No Association of Matrix Metalloproteinase [MMP]-2 (–735C > T) and Tissue Inhibitor of Metalloproteinase [TIMP]-2 (–418G > C) Gene Polymorphisms with Cervical Cancer Susceptibility

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Received: 27 February 2012 / Accepted: 13 June 2012 / Published online: 3 July 2012
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Abstract Matrix metalloproteinase [MMP]-2 and tissue inhibitor of metalloproteinase [TIMP]-2 are emerging as pivotal players in inflammation and carcinogenesis. The present study aimed to evaluate the role of *MMP*-2 (–735C > T) [rs 2285053] and *TIMP*-2 (–418G > C) [rs 8179090] gene polymorphisms in cervical cancer susceptibility in Indian women. We recruited 200 cervical cancer patients from North India and 200 unrelated, age-matched, cancer-free healthy female controls of similar ethnicity. Genomic DNA extraction from peripheral blood samples, collected from the study subjects, was carried out using salting-out method. MMP-2 and TIMP-2 genotyping was performed using polymerase chain reaction-based restriction fragment length polymorphism. Our findings demonstrated no significant association between *MMP*-2 (–735C > T) and *TIMP*-2 (–418G > C) gene polymorphisms and the risk of developing cervical cancer in the study population. Further stratified analysis using a case-only study approach revealed that there was no effect of MMP-2/TIMP-2 polymorphisms on early and advanced stages of cervical cancer. Further MMP-2 and TIMP-2 polymorphisms did not modulate the risk in cervical cancer

patients who smoked tobacco/cigarettes. Overall, the present study demonstrated a lack of association between MMP-2 and TIMP-2 gene polymorphisms and cervical cancer susceptibility in women of Northern India.

Keywords Cervical cancer · Matrix metalloproteinase · Susceptibility · Tissue inhibitor of metalloproteinase

Introduction

Cervical cancer, the second most common cancer in women worldwide, is emerging as a major health concern in recent times [1]. The etiopathogenesis of cervical cancer is indeed complex and therefore, deciphering the underlying cellular/molecular mechanisms in cervical carcinogenesis is one of the primary objectives in cancer research. Although human papillomavirus (HPV) has been implicated as the major etiological agent in cancer of the uterine cervix, yet the viral infection alone does not predispose women to develop cervical cancer; host genetic factors may govern the inter-individual susceptibility and/or determine the risk for progression to invasive carcinoma [2]. Matrix metalloproteinase [MMP]-2 and tissue inhibitor of metalloproteinase [TIMP]-2 have been implicated as pivotal players in inflammation and carcinogenesis.

MMP-2 (also known as 72 kDa gelatinase or type IV collagenase) is a member of the MMP family that primarily hydrolyzes gelatin and type IV collagen [3, 4], the major structural component of basement membrane. This enzyme also has an activity against a spectrum of other proteins, such as growth factor-binding proteins and growth factor receptors.

Furthermore, the production of this proteinase in tumors is not only by cancer cells but also by normal stromal and

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endothelial cells, suggesting that the over-expression of MMP-2 is probably due to transcriptional changes and not gene amplification or an activating mutation. Because the human MMP2 promoter contains a number of cis-acting regulatory elements, the constitutive and induced expression of this proteinase is likely to be subjected to regulation by transcription factors.

Further the activity of MMP-2, among other MMPs, is also regulated by endogenous factors, including a family of anti-proteinases known as tissue inhibitors of metalloproteinases (TIMPs). Of the four members in the TIMP family, TIMP-2 is particularly interesting because of its dual functions in terms of regulating MMP-2 activity [5] and its paradoxical effects on certain cancers [6, 7]. A single nucleotide polymorphism (−418G/C) has also been identified in the promoter of the TIMP2 gene [8].

Although the functional significance of this germ line 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 [9]. It is, therefore, reasonable to postulate that this polymorphism may downregulate 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 [7, 10].

The present study was aimed to evaluate the role of *MMP-2* (−735C > T) [rs 2285053] and *TIMP-2* (−418G > C) [rs 8179090] gene polymorphisms in cervical cancer susceptibility in Indian women.

