Toll-like receptor 3 acts as a suppressor gene in breast cancer initiation and progression: a two-stage association study and functional investigation

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ABSTRACT

Toll-like receptor 3 (TLR3) is a receptor recognizing double-stranded RNA (dsRNA) from viruses as well as from lytic mammalian cells. In the present study, we performed a two-stage association study (n = 3,551) and found that the minor alleles of two SNPs (the T-allele of rs5743312 and the T-allele of rs3775296) conferred increased risks of breast cancer incidence. The adjusted odds ratios (ORs) were 2.281 (P = 7.01 × 10^{-5}) and 2.086 (P = 8.69 × 10^{-5}), respectively. Specifically, the susceptibility variants within TLR3 were significantly associated with larger tumor size (adjusted P-values: 0.004 for rs5743312 and 0.004 for rs3775296). Furthermore, we investigated the biological function of the TLR3 protein in breast cancer cell lines. Notably, the stable expression of TLR3 directly inhibited cell proliferation both in vitro and in vivo. We also verified that TLR3 conferred less invasive phenotypes on breast cancer cells by regulating the mRNA expression of a panel of genes. TLR3-mediated inhibition of proliferation was caused by downregulation of the EGFR/Pi3K/AKT pathway. In summary, our findings strongly suggest that common genetic changes in the TLR3 gene may influence breast cancer susceptibility and development, and TLR3 plays a negative regulatory role in the initiation and progression of human breast cancer cells, at least in part by downregulating the EGFR/Pi3K/AKT pathway.

Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death among women in the West. In some developing countries such as China, breast cancer has increased rapidly in the past three decades. Various mechanisms are involved in the initiation and progression of breast cancer. A large body of evidence suggests that both neovascularization and immune depression play roles in breast cancer etiology for several reasons. Toll-like receptor 3 (TLR3) is a newly defined intersection between immunity and angiogenesis, which suggests its potential functions in cancers. Toll-like receptors (TLRs) have been established as essential factors recognizing various pathogenic microbes that activate innate immunity and adaptive immunity. As a sensor of dsRNA from viruses and mammalian RNA released from lytic cells, TLR3 may be expressed in diverse malignant cell types, and the response to its ligands initiates a signaling cascade resulting in apoptotic cell death. Initial studies illustrated the anticancer properties of TLR3 agonists that display immunostimulatory effects. More current studies have established that TLR3 activation by small interfering RNA may inhibit blood and lymphatic neovascularization. Although several TLR3 downstream mediators may be involved in cancer initiation and progression, the role that TLR3 plays in different cancers is still controversial. The relationships of some pivotal genes in the TLR3-mediated pathway, such as type I IFN, NF-kappaB and IKBKE, with breast tumor biology and prognosis have been well established. However, thus far, few studies have been conducted to illustrate the biological functions of TLR3 in breast cancer cells, and the genetic contribution of TLR3 to breast cancer susceptibility has not yet to be investigated. Accordingly, it is reasonable to further study whether the TLR3-mediated pathway is functional in breast cancer cells.

Results

Genetic variants in TLR3 were associated with breast cancer risk in a hospital-based population

In the present study, we first identified TLR3, IKBKE and TRIF as candidate genes in the TLR3-mediated pathway. Then, we systematically analyzed the genetic variants within these genes in...
a hospital-based population comprising 2,303 unrelated Shanghai women (1,031 sporadic breast cancer cases and 1,272 cancer-free controls). Two alleles and their genotypes were significantly associated with breast cancer, and interestingly, they were all located in the TLR3 gene (Table 1). Increased breast cancer risk was associated with the minor allele and genotypes of two variants in TLR3: one variant was the rs5743312 T allele (OR, 1.173; 95% CI, 1.046–1.315, compared with the C allele, P = 0.006), and the other variant was the rs3775296 +95 T allele (OR, 1.159; 95% CI, 1.036–1.298, compared with the G allele, P = 0.011). Genotypes of both polymorphisms were significantly correlated with breast cancer under a dominant model rather than a recessive model (Table S3). To confirm the associations of genotype with breast cancer, logistic regression analysis was applied with adjustments for age, age at menarche, menopause status, body mass index (BMI) and history of oral contraceptive drugs. The adjusted OR was 2.281 for the TT genotypes of rs5743312 and 2.086 for the TT genotypes of rs3775296 compared with those of major homozygotes. The risk alleles located in TLR3, rather than in IKBKE and TRIF, showed significant associations with breast cancer.

