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ORIGINAL PAPER

A single nucleotide polymorphism in *PIK3CA* gene is inversely associated with P53 protein expression in breast cancer

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Abstract Single nucleotide polymorphism (SNP) rs17849071 was recently reported to be inversely associated with PIK3CA amplification in follicular thyroid cancer, but the main function of this SNP remains unclear. In this study, by using PCR and sequencing method, we explored whether this SNP was associated with P53 expression status and other clinicopathological characteristics in 62 Chinese breast cancer (BCa) patients. In our results, P53 protein accumulation was significantly associated with HER2 overexpression ($P = 0.013$) and Ki-67 expression ($P = 0.007$), which were in accord with previous studies. Besides, there was a significantly inverse relationship between P53 protein expression and rs17849071 GT+GG genotype in Chinese BCa patients ($P = 0.044$). The SNP was not related to other important BCa markers such as estrogen receptor, progestin receptor, and HER2. Among different BCa intrinsic subtypes, no significant differences were found on P53 expression status ($P = 0.356$) or rs17849071 polymorphism (T>G) ($P = 0.813$). In conclusion, SNP rs17849071 GT+GG genotype was inversely associated with P53 protein accumulation in BCa samples. Studies with larger sample size focusing on exploring the relationship of rs17849071 polymorphisms, P53 accumulation, P53 mutations, and PIK3CA amplification might be needed.

Keywords Breast cancer · Single nucleotide polymorphism · p53 · Alpha catalytic subunit of phosphoinositol-3-kinase

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Introduction

Worldwide, over 1.1 million women are diagnosed with breast cancer (BCa) annually, and 410,000 die from the disease each year [1]. BCa is a highly heterogeneous disease. Biomarkers such as estrogen receptor (ER), progesterone receptor (PR), and HER2 can provide useful information for BCa typing and individualized treatment.

In addition to the biomarkers mentioned above, P53 protein, which is encoded by the *TP53* gene, is another frequently used marker of BCa. *TP53* is one of the most frequently mutated genes in human cancers. P53 has many important biological functions, including regulation of the cell cycle, apoptosis, senescence, DNA metabolism, angiogenesis, and cellular differentiation. Mutations of *TP53* frequently lead to accumulation of mutant forms of P53 protein, which can be detected using immunohistochemical staining in clinical practice. P53 protein overexpression is associated with the presence of any *TP53* mutation, especially missense mutations [2]. Several cohort studies have shown that P53 protein accumulation contributes to an increased risk of progression to BCa [3, 4]. Some reports showed that P53 expression might be associated with a significant decrease in BCa survival [5–7]. However, the prognostic value of P53 overexpression in BCa remains controversial.

In addition to the *TP53* gene, alpha catalytic subunit of phosphoinositol-3-kinase (*PIK3CA*) is frequently mutated in BCa [8]. PI3K (phosphatidylinositol 3-kinase) is composed of 85-kDa and 110-kDa subunits. The 85-kDa subunit acts as an adaptor, coupling the 110-kDa subunit (p110) to activated protein tyrosine kinases. PI3K was originally believed to phosphorylate the 3-hydroxy group of inositol phospholipids. PI3KCA activation, either by mutation or gene amplification, initiates a signal transduction pathway that promotes growth, metabolism, and survival in cancer cells [9, 10]. Activation of normal P53 protein may downregulate PIK3CA/Akt signaling [11]. It is also noteworthy that several reports have shown that amplification of *PIK3CA* is frequently associated with *TP53* mutations [11–14].

Recently, Xing et al. reported that SNP rs17849071, which had been shown to be a germline genetic event, was inversely associated with *PIK3CA* amplification in follicular thyroid cancer. However, the main function of this SNP remains unclear. It would be interesting to elucidate the association between the rs17849071 SNP and clinically important characteristics, including P53 protein expression, and to explore the possible function of this SNP. In this study, we investigated the relationship between rs17849071 and the expression of ER, PR, HER2, P53, and Ki-67 in BCa. It was found that SNP rs17849071 was significantly and inversely associated with P53 protein expression in BCa.

