fas −670A>G				
AA	37 (40.22)	146 (42.07)	1.00	0.6976
AG	42 (45.65)	163 (46.97)	1.00 (0.61–1.64)	0.9476
GG	13 (14.13)	38 (10.95)	1.35 (0.65–2.79)	0.4177
AG/GG	55 (59.78)	201 (57.93)	1.07 (0.67–1.71)	0.7480
FasL −844 T>C				
CC	56 (60.87)	202 (58.21)	1.00	0.6928
TC	29 (31.52)	124 (35.73)	0.86 (0.52–1.42)	0.5059
TT	7 (7.61)	21 (6.05)	1.21 (0.49–2.99)	0.6898
TC/TT	36 (39.13)	145 (41.79)	0.91 (0.57–1.45)	0.6455

Significant results are in bold

OR odds ratio, *CI* confidence interval

[a] Adjusted by age

analysis to assess the strength of the association between a polymorphism and breast cancer risk [24]. mRNA expression in tissue samples and sFas and sFasL concentrations in serum were calculated using ANOVA. In controls, genotypic frequencies for each polymorphism were tested for departure from Hardy–Weinberg equilibrium, and $P<0.05$ was considered significant. All statistical analyses were conducted using the Statistical Analysis System (version 11, SAS Institute,

Table 4 Haplotype frequencies of *Fas* −1377G>A and −670A>G polymorphisms in breast cancer patients and healthy controls, and associations with breast cancer risk

−1377G>A	−670A>G	Patients	Controls	OR (95 % CI)	*P* value
G	A	516 (58.7)	373 (42.4)	**1.93 (1.60–2.33)**	**<0.0001**
G	G	23 (2.7)	24 (2.8)	0.96 (0.54–1.71)	0.8938
A	A	55 (6.3)	163 (18.6)	**0.30 (0.21–0.41)**	**<0.0001**
A	G	284 (32.3)	318 (26.2)	0.84 (0.69–1.03)	0.0865

Data are *n* (%). Significant results are in bold

OR odds ratio, *CI* confidence interval

Table 5 Interaction effects between *Fas* (−1377G>A, −670A>G) and *FasL* (−844 T>C) polymorphisms, and associations with breast cancer risk

Genotype		Patients	Controls	OR (95 % CI)[a]	*P* value
Fas −1377G>A	*FasL* −844 T>C				
GG	CC	106 (24.15)	55 (12.53)	1.00	**<0.0010**
GG	TC/TT	62 (14.12)	44 (10.02)	0.76 (0.46–1.27)	0.2239
GA/AA	CC	152 (34.62)	151 (34.40)	**0.52 (0.35–0.78)**	**0.0012**
GA/AA	TC/TT	119 (27.11)	189 (43.05)	**0.32 (0.21–0.48)**	**<0.0010**
Fas −670A>G	*FasL* −844 T>C				
AA	CC	114 (25.97)	72 (16.40)	1.00	**<0.0010**
AA	TC/TT	69 (15.72)	55 (12.53)	0.80 (0.50–1.27)	0.3220
AG/GG	CC	144 (32.80)	134 (30.52)	**0.68 (0.47–0.99)**	**0.0437**
AG/GG	TC/TT	112 (25.51)	178 (40.55)	**0.39 (0.27–0.58)**	**<0.0010**

[a] Adjusted by age. Significant results are in bold

OR odds ratio, *CI* confidence interval

Cary, NC, USA). Haplotype analysis, including frequency distribution, was performed using SHEsis software (http://analysis.bio-x.cn/myAnalysis.php).

Results

Characteristics of the study population

All of the participants in this study were women, with 439 breast cancer patients and 439 healthy, age-matched controls (mean±standard deviation age at diagnosis: cases 52.89±10.78 years; controls 52.95±10.89 years; P=0.933), and none drank alcohol or smoked cigarettes. Demographic data and clinical characteristics of the participants are summarized in Table 1.

Genotype distribution of the *Fas* and *FasL* polymorphisms

Genotype frequencies of the *Fas* −1377G>A, *Fas* −670A>G, and *FasL* −844 T>C polymorphisms among breast cancer patients and controls and their associations with breast cancer risk are presented in Table 2. Briefly, for all three polymorphisms, there was a significant difference among the genotype distribution between cases and controls. Moreover, the three polymorphisms were all associated with breast cancer risk. In addition, the genotype frequencies of the three polymorphisms among controls were consistent with estimates by Hardy–Weinberg equilibrium.