**Associations of individual SNPs in TLR3 with breast cancer risk in a second case-control study**

To further validate the genetic associations observed in the ascertainment study, we analyzed two notable variants in another, independent population and then performed joint analysis. Four hundred and eighty unrelated patients with familial/early-onset breast cancer and 768 unrelated healthy controls from a community-based population were chosen for a second experiment. In this set, we confirmed the highly significant associations of breast cancer with both the rs5743312 polymorphism (OR=95% CI) for T allele versus C allele, 1.379 (1.153–1.673), P = 0.001; OR(95% CI) for TG+TT versus GG, 1.551 (1.225–1.963), P = 2.62 × 10⁻⁴ (Table 2). Because the community screening program is now in progress and epidemiological information is incomplete, we did not adjust for potentially suspected factors in the replication and joint studies. In the joint analysis, both polymorphisms attained highly significant combined P values: 7.01 × 10⁻⁶ for T allele versus C allele and 0.002 for TC+TT versus CC of rs5743312, and 8.69 × 10⁻⁵ for T allele versus G allele and 0.001 for TG+TT versus GG of rs3775296. Compared with their wild-type homozygotes, minor homozygotes of rs5743312 (TT) and rs3775296 (TT) were significantly correlated with breast cancer, with ORs of 1.867 (P = 5.13 × 10⁻⁵) and 1.664 (P = 0.001), respectively (Table 2).

**Genetic association links TLR3 specifically to tumor size**

In addition, we investigated the correlation between polymorphisms within the TLR3 gene and clinicopathologic features, including tumor size and lymph node status. Eight hundred and sixty one unrelated patients were selected from the joint set for analysis, and all the cases had complete information on age, lymph node status and tumor size. It was observed that two variants (rs5743312 and rs3775296) associated with breast cancer initiation were also strongly associated with tumor size. In rs5743312, compared with the CC genotypes, the TC+TT genotype was associated with a significantly increased risk of large tumors (P < 0.001) (OR = 1.576 95% CI 1.24–2.22, P = 7.93 × 10⁻⁴). After adjustments for age and lymph node status, the P values were 0.004 for rs5743312 and 0.004 for rs3775296. However, there were no obvious correlations between the genotype and allele frequencies and lymph node metastasis. When five other SNPs in TLR3 were analyzed, no significant differences between the tested subgroups could be detected (Table 3).

**TLR3 stable overexpression inhibited the growth, redistributed the cell cycle and depressed the motion activity of breast cancer cells in vitro**

To investigate the biological function of TLR3 in human breast cancer cells, we analyzed TLR3 expression using RT-PCR and Western blot in six breast cancer cell lines (the MDA-MB-231-HM cell line was mentioned previously[16]). Among them, MDA-MB-468 cells had the highest TLR3 mRNA and protein expression levels. Compared with the MDA-MB-231-LM cell line, which had the lowest expression, mRNA expression in MDA-MB-468 cells was 37.3-fold higher (Figure 1a,b), and protein expression was 4.9-fold higher (Figure 1c). Then, we transfected the TLR3 expression vector pcDNA™3.1 (+)-TLR3 or empty vector into MDA-MB-231-LM cells and generated stable transfectants. The expression of TLR3 in MDA-MB-231-LM cells was elevated by 10.1-fold at the mRNA level (Figure 1d) and 5.0-fold at the protein level (Figure 1e) compared with expression in MDA-MB-231-Vect cells.
Table 2. Validation of two notable susceptibility SNPs in a second case-control study and in the joint analysis.

| SNPs | Allele or genotype (n = 1,248) | Combined Allele or genotype (n = 3,551) |
|------|--------------------------------|----------------------------------------|
| rs5743312 | C* 652(73.1) 1184(78.9) Reference | 2164(75.0) 3157(79.1) Reference |
| | T* 240(26.9) 316(21.1) 1.379 (1.137–1.673) | 720(25.0) 833(20.9) 1.173 (1.046–1.315) |
| CC | 241(54.0) 474(63.2) Reference | 822(57.0) 1243(62.3) Reference |
| TC+TT | 205(46.0) 276(36.8) 1.461 (1.151–1.854) | 620(43.0) 752(37.7) 1.247 (1.086–1.431) |
| TT | 35(7.8) 40(5.3) 1.721 (1.066–2.780) | 100(6.9) 81(4.1) 1.867 (1.535–2.298) |
| rs3775296 | G 666(72.5) 1170(78.5) Reference | 2185(74.5) 3118(78.5) Reference |
| T | 252(27.5) 320(21.5) 1.383 (1.143–1.674) | 749(25.5) 852(21.5) 1.159 (1.036–1.298) |
| GG | 238(51.9) 466(62.6) Reference | 817(55.7) 1222(61.6) Reference |
| TG+TT | 221(48.1) 279(37.4) 1.551 (1.225–1.963) | 650(44.3) 763(38.4) 1.274 (1.111–1.461) |
| TT | 31(6.8) 41(5.5) 1.480(0.905–2.421) 0.116 | 99(6.8) 89(4.5) 1.664 (1.233–2.245) |

| Tumor size | CC n, (%) | TC+TT n, (%) | OR (95% CI) P \( \chi^2 \) | CC n, (%) | TC+TT n, (%) | OR (95% CI) P \( \chi^2 \) |
|------------|-----------|-------------|----------------|-----------|-------------|----------------|
| 2cm | 327(58.1) 236(41.9) | 1.576 (1.18–2.11) 0.003 0.004 | 333(57.4) 247(42.6) | 1.657 (1.24–2.22) 7.93 × 10\(^{-4}\) 0.004 |
| >2cm | 124(46.8) 141(53.2) | 118(44.9) | 145(55.1) | 185(56.1) | 145(43.9) | 0.844 N.S. N.S. |