Methods and materials

Patients and breast cancer samples

Tumor and tumor-adjacent tissue samples were obtained from 62 BCa patients diagnosed at the Anyang Tumor Hospital (Henan Province, China) between September 2010 and October 2011. The patients were all of Han descent and female. None of the patients had received preoperative hormonal therapy related to their BCa prior to the biopsy/mastectomy procedure. Following surgery, standard immunohistochemical analysis was performed on tumor specimens and the results were confirmed by two experienced clinical pathologists. All tumors were classified according to the World Health Organization Histological Typing of Breast Tumors, and the clinical stage of each patient was determined according to the 5th Edition of the UICC TNM Classification of Malignant Tumors. The characteristics of patients and tumors are shown in Table 1. All patients signed an informed consent form, and the research protocol was approved by the hospital's Institutional Review Board.

Table 1 Patient and tumor characteristics in P53-negative and P53-positive groups

Characteristic	P53-negative group ($n = 29$)	P53-positive group ($n = 33$)	*P*
Median age (y)	54 (range, 38–71)	52 (range, 30–72)	0.074
Stage			0.550
I and II	20 (69.0)	25 (75.8)	
III and IV	9 (31.0)	8 (24.2)	
Maximum diameter (mm)	3.0 (range, 1.5–8.5)	3.0 (range, 1.5–6.0)	0.398
Hormonal status (*n*, %)			0.612
Pre-menopause	11 (37.9)	15 (45.5)	
Post-menopause	18 (62.1)	18 (54.5)	
Estrogen receptor (*n*, %)			0.794
Positive	19 (65.5)	20 (60.6)	
Negative	10 (34.5)	13 (39.4)	
Progesterone receptor (*n*, %)			0.309
Positive	12 (41.4)	19 (57.6)	
Negative	17 (58.6)	14 (42.4)	
HER2 (*n*, %)			0.013
Positive	2 (6.90)	11 (33.3)	
Negative	27 (93.1)	22 (66.7)	
Ki-67 (*n*, %)			
Positive (>20 %)	5 (17.2)	17 (51.5)	0.007
Negative(≤20 %)	24 (82.8)	16 (48.5)	

Immunohistochemical scoring

Histopathological analyses were performed using formalin-fixed, paraffin-embedded tumor tissue. Monoclonal mouse antibodies against ERα, PR, HER2, P53, and Ki-67 (1:50–1:200 dilution, DAKO, Denmark) were used. ER, PR, HER2, P53, and Ki-67 protein status was recorded in the pathology reports. Scoring was conducted using previously published criteria [14]. ER/PR staining was designated as positive when nuclear staining occurred in ≥10 % of tumor cells [15]. To assess HER2 expression, the membrane staining pattern was estimated and scored on a scale of 0–3+. Tumors with scores of ≥2 were considered to be positive for HER2 overexpression. For P53 protein, nuclear staining of tumor specimens was evaluated from a single slide by using a semiquantitative scoring system for intensity and percent of positive nuclei. The system assesses nuclear staining intensity using a 4-level ordered categorical variable (none or <10 % = 0, 10–25 % = 1, 25–50 % = 2, >50 % = 3). Tumors with a score of ≥1 for P53 were considered to be P53 protein-positive. A threshold of ≥20 % was considered to be Ki-67 positive.

Genomic DNA extraction, polymerase chain reaction, and DNA sequencing

DNeasy® Blood & Tissue Kits (QIAGEN 69506) were used to purify DNA from tumor tissues of BCa patients, according to the manufacturer's instructions. A region of the *PIK3CA* gene containing rs17849071 in intron 9 was amplified using the primers 5′-GATTGGTTCTTTCCTG TCTCTG-3′ (forward) and 5′-CCACAAATATCAATTT ACAACCATTG-3′ (reverse) [16]. PCR was performed using a previously described protocol [17]. Briefly, after an initial 3-min denaturing at 95 °C, each temperature was maintained for 40 s for 6 step-down increments, for 2 cycles each. For each of the step-down increments, the denaturing temperature was 95 °C and the extension temperature was 72 °C, with annealing temperatures of 66, 64, 62, 60, 58 and 56 °C. Subsequently, temperatures of 95, 54, and 72 °C were each maintained for 40 s for 30 cycles, followed by a final elongation step at 72 °C for 5 min. PrimeSTAR® HS DNA Polymerase (Takara Code: DR010A) was used to ensure high accuracy and amplification efficiency, according to the manufacturer's instructions. PCR products were subjected to electrophoresis on a 1.5 % agarose gel, along with a molecular size marker (DL2000, TaKaRa), to confirm their quality. The PCR products were then sent to Taihe Biotechnology Co., Ltd. (Beijing, China), where all amplified PCR products were sequenced (ABI 3730xl DNA Analyzer, Applied Biosystems), using the sequencing primer 5′-TTGCTTTTTCTG TAAATCATCTGTG-3′.