Clinical features of cases and controls with *Fas* and *FasL* polymorphisms

The distributions of *Fas* −1377G>A, *Fas* −670A>G, and *FasL* −844C>T genotypes according to the clinical characteristics of patients and their associations with breast cancer risk are presented in Table 3. In brief, no significant association was observed between genotype or combined variant genotype distributions and any of the clinical parameters.

Haplotype analysis between *Fas* −1377G>A and −670A>G polymorphisms

Because the two polymorphisms in *Fas* (−1377G>A and −670A>G) were in linkage disequilibrium with each other, the combined associations of these two polymorphisms with breast cancer were calculated by haplotype analysis. The results indicated that the −1377G/−670A haplotype was associated with an increased breast cancer risk as compared with other haplotypes, whereas the −1377A/−670A haplotype was associated with a reduced breast cancer risk compared with other haplotypes (Table 4).

Interaction effects of *Fas* and *FasL* polymorphisms on breast cancer risk

To further clarify the interaction among different genes, an analysis of gene–gene interactions between *Fas* and *FasL* in breast cancer risk was conducted (Table 5). Briefly, significant interactions between *Fas* −1377G>A and *FasL* −844 T>C (P<0.001), as well as *Fas* −670A>G and *FasL* −844 T>C

($P<0.001$), were found. The results showed that the *Fas* −1377GA/AA and *FasL* −844CC genotypes were associated with a reduced risk of breast cancer, as were the *Fas* −1377GA/AA and *FasL* −844TC/TT genotypes, the *Fas* −670AG/GG and *FasL* −844CC genotypes, and the *Fas* −670AG/GG and *FasL* −844TC/TT genotypes.

Fas expression in tumor tissues and adjacent normal tissues

qRT-PCR was used to determine *Fas* mRNA levels in breast cancer and adjacent normal tissues. The results showed no significant differences in the mRNA levels of *Fas* between breast cancer and normal tissues (Fig. 2a; $P=0.532$). There were also no significant associations observed in Fas expression levels by tumor stage (Fig. 2b; $P=0.866$) or lymph node metastasis (Fig. 2c; $P=0.619$). Furthermore, no significant differences were observed for *Fas* expression in tumor tissues when compared by genotypes of three polymorphisms (Fig. 3a–c).

Concentrations of circulating sFas and sFasL in different genotypes carriers

Circulating levels of sFas and sFasL were measured in 180 patients, who were meticulously selected from 439 cases. The associations among *Fas* −670A>G, *Fas* −1377G>A, and *FasL* −844 T>C genotypes and sFas and sFasL are summarized in Table 6. There was a significantly increased sFas expression in the *Fas* −1377GG genotype compared with those in the GA and AA genotypes (Fig. 4a and Table 6), and in the *Fas* −670AA genotype compared with those in the AG and GG genotypes (Fig. 4b and Table 6). However, no significant differences were found in sFas levels among the three genotypes of the *FasL* −844 T>C polymorphism (Table 6). In addition, no significant differences in the levels of circulating sFasL were found among the genotypes of any of the three polymorphisms (Table 6).

Discussion

Fas and its endogenous ligand FasL trigger the death signal cascade and lead to apoptosis [25]. Earlier studies suggested that polymorphisms of *Fas* (−1377G>A, −670A>G) and *FasL* (−844C>T) were associated with breast cancer risk [2–14]; however, these studies reached inconsistent conclusions. In the current study, we observed that the *Fas* −1377A and −670G alleles were linked to a lower breast cancer risk, which is inconsistent with the findings of other studies [2, 10,