| Lymph node | CC n, (%) | TC+TT n, (%) | OR (95% CI) P \( \chi^2 \) | CC n, (%) | TC+TT n, (%) | OR (95% CI) P \( \chi^2 \) |
|------------|-----------|-------------|----------------|-----------|-------------|----------------|
| Negative | 271(53.2) 238(46.8) | 266(51.9) | 247(48.1) | 185(56.1) | 145(43.9) | 0.844 N.S. N.S. |
| Positive | 180(56.4) 139(43.6) | 0.879 N.S. N.S. | 0.844 N.S. N.S. |

\( \chi^2 \): Unadjusted \( P \) value of two-sided \( \chi^2 \) test; \( \gamma \): \( P \) value calculated by logistic regression, adjusted for age, tumor size and lymph node status; N.S.: no significance

To investigate whether overexpression of TLR3 could modulate tumor growth and invasion of MDA-MB-231-LM in vitro, we assessed proliferation and performed an invasion assay with TLR3-transfected, vector-transfected and parental cells. As shown in Figure 2, MDA-MB-231-TLR3 cells had significantly slower proliferation potential compared with that of MDA-MB-231-Vect cells and MDA-MB-231 cells. The growth rate of MDA-MB-231-TLR3 cells was inhibited by parental and vector-transfected cells (\( P < 0.05 \)) in all testing point. Flow cytometry (FCM) analysis was performed to investigate whether TLR3 expression could affect the breast cancer cell cycle. With TLR3 transfection, the proportion of cells in phase G0–G1 relative to MDA-MB-231-Vect cells was dramatically increased from 48.0% to 65.2% and that in phase S was reduced from 40.3% to 25.7% accordingly (\( P < 0.05 \)).

To elucidate the molecular basis underlying TLR3 overexpression in breast cancer cells, we performed serial sectioning and H&E staining. No pulmonary metastasis was observed with either MDA-MB-231-LM cells or MDA-MB-231-TLR3 cells.

**TLR3 overexpression inhibited the growth of orthotopic xenograft tumors**

Additionally, we investigated the effect of TLR3 overexpression on tumor growth in vivo using an orthotopic xenograft tumor model in athymic mice. All mice injected with breast cancer cells developed subcutaneous tumors. However, TLR3 transfectants formed tumors much smaller than those formed by parental and vector-transfected cells (Figure 2b) (\( P < 0.05 \)). We also found that TLR3 upregulation induced less hemorhage and necrosis in primary tumors. To investigate metastatic potential in vivo, we examined the lungs of mice by performing serial sectioning and H&E staining. No pulmonary metastasis was observed with either MDA-MB-231-LM cells or MDA-MB-231-TLR3 cells.

**The molecular mechanisms of TLR3 overexpression in breast cancer cells**

To elucidate the molecular basis underlying TLR3 expression-mediated tumor and metastasis suppression, we further examined the expression changes of a panel of genes, including those involved in well-characterized proliferation, growth arrest, and epithelial–mesenchymal transition-related pathways and some extensively recognized invasion- and metastasis-associated genes.
Quantitative real-time PCR analysis of MDA-MB-231-LM cells, MDA-MB-231-Vect cells and MDA-MB-231-TLR3 cells showed that TLR3 overexpression resulted in the upregulation of Smad2 (2.35-fold) and TGFβ (2.09-fold); the downregulation of EGFR (decreased 72.5%), β-catenin (decreased 65.6%), AKT (decreased 61.7%), Cathepsin-D (decreased 83.5%) and MMP9 (decreased 64.2%); and the stabilization of ERK, p21, MMP2, MMP7, MMP1, bFGF, c-jun, uPA, Cyclin D, Smad3, E-cadherin, VEGF, vimentin, and fibronectin (Figure 3).

Of note, we observed a remarkable feature of TLR3, namely, genetic variants of TLR3 may alter the initiation and development of breast cancer, and TLR3 significantly inhibited the proliferation of breast cancer cells both in vitro and in vivo. We further focused our attention on the EGFR-mediated pathway.
and the molecular basis underlying TLR3-mediated antiproliferation functions. In addition to the real-time PCR analysis described above, Western blot analysis revealed that TLR3 overexpression was constitutive with the downregulation of EGFR and phospho-AKT (p-AKT) in both stable and transiently transfected MDA-MB-231 cells (P < 0.05 or P < 0.01). In contrast, an upregulation tendency without significance was observed for ERK and phospho-ERK (p-ERK) in the same set. (Figure 4)

**TLR3 was less frequently expressed in breast cancer tissues**

We employed a real-time PCR assay to examine the differential expression of TLR3 mRNA in both breast cancer tissues (n = 43) and normal breast tissues (n = 35). TLR3 mRNA levels were normalized to those of GAPDH. The 43 breast cancer tissues and 35 normal tissue specimens were obtained from women receiving surgeries in the Breast Surgery Department. Significantly lower TLR3 mRNA expression levels were observed in breast cancer tissues compared with those in normal breast tissues (Mann-Whitney test P < 0.05, Figure 5).