Materials and Methods

Study Subjects

The present case–control study included 200 histopathologically confirmed patients of cervical cancer of North Indian ethnicity and 200 unrelated, age-matched, cancer-free healthy female controls of similar ethnicity. Peripheral blood samples were collected from cervical cancer cases, classified according to the International Federation of Gynecology and Obstetrics (FIGO) staging system, and controls with no history of malignancy, drug allergy, hypertension, diabetes or cardiovascular disease. A questionnaire-based survey was conducted for assessment of demographic data, cancer stage and tobacco status of patients. Written informed consent was taken from the study subjects prior to enrollment; the study protocol was approved by local ethics review committee.

DNA Extraction

Blood sample (2–3 ml) was drawn from cervical cancer cases and control subjects; blood samples were collected in ethylene diamine tetraacetic acid (EDTA) vials. Genomic DNA was extracted from peripheral blood leucocytes using the salting-out method [11]. DNA quality was determined using 1 % agarose gel electrophoresis followed by staining with ethidium bromide. Purity of DNA was determined by taking the optical density (OD) of the samples at 260 nm and 280 nm using the Nanodrop Analyzer (ND-1000) spectrophotometer (Nano Drop Technologies Inc., Wilmington, DE, USA). DNA samples for which the absorbance ratio of 260/280 corresponded to 1.7 were considered to be of high purity. The DNA quantity was estimated by nano-drop spectrophotometer and expressed in nanogram per microlitre (ng/μl).

MMP-2 (−735C > T) [rs 2285053] and *TIMP-2* (−418G > C) [rs 8179090] Genotyping

Genotyping of the SNPs; *MMP2* (−1306) C/T; (735) C/T and *TIMP2* (−418) G/C; (−303) C/T was successful for 200 cases and 200 controls. The genotype variants were analyzed using polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) methodology for all the SNPs. Primer sequences, PCR conditions and the restriction enzymes used have been described elsewhere for both *MMP2* and *TIMP2* [12–15]. Positive and negative controls were used in each genotyping assay, and 5 % of the samples were randomly selected and run in duplicates with 100 % concordance. Some of the samples selected randomly were also subjected to sequencing for validation. The results were reproducible with no discrepancy in genotyping.

Statistical Analysis

The sample size was calculated using QUANTO software, version 1.1 (<http://hydra.usc.edu/gxe>). Deviation from Hardy–Weinberg equilibrium in controls was assessed by the χ^2 goodness of fit test; Chi-square analysis was used to determine differences in genotypic and allelic frequencies. Age-variable was expressed as mean $\pm$ standard deviation (s.d.). Logistic regression analysis was carried out to estimate age-adjusted odds ratio (OR); tests of statistical significance were two-sided and taken as significant when *P* value was less than 0.05. All statistical analyses were performed using Statistical Package for Social Sciences version 15.0 (SPSS, Chicago, IL, USA).

Results

Demographic and Clinical Characteristics of Study Subjects

The present study included 400 study subjects i.e., 200 histopathologically confirmed cases of cervical cancer and 200 unrelated, cancer-free, healthy controls. Cervical cancer patients and control subjects were of similar ethnicity and comparable in age (48.27 ± 10.04 vs. 46.95 ± 9.81 years). Clinical diagnosis/staging of cervical cancer patients was performed as per the guidelines outlined by the International Federation of Gynecology and Obstetrics (FIGO). FIGO stages I, II, III and IV were diagnosed in 200 cervical cancer cases; 21 patients (10.5 %) were assigned stage I while clinical stages II, III and IV were diagnosed in 71 (35.5 %), 104 (52.0 %) and 4 (2.0 %) patients, respectively. Majority of cervical cancer patients (52 %) had stage III. In our cancer patients, 55 (27.5 %) were tobacco users and 145 (72.5 %) were non-users.

MMP-2 (−735C > T) [rs 2285053] and *TIMP-2* (−418G > C) [rs 8179090] Gene Polymorphisms and Cervical Cancer Susceptibility

In the present case–control study, the association of *MMP-2* (−735C > T) and *TIMP-2* (−418G > C) gene polymorphisms with the risk of developing cervical cancer was investigated. A total of 200 histopathologically confirmed cases of cervical cancer and 200 cancer-free, unrelated, healthy controls were included in the analysis. Genotype and allele frequency distributions of *MMP-2* (−735C > T) and *TIMP-2* (−418G > C) gene polymorphisms were assessed. All the cases and controls were adjusted for age.

The frequencies were observed to be in Hardy–Weinberg equilibrium (HWE) in the control population. The observed genotype and allele frequency distribution of *MMP-2* and *TIMP-2* gene polymorphisms between cases and controls are depicted in Table 1.

