Substantial reduction in risk of breast cancer associated with genetic polymorphisms in the promoters of the matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 genes

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Introduction

The matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) have been shown to play important roles in multiple ways in all stages of cancer initiation and development. Single nucleotide polymorphisms identified in the promoters of MMP2 (−1306C → T) and TIMP2 (−418G → C) abolish the Sp1-binding site and thus may down-regulate expression of the genes. This case-control study examined the contribution of these functional polymorphisms to susceptibility to development and metastasis of breast cancer. MMP2 genotypes were determined by PCR-based denaturing high performance liquid chromatography analysis and TIMP2 genotypes identified by a PCR-RFLP method in 462 breast cancer patients and 509 frequency matched control women. We found that the variant MMP2 genotype (−1306CT or TT) was associated with substantially reduced risk of breast cancer [odds ratio (OR), 0.46; 95% confidence interval (95% CI), 0.34–0.63], compared with the CC genotype. For TIMP2, a moderately reduced risk of the cancer (OR, 0.76; 95% CI, 0.58–0.99) was also associated with the variant allele (−418GC or CC), compared with the GG common allele. Furthermore, it seemed that the polymorphisms in the two genes had some additive effect and the reduced risk related to the polymorphisms appeared to be more pronounced in younger subjects. However, no significant associations were observed between the polymorphisms in the two genes and tumor stage and metastasis of the cancer at the time of diagnosis. These findings suggest that the presence of the variant allele in the promoter of MMP2 or TIMP2 may be a protective factor for the development of breast cancer.

Abbreviations: CI, confidence interval; DHPLC, denaturing high performance liquid chromatography; FGF, fibroblast growth factor; IGF, insulin-like growth factor; MMPs, matrix metalloproteinases; OR, odds ratio; TIMPs, tissue inhibitor of metalloproteinases; TNM, UICC Tumor–Node–Metastasis classification.
Although the functional significance of this germline polymorphism is currently unknown, down-regulation of the transcriptional activity due to the variant has been suggested because the G → C substitution is located within the consensus sequence for the Sp1-binding site in the promoter region of TIMP2 (23) and this polymorphism has been associated with chronic obstructive pulmonary disease (22). It is, therefore, reasonable to postulate that this polymorphism may down-regulate TIMP-2 expression and consequently cause an imbalance between the activities of TIMP-2 and MMP-2, which is believed to have a significant impact on cancer development and progression (21,24–26).

We have previously reported that the frequency of the MMP2 −1306CC genotype was over-represented in patients with lung cancer compared with cancer-free controls and this genotype was associated with a several fold increased risk of lung cancer alone or in conjunction with smoking exposure (27). We have also demonstrated a significant association between the MMP2 polymorphism and risk of development but not metastasis of gastric cardia adenocarcinoma (28). These findings raise the hypothesis that the MMP2 genotype that produces an increased level of MMP-2 over a lifetime might render the carriers more susceptible to tumorigenesis. The objective of this study was to examine the possible correlation between MMP2 and TIMP2 promoter polymorphisms and predisposition to the development and metastasis of breast cancer.

Materials and methods

Study subjects
This study included 462 incident breast cancer patients and 509 healthy population controls. All subjects were ethnically homogeneous Han Chinese women. Patients with breast cancer were consecutively recruited from January 1997 to November 2001, at the Cancer Hospital, Chinese Academy of Medical Sciences (Beijing). All eligible patients diagnosed at the hospital during the study period were recruited, with a response rate of 90%. Patients were from Beijing city and its surrounding regions and there were no age, stage or histology restrictions. The presence (M+) or absence (M−) of detectable metastases was evaluated according to the UICC Tumor–Node–Metastasis classification (TNM) for breast cancer at diagnosis (29). The clinical and histochromic features of the patients are summarized in Table I. Population controls were cancer-free women living in the Beijing region; they were selected from a nutritional survey conducted in the same period as the cases were collected (27). The control subjects were randomly selected from a database consisting of 2500 individuals based on a physical examination. The selection criteria included no history of cancer and frequency matching by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute.