**Discussion**

As mentioned above, first, we scrutinized sixteen genes within the TLR3-mediated pathway to understand their relationships with inherited genetic variation to breast cancer risk. In the first case-control study, we observed a significant association between two variations within the TLR3 gene and breast cancer. The association was successfully verified in another independent Chinese population, and the combined P value between these two promoters (Supplementary data, Fig. S1).

SNPs are the most common type of genetic variation among people. SNPs may change the encoded amino acids (nonsynonymous) or can be silent (synonymous) or simply occur in the noncoding regions. Most of these variations have no correlation with any disease. Some of them can act as biological markers to locate genes that are associated with disease. Few of them may play a more direct role in disease by affecting the gene’s function when SNPs occur within a gene or in a regulatory region near a gene. These functional SNPs may influence promoter activity (gene expression), messenger RNA (mRNA) conformation (stability), and subcellular localization of mRNAs and/or proteins and hence may produce disease. In this study, the two SNPs are only markers of the TLR3 gene and are not causative polymorphisms. The first SNP, rs5743312, is in the intronic region after exon 2. We used the Splicing-based Analysis of Variants (SPANR) online tool (http://tools.genes.toronto.edu/) to investigate its function in alternative splicing processes. The maximum difference in the percent of transcripts with the exon spliced in (PSI) across tissues (dSPI) of this variant was only −0.09. |dSPI| ≥ 5 is the threshold to determine if a variant is predicted to disrupt splicing, rs5743312 is unlikely to lead to alternative splicing. The second SNP, rs3775296, is in the 5’ untranslated region (UTR) of the TLR3 gene. To investigate its influence on gene expression, we used the pGL3-Basic reporter vector to construct wild-type and 12459C>A (rs3775296) dual-luciferase reporter plasmids (wild-type and 12459C>A vectors). However, no difference in luciferase activity could be observed between these two promoters (Supplementary data, Fig. S1). Therefore, this SNP is not involved in regulation of the TLR3 gene.

TLR3 is a member of the Toll-like receptor family, which plays a fundamental role in pathogen recognition and activation of innate immunity. TLR3 recognizes double-stranded RNA associated with viral infection and induces the activation...
In TLR3 signaling, the pivotal genes NF-κB and IKBKE have relationships with breast tumor biology and prognosis. However, few studies have been conducted to study the biological functions of TLR3 in breast cancer cells. Some studies have reported that TLR3 activation by extrinsic agonists can directly induce apoptosis in breast cancer cells. Nevertheless, endogenous expression of TLR3 and the genetic association between TLR3 and breast cancer remains unclear. Here, we hypothesized that TLR3 is a candidate susceptibility gene for breast cancer. Our analysis was designed as an exhaustive genetic association study using a tagging polymorphism strategy. In the first sporadic breast cancer set, we investigated the associations of a panel of SNPs within TLR3 signaling and successfully found two common polymorphisms (rs5743312 and rs3775296) that were associated with breast cancer risk, and interestingly, they were all located in the TLR3 gene. The role of IKBKE in breast cancer biology and prognosis has recently been well documented, and the hypothesis that variants in IKBKE might contribute to breast cancer risk is appealing. However, our

Figure 4. TLR3 was involved in the EGFR-mediated pathway. EGFR signaling was inhibited by the endogenous expression of TLR3. Western blot analysis of the expression of key factors in the EGFR-mediated pathway in MDA-MB-231 and its TLR3 transfectants. 1–4: MDA-MB-231LM cells transiently transfected with pcDNA3.1(+), MDA-MB-231LM cells transiently transfected with pcDNA3.1 (+)-TLR3, MDA-MB-231-Vect cells, and MDA-MB-231-TLR3 cells. GAPDH was used as an internal control.