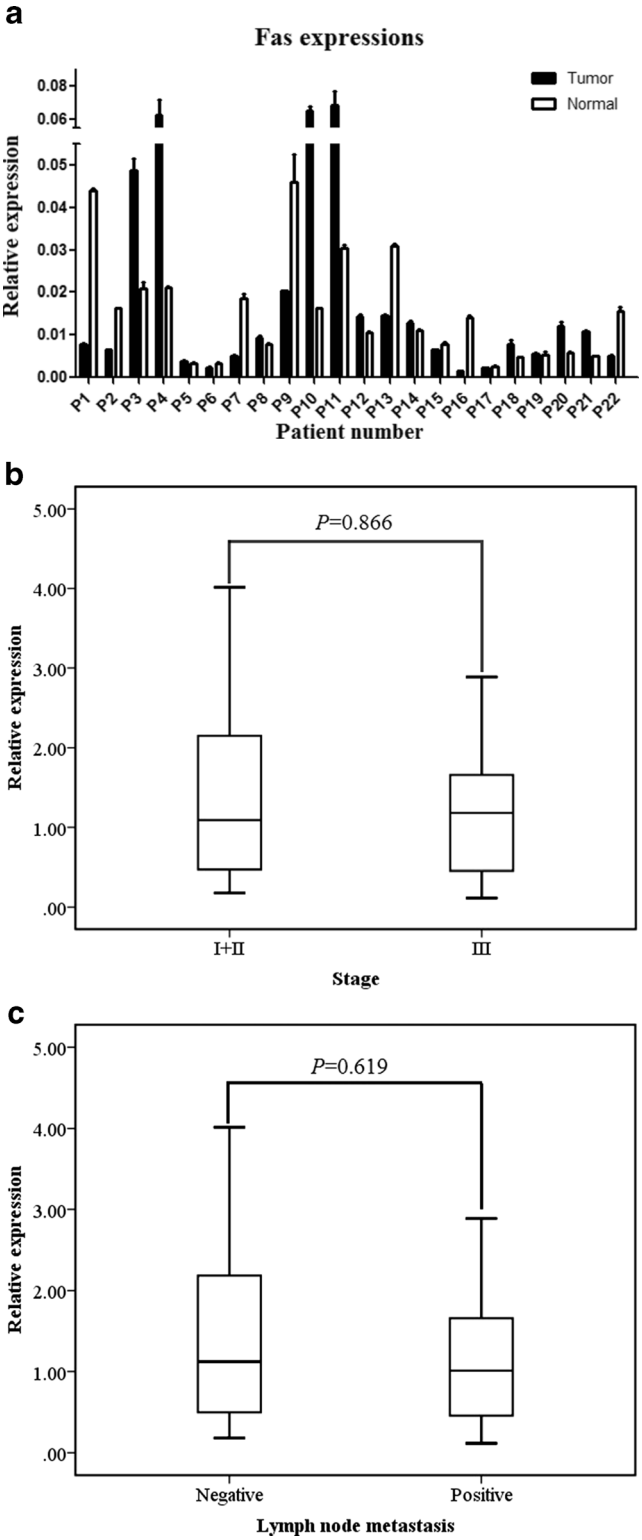

Fig. 2 Relative *Fas* expression in breast cancer tissues and adjacent normal tissues. **a** *Fas* expression in 22 breast cancer tissue and 22 matched adjacent normal tissue samples were measured by qRT-PCR. *Fas* expression according to **b** tumor stage and **c** lymph node metastasis