Polymorphism analysis
Genomic DNA was isolated from the peripheral blood lymphocytes of the study subjects. Genotypes were analyzed using PCR-based methods as described below. Genotyping was performed without knowledge of subjects’ case/control status. A 15% masked, random sample of cases and controls was tested twice by different persons and the results were concordant for all masked duplicate sets.

MMP2 genotypes were determined by PCR-based denaturing high performance liquid chromatography (DHLPC) analysis and directly DNA sequenced as described previously (27,28). The primers used to amplify a 295 bp fragment of the MMP2 promoter containing the −1306 G/C site were: MMP2-2F, 5'-CGT CTC TTG TTG GCT GGT CA; TIMP-2R, 5'-CCT TCA GCT. Amplification was accomplished with a 25 μl reaction mixture containing −100 ng template DNA, 0.5 μM each primer, 0.2 mM dNTP, 2.0 mM MgCl2, 1.5 mM MgCl2, and 1.2 U Taq DNA polymerase with 1× reaction buffer (Promega). The PCR profile consisted of an initial melting step of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C, with a final elongation of 7 min at 72°C. DHPLC analysis was performed on a Transgenomic WAVE System (Transgenomic Inc., Omaha, NE) identical to that described previously (27,28).

The genotypes of TIMP2 at the −418 G/C site were analyzed by a PCR-RFLP method on the basis of that reported previously (22). The primers used, which produced a 304 bp fragment containing the −418 G/C site, were: TIMP-2F, 5'-CGT CTC TTG TTG GCT GGT CA; TIMP-2R, 5'-CCT TCA GCT CGA CTC TGG AG. Amplification was accomplished with a 25 μl reaction mixture containing −100 ng template DNA, 0.5 μM each primer, 0.2 mM dNTP, 2.0 mM MgCl2, and 1.2 U Taq DNA polymerase with 1× reaction buffer (Promega). The PCR profile consisted of an initial melting step of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C, with a final elongation step of 7 min at 72°C. The 304 bp PCR products were then subject to digestion with BsoBI (New England Biolabs, Beverly, MA) and separated on a 2.5% agarose gel. The G allele had two BsoBI restriction sites and resulted in three bands (230, 51 and 51 bp). The C allele lacked one BsoBI restriction site and thus produced two fragments of 283 and 51 bp.

Statistical analysis
The χ² test was used to examine the differences in the distributions of genotypes between cases and controls. The association between the MMP2 or TIMP2 polymorphisms and risk of breast cancer was estimated by odds ratios (ORs) and their 95% confidence intervals (95% CIs), which were calculated by unconditional logistic regression models. We tested the null hypotheses of additivity and multiplicative effects and evaluated the departures from additive and multiplicative interaction models (31).

Results
The allele frequencies and genotype distributions of MMP2 and TIMP2 in patients and controls are summarized in Table II.
The allele frequencies for the MMP2 −1306C and −1306T were 83.7 and 16.3% in patients, compared with 91.0 and 9.0% in patients ($P < 0.00001$). The genotype distributions in patients were 82.5 (CC), 17.1 (CT) and 0.4% (TT) and in controls were 68.6 (CC), 30.2 (CT) and 1.2% (TT), neither of which deviated from those expected from the Hardy–Weinberg equilibrium ($P = 0.678$ and 0.179, respectively).

Although the TT homozygote patients failed to demonstrate a difference from controls due to the extreme rarity of this genotype in both patients and controls, the frequency of the heterozygote (CT) was significantly lower in patients than in controls (17.1 versus 30.2%; $\chi^2 = 22.98$, $P < 0.00001$). The frequencies of the alleles TIMP2 −418G and −418C were 83.5 and 16.5% in patients and 80.3 and 19.7% in controls, a difference which was only borderline significant ($P = 0.060$).