Figure 5. Differential TLR3 expression in breast cancer tissues and normal breast tissues. Relative TLR3 mRNA expression was detected in breast cancer tissues (n = 43) and normal breast tissues (n = 35). The horizontal lines represent the mean values. The mean TLR3 mRNA expression level in normal breast tissues was significantly higher than that in breast cancer tissues (P-value of Mann-Whitney test<0.05).
data convincingly demonstrate that none of the tSNPs that effectively capture 89.7% of the IKBKE gene region is likely to have a major influence on breast cancer risk, suggesting that the IKBKE genotype is not implicated in early events in the pathogenesis of breast cancer. Likewise, none of the potential functional loci of TRIF is associated with breast cancer risk in both population sets. Currently, the pathway-based approach rather than the single SNP-based approach is more frequently used for association studies because SNPs with opposite effects may coexist in one pathway. Among our pathway-based SNPs, two susceptibility loci were centralized in TLR3. None of any suspected variants within IKBKE and TRIF was observed to be associated with breast cancer risk in both population sets. Only one SNP in TLR3 (c.1377C>T, rs3775290) has been previously evaluated in relation to breast cancer risk in Croatia. However, this study found no association between this SNP and breast cancer risk, partly because of the limitations imposed by the small sample size (130 cases versus 101 controls) and the limited numbers of SNPs and alleles. Although we did not study rs3775290 in the present study, another tSNP, rs13126816, which can tag rs3775290, was recruited, and in accordance with the previous study, there was no association between this SNP and breast cancer susceptibility. We confirmed the association of breast cancer with the susceptibility SNPs rs5743312 and rs3775296 in another, independent set of familial/early-onset breast cancer patients and community-based controls. Of note, no mutations in high penetrance genes were detected in patients with familial/early-onset breast cancer. Thus, the observed increased risk was largely recognized as the influence of the two observed susceptibility SNPs specifically. Combined analysis of the ascertainment and replication studies demonstrated a highly significant combined P value (10^-5). These findings suggest that rs5743312 and rs3775296 of TLR3 are associated with both sporadic breast cancers and familial/early-onset breast cancers, implying that sporadic and familial/early-onset breast cancer share, at least partially, the same low-penetrance genetic susceptibility. Combined evidence proved that there are common variants in the human genome with modest effects on common disease risk. Thus, the observed 17–55% increase in risk in this study, although modest, is consistent with the hypothesis of common variants.

We investigated the relations of gene polymorphisms with the various clinicopathological features of patients with breast cancer, and of interest, the two SNPs associated with breast cancer initiation in the TLR3 gene also showed poor correlation with parameters of prognosis. The T allele of rs5743312 and the T allele of rs3775296 were more frequently found in patients with large tumors. After adjustment for age and node status, these associations were retained, meaning rs5743312 and rs3775296 are independent predictors of advanced stage and poor prognosis. This result is in keeping with our observation that identified a significant association between the two SNP genotypes and breast cancer susceptibility. Of note, our results have been adjusted for suspected breast cancer risk factors. Adjustments may reveal an independent effect of genetic changes on the significance of our results. In addition, the samples in the present study are free of germine mutations. This result provides convincing evidence that the germline variants rs5743312 and rs3775296 have risk effects on the initiation and development of breast cancer.

Breast cancer appears to be a consequence of both genetic and environmental influences. In linkage analysis and association studies, several susceptibility loci and genes, including BRCA1, BRCA2, ATM and TP53, have been identified as the causal factors of inherited predisposition to breast cancer. Considering the close functional relationships between both PALB2 and BRCA2 and BRIP1 and BRCA1, it is conceivable that mutations and polymorphisms in PALB2 and BRIP1 may account for a proportion of BRCA1/BRCA2-negative breast cancers. Despite these significant advances, the identified genes and loci have failed to satisfactorily explain rates of inheritance. For most familial as well as sporadic cases, a substantial component of risk may be multiple low-penetrance genes. Recently, large-scale genome-wide association studies identified several new, independent, low-penetrance susceptibility loci that are strongly associated with breast cancer in populations of diverse ethnicity. Although whole-genome screening of cancer susceptibility loci is an available methodology, the classic candidate gene strategy of investigating carcinogenesis-related pathways is still frequently employed. Since the two SNPs in this study are only biological markers to locate gene that are associated with disease, we studied the molecular changes in breast cancer cells after TLR3 plasmid transfection. In addition to the novel breast cancer susceptibility loci in TLR3, another important observation is that overexpression of TLR3 inhibits proliferation and invasion both in vitro and in vivo. We established TLR3-overexpressing cell clones, designated MDA-MB-231-TLR3, by stable transfection. We noticed that TLR3 significantly inhibited proliferation and invasion in vitro, which is consistent with those studies provoking TLR3 by using an extrinsic ligand-mimic. In vivo, we also observed that TLR3 significantly inhibited the growth of orthotopic xenografts. The influence of TLR3 on the cell cycle distribution could be observed, and its effects on the mRNA expression of many genes (including EGFR, AKT, β-catenin, Cathepsin-D, MMP9, Smad2, and TGFβ) were significant. EGFR activation has been well established as one of the major tumor proliferation signaling pathways. EGFR transactivation recruits the p85 subunit of PI3K, where the lipid kinase facilitates the activation of key regulatory kinases such as phosphor-Akt. The PI3K/Akt pathway plays an integral role in cell survival and can activate NF-kappaB in a cell type- and stimulus-specific manner. In this study, we observed that TLR3 may efficiently block key factors of EGFR signaling inside cells, and TLR3-mediated proliferation and invasion downregulation occur at least in part through an inhibited EGFR-dependent signaling pathway. The Ras/Erk pathway is another EGFR-mediated pathway related to carcinogenesis. In contrast, upregulation without significance was observed in ERK and phospho-ERK (p-ERK) in the same set. In our study, we observed that overexpression of TLR3 had the tendency to activate the ERK pathway, which is supported by previous reports.