Statistical analysis

Fisher's exact test or chi-squared tests were used to assess associations among categorical variables. All P values were two-sided, and results were considered statistically significant at $P < 0.05$. Analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

Results

P53 protein accumulation was significantly associated with HER2 overexpression and Ki-67 expression in BCa

Correlations between patient and tumor characteristics and P53 expression status are shown in Table 1. The BCa patients were all female, with a median age of 52.6 (range, 30–72) years. Most tumors were infiltrating ductal carcinomas ($n = 60$, 96.8 %), and 2 were infiltrating lobular carcinomas ($n = 2$, 3.2 %). Most tumors were in stage II ($n = 45$, 72.6 %). P53 protein accumulation was significantly associated with HER2 overexpression ($P = 0.013$) and Ki-67 expression ($P = 0.007$), but not with disease stage, maximum diameter, or ER or PR expression status.

Inverse relationship between heterozygous genotype GT, homozygous genotype of the minor allele G at rs17849071, and P53 expression in BCa

As shown in Fig. 1, in the 62 BCa specimens, three genotypes were identified by direct sequencing of intron 9 of the *PIK3CA* gene. The homozygous genotype TT (Fig. 1a) accounted for 88.7 % ($n = 55$) of cases, the heterozygous genotype GT (Fig. 1b) accounted for 9.7 % ($n = 6$) of cases, and the homozygous genotype of the minor allele G (Fig. 1c) at rs17849071 accounted for 1.6 % ($n = 1$) of cases. As Xing et al. reported, this SNP is a germline genetic event and it shows high frequency in health control [17]. We performed several PCR and gene sequencing tests in tumors, tumor-adjacent tissue, and blood samples from the same BCa patients and found the same results (data not shown). Of the 29 P53 protein-negative tumor samples, the GT/GG genotype accounted for 20.7 % ($n = 6$) of cases, but among the 33 P53 protein-positive samples, only 1 (3.0 %) sample with the GT/GG genotype was identified. As shown in Fig. 1d, P53 protein expression status was significantly and inversely associated with the heterozygous genotype GT, and the homozygous genotype of the minor allele G at rs17849071 in BCa specimens ($P = 0.044$).

The association between rs17849071 polymorphism (T>G) and breast tumor characteristics

As shown in Table 2, six cases heterozygous for the minor allele G and the major allele T and one case homozygous for of the minor allele G at rs17849071 were identified among the 62 Chinese BCa patients. The other 55 subjects were all homozygous for the major allele, T, at rs17849071. No significant association was observed between the GT/GG genotype and ER, PR, HER2, or Ki-67 expression status. However, in the TT genotype group, the percentage of P53-positive subjects was strikingly higher than that in the GT/GG genotype group (58.2 vs. 14.3 %).

The distribution of P53 expression and rs17849071 in the clinicopathological surrogate subtypes of BCa

As suggested by the Expert Panel of the 13th St. Gallen International Breast Cancer Conference (2013), BCa can be divided into four main intrinsic subtypes [18]. As shown in Table 3, the percentage of P53-negative samples was highest in the Luminal A subtype (57.1 %) and lowest in