The distributions of three TIMP2 genotypes in patients and controls also fitted the Hardy–Weinberg equilibrium law ($P = 0.959$ and 0.462, respectively). The frequencies of the three TIMP2 genotypes in patients were 69.5 (GG), 28.1 (GC) and 2.4% (CC), which were not significantly different from those in controls ($\chi^2 = 4.20$, $df = 2$, $P = 0.123$). However, as shown in Table II, 28.1% of patients carried the GC genotype, and this difference from controls due to the extreme rarity of this genotype was only borderline significant ($P = 0.060$).

The results of the analysis of a potential combined effect between the MMP2 −1306 and TIMP2 −418 genotypes on risk of breast cancer are shown in Table III. Although no significant interaction between the polymorphisms of the two genes was observed, patients with the MMP2 −1306CT or TT genotype appeared to be less likely to carry the TIMP2 −418GC or CC genotype compared with controls (6.0 versus 12.8%; $P = 0.0002$). Among subjects carrying both MMP2 variant and TIMP2 variant genotypes, the OR for breast cancer was 0.36 (95% CI, 0.23–0.59), while the ORs were 0.47 (95% CI, 0.32–0.69) among subjects carrying the MMP2 variant and TIMP2 wild-type genotypes and 0.78 (95% CI, 0.57–1.07) among subjects carrying the MMP2 wild-type and TIMP2 variant genotypes, respectively ($\chi^2 = 27.5$, $P < 0.00001$, test for trend). These data may suggest an additive effect between the TIMP2 variant and MMP2 variant alleles on reduced risk of developing breast cancer according to the statistical models (31).

The effects of the MMP2 and TIMP2 polymorphisms were additionally examined with stratification by age, tumor size, metastatic status and common prognostic factors such as estrogen and progesterone receptors. It was found that reduced risk of breast cancer associated with the MMP2 variant (−1306CT or TT) genotypes was more pronounced in subjects who were younger (≤55 years) at diagnosis (OR, 0.42; 95% CI, 0.29–0.61), compared with that in subjects who were
Discussion

Breast cancer is one of the leading causes of cancer death among women in both China and Western countries. Although 10–15% of breast cancer patients have some family history of the disease, only 5% can be accounted for by highly penetrant germline mutations in genes such as BRCA1 and BRCA2 (32). It has been shown that first degree relatives of breast cancer patients have a 2-fold elevated risk over the general population (33), most of which cannot be explained by BRCA1/2 (34). Although some of the familial risk may be due to shared environmental factors, there may be other common, low penetrance genetic variants affecting susceptibility to breast cancer. Because the MMP2/TIMP2 system has a significant impact on the development and progression of cancer, including breast cancer, and because genetic polymorphisms in the promoters of MMP2 and perhaps TIMP2 are correlated with decreased enzyme activity (vide supra), we sought to determine whether these polymorphisms may be associated with varying risk of breast cancer. On the basis of analysis of 462 patients with breast cancer and 509 controls, we found that the MMP2 and TIMP2 polymorphisms influenced risk of developing but not TNM stage and metastasis of breast cancer. Subjects carrying the variant genotypes of MMP2 or TIMP2 were at a moderately reduced risk of cancer. In addition, it appeared that the polymorphisms in the two genes had some additive effect with regard to reducing breast cancer risk and the protective effects were more pronounced in younger subjects (<55 years old), which is in line with the conception that genetic susceptibility is often associated with an early age of disease onset (35–37). To the best of our knowledge, this is the first study to examine the relationship between the MMP2 and TIMP2 polymorphisms and breast cancer risk. These results are consistent with our previous findings for the MMP2 polymorphism in lung and gastric cardia cancer studies (27,28).