Previous studies showed that TLR3 can activate EGFR via a signaling cascade to produce innate immune response signaling in bronchial epithelial cells. However, in this study,
we showed that the endogenous expression of TLR3 inhibited the EGFR pathway. One possible explanation for these differing results is the various functions of TLR3 in diverse cell types. Another explanation is the methods used to activate TLR3 in diverse studies, which means that extrinsic stimulation and endogenous expression of TLR3 play different roles in EGFR signaling activation. dsRNA, a specific ligand of TLR3, serves not only as a ligand of TLR3 but also of TLR2, RIG-I/MDA5, PKR, NALP3 and Dicer in the RNAi system along with as-yet-unidentified receptors. Consequently, TLR3 specificity was undermined by using an extrinsic ligand. Otherwise, our finding may suggest a unique negative regulation of EGFR signaling that occurs specifically through endogenous expression TLR3.

Another interesting finding is low TLR3 expression in breast cancer samples. In this study, we also examined TLR3 gene expression in 43 cases of human breast cancer samples and 35 cases of normal human breast tissues by real-time PCR. The normal tissues had significantly higher TLR3 expression than that of the cancer tissues, indicating the protective effect of TLR3 against breast cancer. One explanation is that exogenous and endogenous dsRNAs in the human body can kill breast cancer cells by binding to TLR3 and inducing apoptosis. If there is lower expression of TLR3, there is reduced killing of tumor cells.

As a hospital-based study, this investigation may be subject to type I error resulting from population selection bias. However, adequate sample size, a solid genotyping technique, conservative corrections, successful replication and functional demonstration are capable of minimizing the impact of a type I error. Another potential limitation of this study is that results are reported for a single gene. Breast cancer risk may be a complex function of genotypes across several genes. For example, a mutation in one gene may only increase risk in the presence of a mutation in a different gene. Although a gene involved in such gene-gene interactions can be discovered using a marginal test (ignoring the other genes), incorporating information about other genes may improve the power to detect associations, offering the opportunity to investigate the combined contribution of multiple genes to the risk of breast cancer. Furthermore, in our subsequent studies, we will investigate the associations between the two identified SNPs and clinical characteristics, tumor characteristics such as histological type, responses to treatment, and type of cancer.

The clinical, genetic, and functional data presented here implicate TLR3 as a predisposing and suppressor gene in breast cancer. TLR3 plays a negative regulatory role in the proliferation of human breast cancer cells at different stages of the disease, and its mechanism, at least in part, involves downregulation of the EGFR/P13K/AKT pathway.

Materials and methods

Our study's involvement with human subjects complies with the Declaration of Helsinki.

Subjects for ascertainment and replication studies

In the first hospital-based case-control study, 2,303 genetically unrelated Han Chinese women in Shanghai and surrounding regions were recruited between January 2004 and December 2008 in Shanghai Cancer Hospital. All 1,031 eligible patients were pathologically confirmed to be patients with primary sporadic breast cancers (890 invasive breast cancers and 141 ductal cancers in situ with or without microinvasion). Patients with distant metastatic breast cancer or other cancers were excluded. The 1,272 control subjects, matched to the patients based on age and geographical regions, were chosen from women who had come to the outpatient department of Shanghai Cancer Hospital, mainly for breast cancer screening. The selected controls were confirmed to be free of breast cancer by comprehensive physical examinations, mammography, ultrasonography and biopsy if necessary. Some controls were diagnosed as benign disorders by biopsy and pathologically confirmed as fibrocystic diseases, which conferred no increased risk for breast cancer according to the current evidence. Women with cancer history were excluded from the control group. All participants had complete epidemiological and clinicopathological information stored in a computerized database established by the Department of Breast Surgery of the Shanghai Cancer Hospital as described previously. In brief, patients and controls in the ascertainment study were comparable in age [mean age ± standard deviation (SD) for the patients was 48.6 ± 10.0 years versus 47.0 ± 9.3 years for the controls, P > 0.05], age at menarche (patients 15.0 ± 1.8 years versus controls 15.0 ± 1.8 years, P > 0.05), menopausal status (41.9% of patients versus 39.1% of controls were postmenopausal, P > 0.05), BMI (patients 23.2 ± 3.0 versus controls 23.2 ± 2.6, P > 0.05) and family history (9.8% of patients versus 10.1% of controls with breast cancer family history, P > 0.05). Because the majority of Chinese women are nonsmokers and nondrinkers, this study was restricted to women who were neither cigarette smokers nor alcohol consumers. The study was approved by the Scientific and Ethical Committee of the Cancer Hospital of Fudan University. Informed consent was obtained from each subject involved.