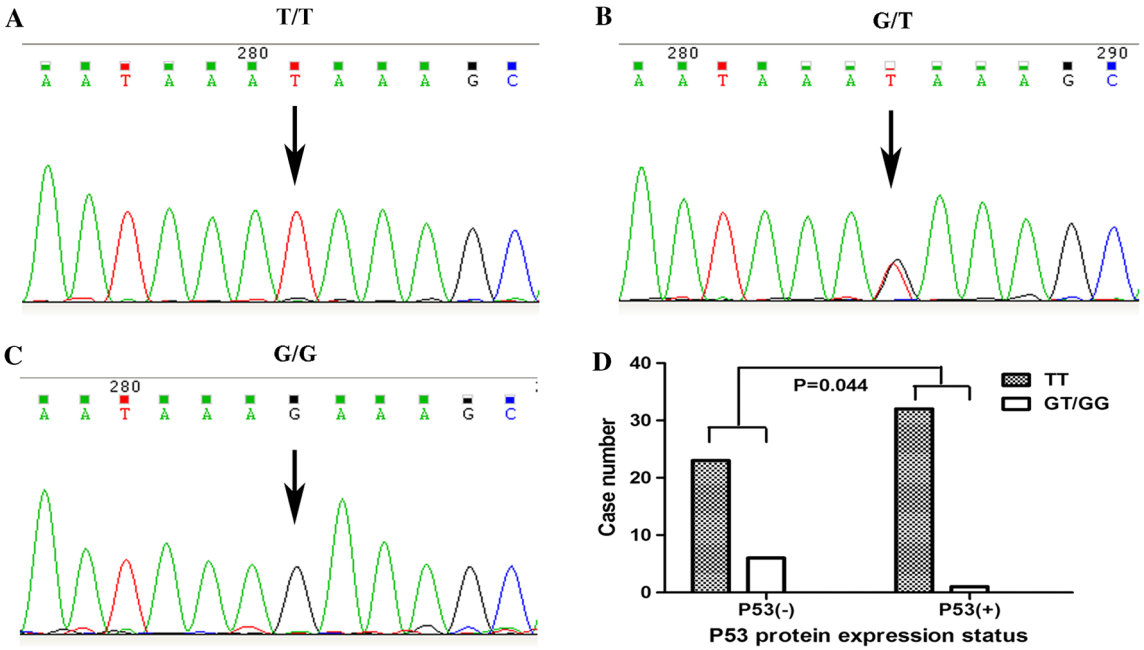

Fig. 1 Inverse relationship between the heterozygous genotype GT, the homozygous genotype of the minor allele G at rs17849071 and P53 expression in breast tumors. The homozygous genotype of the major allele T at rs17849071 (**a**). The heterozygous genotype of minor allele G and major allele T at rs17849071 (**b**). And the homozygous genotype of the minor allele G at rs17849071 (**c**). **d** shows that in the P53-negative group ($n = 29$), a larger number of cases and a higher percentage of heterozygous genotype GT and minor homozygous genotype GG ($n = 6$, 20.7 %) were found. In the P53-positive group ($n = 33$), only one heterozygote GT was identified ($n = 1$, 3.03 %). The difference was statistically significant ($P = 0.044$)

Table 2 The association between rs17849071 polymorphism (T>G) and breast tumor characteristics in a Chinese population

Category	TT ($n = 55$)	GT/GG ($n = 7$)	P
Estrogen receptor (n, %)			1.000
Positive	34 (61.8)	5 (71.4)	
Negative	21 (38.2)	2 (28.6)	
Progesterone receptor (n, %)			1.000
Positive	27 (49.1)	4 (57.1)	
Negative	28 (50.9)	3 (42.9)	
HER2 (n, %)			1.000
Positive	12 (21.8)	1 (14.3)	
Negative	43 (78.2)	6 (85.7)	
Ki-67 (n, %)			
Positive (>20 %)	21 (38.2)	1 (14.3)	0.405
Negative (≤20 %)	34 (61.8)	6 (85.7)	
P53 (n, %)			
Positive	32 (58.2)	1 (14.3)	0.044
Negative	23 (41.8)	6 (85.7)	

the HER2 overexpression (20.0 %) subtype. The rs17849071 GT/GG genotype is most common in the Luminal A intrinsic subtype (14.3 %) and least common in the HER2 overexpression (0 %) subtype. However, with our relatively small sample size, no significant differences were observed in P53 expression status ($P = 0.356$) or rs17849071 polymorphism (T>G) ($P = 0.813$) among the different BCa intrinsic subtypes.

Discussion

TP53 mutation is a hallmark of cancer. It occurs in almost half of all human cancers and always results in the expression of a mutant P53 protein that has acquired transforming activity [19]. The majority of *TP53* mutations result in the substitution of single amino acids in the central region of the P53 protein, generating a spectrum of variants [20]. P53 protein overexpression is associated with the presence of any *TP53* mutation, especially missense mutations [2]. When mutations occur, the normal P53 tumor-suppressive functions are disrupted and P53 can instead acquire oncogenic properties via gain-of-function mechanisms.