Our result showing a protective effect of the MMP2 polymorphism against the risk of breast cancer is supported by several lines of previous findings. Firstly, it was reported that the −1306C→T transition in the promoter region of MMP2 leads to a strikingly lower promoter activity with the T allele due to disruption of an Sp1-binding site (15). Deletion or site-directed mutagenesis analysis of the MMP2 promoter has also shown that the Sp1 site, among other promoter elements such as AP-2, is critical for constitutive activity of this gene (14). On the other hand, a recent study demonstrated that a reduction in Sp1 DNA binding activity or phosphorylation by non-steroidal anti-inflammatory drugs suppresses MMP2 expression (38). These data clearly suggest that absence of the Sp1 consensus sequence in the MMP2 −1306T allele would produce a lower level of MMP-2 protein in individuals carrying the CT or TT genotype than those carrying the CC genotype. Because MMP-2 has been shown to play an important role in the development of cancers, including breast cancer (8–12), reduced expression would be expected to be associated with a reduced risk of cancer. Secondly, several studies with genetically modified animals have associated a low level of constitutive expression of MMP-2 with reduced risk of tumor formation. It was found that when induced by carcinogenic stimulus, mice that lack the Mmp2 or Mmp9 gene developed fewer tumors than wild-type mice (39). Cancer cells injected via a vein were found to be less capable of colonizing the lungs of Mmp2 knockout mice than the lungs of wild-type mice (40). Of particular interest, transgenic mice that overexpress MT-MMP-1, a known activator of pro-MMP-2, were at increased risk of mammary tumor formation and metastasis (41). In addition, functional polymorphisms in some other MMP genes have also been linked to varying susceptibility to certain cancers (42–45) and a single adenosine insertion polymorphism in the MMP3 promoter (6A allele), which has half the transcriptional activity of the 5A allele, has been associated with a reduced risk of breast cancer (46).

A provocative finding from this molecular epidemiological study was the observation that a moderately decreased risk of breast cancer was associated with the TIMP2 −418GC→C polymorphism, which is located within the Sp1-binding site in the promoter of the gene and presumably affects transcriptional activity (22,23). Because TIMP-2 is considered an endogenous inhibitor of MMP-2, the reason why the variant alleles are less susceptible to breast cancer may not be immediately evident. However, accumulating evidence indicates that, in addition to the inhibitory effect on MMP-2, TIMP-2 may act as a multifunctional molecule, which promotes tumor cell growth and tumor angiogenesis and inhibits tumor cell apoptosis (1,47). These effects of TIMP-2 through MMP-2-dependent or -independent pathways make it paradoxical in carcinogenesis. In fact, several clinical investigations have correlated high levels of TIMP-2 with proliferation and/or progression of breast cancer (48–50) and other cancers (21,51). Furthermore, it is worth noting that other members of the TIMP family, such as TIMP-1 and TIMP-4, also have promotive effects on the growth of breast cancer cells (52,53). Our results in the present study are parallel to these previous findings and suggest that lower constitutive expression of TIMP-2 might render the hosts less susceptible to breast cancer, probably through the MMP-independent pathway.

Despite the existence of conflicting results, it is generally believed that local overexpression of MMP-2 promotes and TIMP-2 inhibits cancer invasion and metastasis. Several studies have suggested that genetic polymorphisms in the promoter of MMP1 (1G/2G) or MMP3 (5A/6A), which alter the transcription activity of the genes, may influence invasiveness or metastasis of some types of cancer such as melanoma (54), colorectal cancer (55) and breast cancer (46). However, in the present study, neither the MMP2 nor TIMP2 genotype was significantly correlated with tumor stage or metastatic status of breast cancer at the time of diagnosis. These results suggest that the examined polymorphisms in MMP2 and TIMP2 might not play the major role as a relevant genetic factor suppressing or inducing local expression of MMP-2 and TIMP-2 and, therefore, might not serve as a sole risk marker of metastatic disease. However, our data on breast cancer metastasis have some limitations because they were obtained at the time of diagnosis. Further examinations of larger patient series with prospectively followed-up clinical outcome, especially the survival rate, may be required.
In conclusion, our study suggests that presence of the variant allele in the promoter of MMP2 or TIMP2 may be a protective factor for the development but not metastasis of breast cancer in Chinese women. This activity might result from the genetically determined balance of MMP-2 and TIMP-2, which seems to have the ability to suppress breast cancer cell growth.

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References


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