We further validated SNPs that showed significant associations with breast cancer in a second independent population of familial/early-onset breast cancer cases and community-based controls. Since 2000, Shanghai Cancer Hospital has conducted a multicenter hospital-based BRCA mutation screening project to obtain a full understanding of the contribution of BRCA1/2 mutations to Han Chinese hereditary breast cancer. Approximately 600 cases from five medical centers in China have been recruited since 2000. The eligibility criteria have been published previously. In the present study, we chose 480 unrelated familial (at least one first- or second-degree relative was a breast cancer patient) and/or early-onset (breast cancer diagnosed younger than 35 years old) cases from Shanghai City and its surrounding regions. All selected familial/early-onset cases had been tested for BRCA1, BRCA2, BRIP1 and PALB2 germline mutations, and no deleterious genetic changes were found. Additionally, 768 cancer-free women were identified as controls from a community-based breast
cancer screening project initiated in May 2008 by the Shanghai Cancer Hospital and the Shanghai Municipal Center for Disease Control and Prevention (CDC). General information about the project has been described previously. The project is currently ongoing and has involved more than 10,000 women, 5,000 of whom provided blood genomic DNA samples. Because epidemiological information derived from questionnaires was not completely input into the database, we did not make adjustments for potentially suspected factors in the replication study.

**DNA/RNA preparation**

Genomic DNA for genotyping was extracted from 3 to 5 ml of the study participants’ peripheral blood leukocytes using a PureGene DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN, USA) according to the manufacturer’s protocol and then stored at −20°C. Breast tissue samples obtained from women receiving surgeries in the Breast Surgery Department of the Shanghai Cancer Hospital were snap-frozen in liquid nitrogen and stored at −80°C in an established breast tissue bank. mRNA was extracted from frozen breast tissue specimens, as well as cultured cells, and reverse-transcribed. General PCR was performed as described previously. Here, we defined breast tissue samples from healthy cancer-free women without any family history or personal history of cancer as ‘nonsusceptible’ normal breast tissues.

**Candidate genes and SNP selection in the trlr3-mediated pathway**

In the TLR3 signaling pathway, we chose three genes, TLR3, TRIF (a TLR3-specific adaptor molecule) and IKBKE, as candidate genes. In the TLR3 gene, SNPs spanning a 17.0-kb region from 0.8 kb upstream of the 5’-flanking region to 0.5 kb downstream of the 3’-flanking region were surveyed in NCB1-dbSNP and the International HapMap database of the Han Chinese population (HapMap Data Rel 21a/phaseII Jan07). Tagging SNPs (tSNPs) were selected using the pairwise method under the restrictions of MAFs>0.05 and r² > 0.8 to identify tSNPs that efficiently capture common variants and tag most unknown variants. A total of ten tSNPs (rs11730143, rs7657186, rs13126816, rs6552950, rs5743312, rs3775296, rs3775291, rs10025405, rs11721827 and rs76686666) that captured all alleles were identified. Because rs11721827, rs6552950 and rs10025405 were located in introns or could be captured by other SNPs, we excluded them from further genotyping. Finally, we chose seven representative tSNPs that effectively captured all SNPs in TLR3. In addition, variants in TLR3 with potential functional effects (such as causing amino acid changes or alternative splicing or locations in putative transcription factor binding sites) were chosen for genotyping whenever possible. We used potentially functional polymorphisms with MAFs>1%. Therefore, we added an additional SNP, rs5743316, located in an exon for further genotyping. Finally, for all eight candidate SNPs, three potentially functional polymorphisms were selected, including two missense SNPs, rs3775291 (Leu412Phe) and rs5743316 (Asn284Ile), and one SNP (rs3775296) with suspected functional change in the 5’ UTR region (Figure 6).

**SNP genotyping**

In the first stage, genotyping of the sixteen selected SNPs was performed with the 12-plex SNPstream system (Beckman Coulter, Fullerton, CA, USA) and was carried out by the Chinese National Human Genome Center (Shanghai, China). Call rates for genotyping ranged between 91 and 100%. After the first stage of genotyping, one missense SNP in TLR3, rs5743316 (Asn284Ile), was excluded because no polymorphism was observed in the Shanghai population, and one intron tSNP in TLR3, rs76686666, was excluded because of a lower call rate.

In the validation study, rs3775296 and rs5743312 were genotyped with the same methods. The sequences of primers and probes for each SNP are provided in Table S1.

**Cell lines and mice**

The human breast cancer cell lines MDA-MB-468, MCF-7, T-47D, MDA-MB-231, 293T and SUM-149 were obtained from the American Type Culture Collection and grown in complete growth medium as recommended by the manufacturer and described previously. Female BALB/c-nu/nu nude mice, 4–6 weeks old, were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China), and raised in laminar flow cabinets under specific pathogen-free conditions with food and water ad libitum. All experiments in mice were conducted in accordance with the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals. The study protocol was also approved by the Institutional Animal Care and Use Committee of Fudan University (Shanghai, China).
Plasmid constructs and stable transfection

A human TLR3 expression vector was constructed using the pcDNA3.1 Directional TOPO Expression Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The entire open reading frame of the human TLR3 gene was amplified from 293T cells by RT-PCR using gene-specific primers (upstream primer, 5’-CCCAAGCTTGGTAGCATCAGAAATCTCAATC-3’, with an added HindIII site underlined; downstream primer, 5’-CGGAAATTCCGATTTAATAATTTTAGCATCAGAG-3’, with an added EcoRI site underlined). All constructs were verified before use by direct sequencing. MDA-MB-231 cells were transfected by Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. After selection in the presence of 1,000 µg/ml geneticin (G418 sulfate; Invitrogen) for 4 weeks, stably transfected TLR3 and vector clones derived from MDA-MB-231-LM were named MDA-MB-231-TLR3 and MDA-MB-231-Vec, respectively. RT-PCR and Western blotting analyses were performed to measure expression levels.