Kim et al. [21] showed that overexpression of P53 is a prognostic marker for hormone receptor-positive BCa, especially in pre-menopausal women. Some reports have also shown that response to endocrine therapy is negatively associated with P53 expression status [22–24]. In the present study, we did not evaluate the prognostic value of

Table 3 The distribution of rs17849071 in the clinicopathological surrogate subtypes of breast cancer

Intrinsic subtype	Luminal A	Luminal B	HER2 overexpression	Basal-like	*P*
P53+ (%)	9 (42.9)	13 (61.9)	4 (80.0)	7 (46.7)	0.356
P53− (%)	12 (57.1)	8 (38.1)	1 (20.0)	8 (53.3)	
TT (%)	18 (85.7)	19 (90.5)	5 (100)	13 (86.7)	0.813
GT/GG (%)	3 (14.3)	2 (9.5)	0 (0)	2 (13.3)	

P53 expression, but found that the percentage of P53 positive was high in the HER2 overexpression subtype, which is in accordance with previously published reports [25]. It should be noted that in our study, the percentage of P53 accumulation was even higher (87.5 %) in the Luminal B-like (HER2-positive) subtype (data not shown). These results may be explained by the relationship between P53 and HER2 expression. Yamashita et al. [26] reported that the coexistence of HER2 overexpression and P53 protein accumulation is a strong prognostic molecular marker in BCa; the authors also demonstrated a significant association between P53 and HER2 [26]. These data are in accordance with our results.

The most notable result of our study was the inverse relationship between P53 protein expression and the rs17849071 GT+GG genotype in BCa patients. This rs17849071 SNP was not associated with other clinical characteristics such as ER, PR, or HER2 expression. To date, the only data available for rs17849071 indicated that in follicular thyroid cancer, the occurrence of rs17849071 G/T was strikingly low, and that the heterozygous genotype (GT) was inversely associated with *PIK3CA* amplification in thyroid tumors [17]. Evaluating the relationship between P53 protein expression and *PIK3CA* amplification may further illuminate the mechanisms underlying the observed results.

PIK3CA is the second most frequently mutated gene in BCa, following the *TP53* gene. *PIK3CA* is also associated with various types of cancer [13, 27]. *PIK3CA* amplification is believed to enhance PI3 K signaling and Akt activity and subsequently to promote cellular proliferation and survival. Typical markers of PI3 K pathway activation, such as pAKT, pS6, and p4EBP1, are highly expressed in the basal-like and *HER2E* mRNA subtypes and are correlated with *PIK3CA* amplification [8].

Singh et al. [28] reported that P53 negatively regulates *PIK3CA* transcript and protein levels in a PTEN-independent manner. Furthermore, Astanehe et al. [29] reported that P53 can directly bind to the *PIK3CA* promoter and inhibit its activity. Inactivation of P53 may lead to subsequent upregulation of *PIK3CA* and its downstream signaling. In a genomic landscape study, *TP53* mutations were found to be closely related to copy number gains of 3q26, where *PIK3CA* was located [30]. In fact, many studies have shown that *TP53* mutations are positively associated with *PIK3CA* amplification in various types of tumors [14, 29, 31]. In this study, we showed that the rs17849071 SNP is inversely associated with P53 protein accumulation in BCa. Further studies investigating whether this SNP is also inversely associated with *TP53* mutations, as well as PIK3CA amplification in BCa are warranted.

The following limitations of the present study should be considered. The sample size was relatively small, and the results should be confirmed in larger studies. Furthermore, although immunohistochemical staining is a widely used method, it is semiquantitative and subjective, and the results depend on the threshold set during scoring. It should be noted that some types of *TP53* mutations might also lead to negative results of P53 protein detection by IHC. Direct detection of *TP53* mutations in tumors may provide more accurate data.

In summary, we analyzed associations between breast tumor characteristics and P53 expression status and found that P53 protein accumulation is significantly associated with HER2 overexpression and Ki-67 expression. We further evaluated the association between the rs17849071 polymorphism (T>G) and breast tumor characteristics and found a significant inverse relationship between P53 protein expression and the rs17849071 GT+GG genotype in Chinese BCa patients. Future studies employing larger sample sizes will be necessary to confirm these results and to evaluate the relationship between the rs17849071 SNP and the *TP53* mutation and *PIK3CA* amplification in BCa patients. It will also be necessary to evaluate the potential prognostic value of this SNP in BCa, especially in various BCa subtypes. Meanwhile, studies that explore the potential molecular mechanisms linking rs17849071 to P53 protein overexpression are warranted.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical Standards We declare that the experiments comply with the current laws of China.

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