The findings did not reveal any significant association between *MMP-2* and *TIMP-2* gene polymorphisms and the risk of developing cervical cancer at the genotype and allele level in the study population. Furthermore, no significant association was observed upon carrier analysis using dominant model.

MMP-2 (−735C > T) [rs 2285053] and *TIMP-2* (−418G > C) [rs 8179090] Gene Polymorphisms and Stages of Cervical Cancer

A case-only analysis was carried out to investigate whether any possible association exists between *MMP-2* (−735C > T) and *TIMP-2* (−418G > C) gene polymorphisms and stages of cervical cancer; stages I and II were combined into early stage ($n = 92$ cases), and stages III and IV into advanced stage ($n = 108$ cases). However, our findings did not demonstrate any significant association of *MMP-2* and *TIMP-2* gene polymorphisms with clinical stages of cervical cancer (Table 2).

MMP-2 (−735C > T) [rs 2285053] and *TIMP-2* (−418G > C) [rs 8179090] Gene Polymorphisms and Tobacco Usage Among Cervical Cancer Patients

In the present study, 55 (27.5 %) cervical cancer patients were tobacco users and 145 (72.5 %) out of a total of 200 patients were non-users. Using a case-only study approach,

Table 1 Genotype and allele frequency distribution of *MMP-2* (−735C > T) [rs 2285053] and *TIMP-2* (−418G > C) [rs 8179090] gene polymorphisms in cervical cancer cases and controls

MMP/TIMP gene polymorphisms	Cases (%) n = 200	Controls (%) n = 200	P value	OR (95 %CI)
<i>MMP-2</i> (rs 2285053)				
CC	163 (81.5)	173 (86.5)	–	Reference
CT	34 (17.0)	25 (12.5)	0.172	1.48 (0.84–2.59)
TT	3 (1.5)	2 (1.0)	0.659	1.50 (0.25–9.14)
CT + TT	37 (18.5)	27 (13.5)	0.156	1.48 (0.86–2.55)
C*	360 (90.0)	371 (92.8)	–	Reference
T*	40 (10.0)	29 (7.2)	0.157	1.44 (0.87–2.37)
<i>TIMP-2</i> (rs 8179090)				
GG	150 (75.0)	165 (82.5)	–	Reference
GC	49 (24.5)	35 (17.5)	0.104	1.50 (0.92–2.45)
CC	1 (0.5)	0	1.000	–
GC + CC	50 (25.0)	35 (17.5)	0.087	1.53 (0.94–2.49)
G*	349 (87.3)	365 (91.3)	–	Reference
C*	51 (12.7)	35 (8.7)	0.089	1.49 (0.94–2.34)

* Total number of chromosomes in cases = 400 and controls = 400
OR age-adjusted odds ratio, CI confidence interval

Table 2 Association of *MMP-2* (−735C > T) [rs2285053] and *TIMP-2* (−418G > C) [rs8179090] gene polymorphisms with early (I + II) vs advanced (III + IV) stages of cervical cancer

Total number of cervical cancer cases of early stages (I + II) = 92 and of advanced stages (III + IV) = 108

OR age-adjusted odds ratio, CI confidence interval

MMP/TIMP gene polymorphisms	Early stages (I + II) n (%)	Advanced stages (III + IV) n (%)	P value	OR (95 %CI)
MMP 2 (rs 2285053)				
CC	72 (78.3)	91 (84.2)	–	Reference
CT	20 (21.7)	14 (13.0)	0.132	0.56 (0.26–1.19)
TT	0	3 (2.8)	0.999	1.25 (0.00)
TIMP 2 (rs 8179090)				
GG	71 (77.2)	79 (73.1)	–	Reference
GC	20 (21.7)	29 (26.9)	0.475	1.27 (0.66–2.46)
CC	1 (1.1)	0	1.000	0.00 (0.00)

Table 3 Association of *MMP-2* (−735C > T) [rs2285053] and *TIMP-2* (−418G > C) [rs8179090] gene polymorphisms with tobacco usage among cervical cancer patients

Total number of tobacco users among cervical cancer cases = 55 and total number of non-users = 145 cases