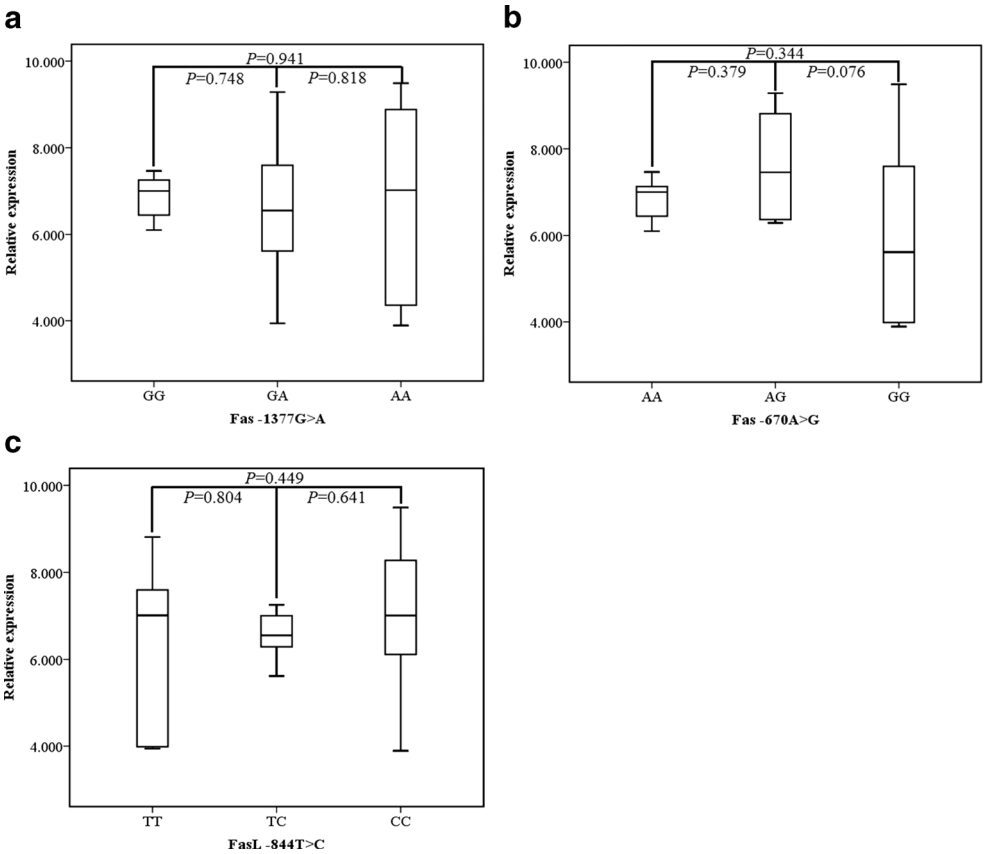

Fig. 3 Relative *Fas* expression in breast cancer tissues in three genotypes of *Fas* and *FasL* polymorphisms. *Fas* expression in three genotypes of **a** *Fas* −1377G>A polymorphism, **b** *Fas* −670A>G polymorphism, and **c** *FasL* −844 T>C polymorphism in breast cancer tissues

12, 14], and that *FasL* −844TC genotypes were also significantly associated with a lower breast cancer risk.

Shao et al. reported that the genotype of *Fas* −1377GA and the *Fas* −670AG or GG genotype were associated with decreased prostate cancer risk [26]. Such associations were earlier identified by Li et al. in cutaneous malignant melanoma [27]. Similarly, the current study found that the *Fas* −1377AA and −670GG genotypes were associated with decreased breast cancer risk. However, contradictory conclusions have been reported by other studies, as follows. For the *Fas* −1377G>A polymorphism, previous studies reported that people who carry the A allele have a higher breast cancer risk than those carrying the G allele [2, 10, 11, 14], and for the *Fas* −670A>G polymorphism, Hashemi et al. reported that the *Fas* −670GG genotype was associated with an increased risk of breast cancer [12].

The aforementioned inconsistent study results might be connected to previous evidence that the *Fas* −1377G>A change can damage its binding element Sp1 and lead to decreased levels of Fas, while the *Fas* −670A>G change can damage its binding element STAT1 with the same result [28, 29]. Therefore, polymorphisms in *Fas* may be associated with an increased risk of breast cancer by downregulating the capacity for apoptosis. However, recent evidence indicates that Fas is a double-edged sword, because it can transmit proliferation and activation signals and convert a tumor suppressor into a tumor promoter at certain levels of FasL [30]. Moreover, activated Fas can mobilize and accumulate immune suppressive cells (e.g., myeloid-derived suppressor cells, regulatory T cells) in tumor tissue, where they can exert a tumor-promoting effect by inhibiting T and natural killer cell proliferation and activation, and contribute to tumor angiogenesis [30–33]. In addition, the tumor-promoting effect of Fas has been confirmed in vitro and in vivo [34–38].

On the other hand, previous studies have suggested that the −1377G and −670A alleles may upregulate the production of sFas, which can protect malignant cells from Fas-mediated apoptosis by inhibiting Fas receptor–ligand binding in the extracellular space [26, 39–42], consistent with the results in this study. In addition, the *Fas* −1377G and −670A alleles were associated with higher levels of sFas, which inhibits Fas-mediated apoptosis by neutralizing FasL or antibody [15]. However, the present study did not observe any association of Fas in breast tissue with *Fas* polymorphisms. Thus, we believe that the association of *Fas* polymorphisms with breast cancer risk may be the result of altered concentrations of sFas in serum, rather than of Fas expression in tumor tissue. Furthermore, the association of the *Fas* −1377G>A and −670A>

Table 6 Associations of *Fas* and *FasL* polymorphisms and serum concentrations of sFas and sFasL

Genotype	Patients, *n* (%)	Mean sFas/sFasL level (ng/mL)	SD	*P* value
sFas				
Fas −1377G>A				
GG	68 (37.78)	1.166	1.365	**0.002**
GA	83 (46.11)	0.695	0.257	
AA	29 (16.11)	0.655	0.278	
Fas −670A>G				
AA	69 (38.33)	1.156	1.358	**0.002**
AG	81 (45.00)	0.695	0.258	
GG	30 (16.67)	0.663	0.276	
FasL −844 T>C				
CC	99 (55.00)	0.829	0.823	0.819
TC	63 (35.00)	0.920	1.094	
TT	18 (10.00)	0.881	0.280	
sFasL				
Fas −1377G>A				
GG	64 (37.78)	0.727	0.294	0.785
GA	88 (46.11)	0.745	0.330	
AA	20 (16.11)	0.692	0.338	
Fas −670A>G				
AA	65 (38.33)	0.727	0.291	0.832
AG	86 (45.00)	0.744	0.334	
GG	21 (16.67)	0.699	0.331	
FasL −844 T>C				
CC	94 (55.00)	0.762	0.337	0.270
TC	61 (35.00)	0.712	0.308	
TT	17 (10.00)	0.638	0.196	