Real-time PCR and western blotting analyses

cDNA-specific primers were designed using Primer Premier 5.00 (Premier Biosoft International, Palo Alto, CA, USA) for human genes. cDNA was then subjected to quantitative real-time PCR. The expression of human genes was analyzed by a SYBR Green fluorescence-based assay (Takara Bio, Otsu, Japan) in a fluorescence temperature cycler (Opticon; MJ Research, Watertown, MA, USA) using the standard curve method as described previously or a modified 2^(-ΔΔCt) method. All samples were tested in triplicate, and the mean values were determined. The primer sequences and PCR conditions are described in Supplemental Table S2. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control.

Western blotting analysis was carried out according to standard protocols. Briefly, equal amounts of total protein from different cells were separated by 10% SDS-PAGE and then incubated with relevant monoclonal antibodies against target proteins (the TLR3 antibody was from GeneTex, Inc., and the others were from Santa Cruz Biotechnology, Inc.). Target proteins were detected by an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposure to FujiFilm Las-3000. Protein expression of the GAPDH gene was measured for normalization. Images were analyzed by BandScan V5.0 software.

Cell proliferation assays and cell cycle analysis

A total of 5 × 10^3 cells were plated in duplicate wells of 96-well plates and allowed to adhere overnight. After 48 h, indices of cell proliferation were determined with Cell Counting Kit-8 (Dojindo, Molecular Technologies, Gaithersburg, MD). For cell cycle analysis, cells at the exponential growth phase were harvested, and single-cell suspensions containing at least 1 × 10^6 cells were treated following the standardized protocol; the cell cycle was analyzed by flow cytometry.

Wound closure assay

Cells were plated in the wells of 6-well plates. Confluent cell monolayers were wounded by manually drawing a furrow across each monolayer with a 20-µl pipette tip. The cell culture medium was then replaced with fresh medium, and wound closure was monitored by phase contrast microscopy at various times. The wound area at each time point after wounding was quantified using Quantity One 1-D (Bio-Rad Laboratories, Inc.) software.

Lentivirus transduction

The lentivirus of the TLR3 shRNAs (PKO.1 vector, target sequences: sh1-TCACGCAATTGGAAGATTATT; sh2-CCG CCACTTTCAAAGGTA) and TLR3 overexpressing (NM_003265, pCDH vector) or their vector control lentivirus were from Shanghai BaiXu Co., Ltd. (Shanghai, China). Viral infection was performed in 6-well plates using 2 × 10^6 cells in a total volume of 0.5 mL of lentiviral supernatant containing 8 µg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). Two days after infection, puromycin (Sigma-Aldrich, St. Louis, MO, USA) was added to the media at 1 µg/mL and selected for one week. The expression levels of the target genes were determined by Western blot analysis.

Assessment of tumorigenicity in vivo

The tumorigenicity and spontaneous metastatic capability of the cell lines were determined by injecting cells (1 × 10^6/mL cells) subcutaneously into the mammary fat pad, as described previously. Animals were divided into two groups, MDA-MB-231-Vec and MDA-MB-231-TLR3, and each group had six mice. Tumor length and width were measured weekly with calipers. Tumor volume was calculated by the formula (a × b^2) × 0.5, where a and b mean the long and short dimensions, respectively. Mice were sacrificed and autopsied when tumors reached 1.5 cm in diameter. The tumors were then removed and weighed. To evaluate metastatic potential, whole lungs of mice were sectioned at 50-µm intervals and stained with hematoxylin and eosin.

Statistical analysis

The observed genotype frequencies in the controls were tested for Hardy–Weinberg equilibrium (HWE) using χ^2 tests for each SNP locus. Tests of association were conducted using 2 × 2 or 2 × 3 Pearson’s χ^2 test or Fisher’s exact test. The significance of association results were corrected using permutation tests. To compare continuous variables, a Student’s t test or Mann–Whitney test was used between two groups, and one-way ANOVA or the Kruskal–Wallis test was used among three or more groups. The OR and its 95% CI for an association between a single locus and breast cancer risk or a tumor characteristic were calculated by logistic regression, adjusting for suspected factors if available, to determine the value of the genotype as an independent prognostic marker. All statistical tests and corresponding P values were two-sided. Differences were considered significant at P < 0.05. Statistical analysis was performed using
Conflict of interest statement
All authors declared no potential conflicts of interest.

Funding
This research was supported by grants from the National Natural Science Foundation of China [81102003].

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