Significant results are in bold

SD standard deviation

G diplotypes with breast cancer risk revealed a markedly lower risk for developing breast cancer in carriers of *Fas* −1377A/−670A, whereas the diplotype −1377G/−670A was associated with an increased risk of breast cancer in this study. These results might indicate that these two polymorphisms are in linkage disequilibrium, consistent with previous studies [10, 14].

For the *FasL* −844 T>C polymorphism, the present results showed a lower risk of breast cancer in TC compared with CC genotype carriers. Hashemi et al. reported an increased risk of breast cancer in CC compared with TT genotype carriers (OR=3.18, 95 % CI=1.21–8.33) in an Iranian population [12]. Zhang et al. found the TC genotype to be associated with a decreased breast cancer risk (OR=0.76, 95 % CI= 0.62–0.94) in a Chinese population [10]. Furthermore, Wang et al. found the CC genotype to be associated with an increased breast cancer risk (OR=1.92, 95 % CI=1.46–2.54) compared with the TT/TC genotype in a Chinese population [2]. As previously mentioned, the −844 T>C mutation has been associated with elevated expression of both sFasL and its membrane types [10] due to connection with its binding motif CAAT/enhancer-binding protein β transcription factor, which was associated with a higher breast cancer risk [43]. The study of gene–gene interactions between *Fas* and *FasL* showed a markedly lower risk of breast cancer in those carrying the *Fas* −1377GA/AA genotype and the *FasL* −844TC/TT genotype compared with −1377GA/AA and −844TC/TT alone. The same phenomenon was observed for the *Fas* −670AG/GG and −844TC/TT genotypes, indicating a strong synergistic effect between these two genes with respect to breast cancer.

Due to different genetic backgrounds between individuals, it is of great importance to choose the anticancer drugs [44, 45]. It was reported that GSTs not only participate in drug detoxification but also is involved in the control of apoptosis by inhibition of JNK signaling pathway [46]. As thus, they have become the focus of chemotherapy resistance research, in which GSTP1 was the most extensively investigated in breast cancer [47, 48]. Previous studies showed that GSTP1 expression might play a vital role in drug resistance in breast

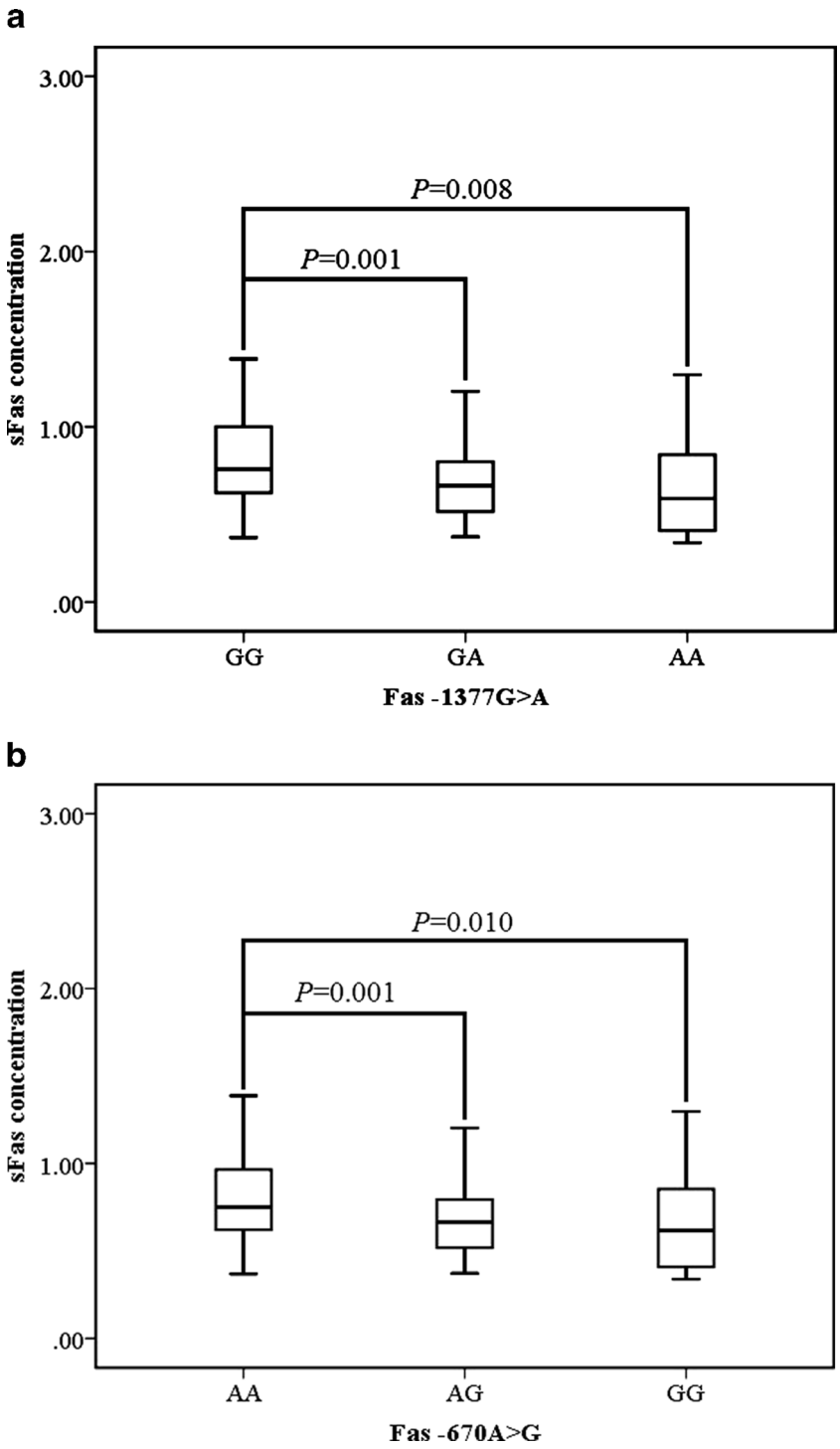

Fig. 4 Association of *Fas* −1377G>A (**a**) and *Fas* −670A>G (**b**) polymorphisms and concentrations of sFas in patients' serum. **a** The GG genotype was associated with higher sFas concentrations than GA or AA (P=0.001 and P=0.008, respectively). **b** The AA genotype was associated with higher sFas concentration than AG and GG (P=0.001 and P=0.010, respectively)

cancer [47–49]. The *GSTP1*−313A>G (rs1695) polymorphism is reported to reduce enzyme activity in removal of chemotherapy agents [50–52]. Moreover, the −313GG or AG genotype was reported to correlate significantly with unfavorable prognosis for breast cancer patients treated with alkylating agents [53]. Besides, the P-glycoprotein encoded by the multidrug resistance gene *MDR1* is important in efflux transport of the chemotherapeutic agent, doxorubicin, used in breast cancer treatment. The polymorphism in exon 26, −3435C>T, was found to be a considerable advantage in neoadjuvant chemotherapy. Kafka et al. [54] reported that −3435TT was associated with a clinical response for treating

breast cancer patients with anthracycline alone or in combination with taxane. In contrast, a recent study reported that the −3435CC was related to an enhanced response after neoadjuvant therapy and longer time to progression after anthracycline-based chemotherapy.

This study has some limitations. First, it is difficult to determine the effect of environmental factors such as diet, use of oral contraceptives, occupational exposures, and physical exercise, which can influence breast cancer risk via gene–environment interactions. Second, although our patient population was not small, it was still insufficient for further subgroup analysis and may limit the statistical power of our study.

Conclusions

In conclusion, this study provides evidence that *Fas* −1377G>A, *Fas* −670A>G, and *FasL* −844 T>C polymorphisms are associated with altered breast cancer risk in the Chinese population. Two polymorphisms of *Fas* were in linkage disequilibrium, and two *Fas* polymorphisms and a *FasL* polymorphism had an interaction effect on breast cancer risk, respectively. Moreover, the *Fas* −1377GG and −670AA genotypes were associated with higher sFas concentrations than other genotypes, indicating that *Fas* polymorphisms may be associated with altered breast cancer risk mainly by regulating the concentration of sFas. These conclusions need to be validated in large and multiethnic populations.

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Conflict of interest None.

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