OR age-adjusted odds ratio, CI confidence interval

MMP/TIMP gene polymorphisms	Non-users n (%)	Users n (%)	P value	OR (95 %CI)
MMP 2 (rs 2285053)				
CC	116 (80.0)	47 (85.5)	–	Reference
CT	27 (18.6)	7 (12.7)	0.417	0.69 (0.28–1.70)
TT	2 (1.4)	1 (1.8)	0.943	1.09 (0.09–12.60)
TIMP 2 (rs 8179090)				
GG	114 (78.6)	36 (65.5)	–	Reference
GC	31 (21.4)	18 (32.7)	0.139	1.70 (0.84–3.43)
CC	0	1 (1.8)	1.000	–

we observed that there was no significant association between *MMP-2* (−735C > T) and *TIMP-2* (−418G > C) gene polymorphisms and modulation of cervical cancer risk due to tobacco usage in the study population (Table 3).

Discussion

In the present case–control study designed in the state of Uttar Pradesh in North India, we aimed to evaluate the role of *MMP-2* and *TIMP-2* gene polymorphisms in the risk of developing cervical cancer. Our findings demonstrated a lack of association between *MMP-2* (−735C > T) [rs 2285053] and *TIMP-2* (−418G > C) [rs 8179090] gene polymorphisms and cervical cancer susceptibility in women of Northern India. Further stratified analysis using a case-only study approach did not demonstrate any association of the studied gene polymorphisms with cancer stages (early vs. advanced) as well as tobacco usage in patients of cervical cancer.

Cervical cancer, a major health problem among women worldwide, is linked to persistent infection by human papillomaviruses (HPV) [16]. The high-risk variants of human papillomavirus such as HPV 16 and 18 are major etiological agents of cervical cancer [17]. Host genetic and

environmental factors are likely to play an important role in human diseases. Deciphering the cellular and molecular mechanisms involved in the etiology of cervical cancer has been a major thrust area in medical research in recent times. Analysis of potentially functional polymorphisms in candidate genes has emerged as a powerful approach in understanding the complex relationship between genotype and phenotype. In this context, association analyses can be used to explore the role of genetic polymorphisms in susceptibility to various cancers, including cervical cancer.

Tumor cells produce enzymes that destroy the matrix barriers surrounding the tumor, permitting invasion into surrounding connective tissues, entry and exit from blood vessels, and metastasis to distant organs. Enzymes that degrade the extracellular matrix (ECM) have long been viewed as essential for tumor progression. MMPs are able to degrade virtually all ECM components. Therefore, classically, MMPs were recognized as being produced and secreted by tumor cells, degrading basement membrane and extracellular matrix components, thereby facilitating tumor cell invasion and metastasis.

We did not find any association between *MMP2* (735) C/T polymorphism and cervical cancer. Contrarily Vasku et al. [18] had found that variant allele frequency of the −735C > T *MMP-2* polymorphism was significantly

higher in patients with chronic heart failure than in subjects without clinical signs of cardiovascular disease. Cotignola et al. [19] reported patients with esophageal squamous cell carcinoma carrying the $-735CC$ genotypes had an increased risk of developing cancer.

The findings did not reveal any significant association between TIMP-2 gene polymorphisms and the risk of developing cervical cancer at the genotype and allele level in the study population. While in case of breast cancer, Zhou and colleagues [14] found a reduced risk for the variant allele compared to the common allele. In contrast, the variant allele has been associated with an increased risk of head and neck cancer [20], oral squamous cell cancer [21], and gastric cancer [22] in other studies.

To the best of our knowledge, this is perhaps the first study exploring the role of *MMP-2* ($-735C > T$) and *TIMP-2* ($-418 G > C$) gene polymorphisms in cervical cancer susceptibility in Indian women. Although our findings demonstrated that there was a lack of association between the studied gene polymorphisms and cervical cancer susceptibility, yet the present study further emphasizes the emerging significance of genetic variants in *MMP-2*/*TIMP-2* and the risk of developing cervical cancer. Our study had certain strengths as well as limitations. The possibility of population admixture was ruled out as the study subjects enrolled were of same ethnicity i.e., North Indian. As we also sought to investigate whether *MMP-2* and *TIMP-2* gene polymorphisms had any significant role in clinical stages of cervical cancer, we accordingly excluded post-operative cases of cervical cancer; therefore, only de novo cervical cancer patients prior to chemo/radiotherapy were included in the present study. Moreover, the diagnosis and clinical staging (FIGO stages I–IV) of cervical cancer in the study population was accurate. We also conducted a questionnaire-based personal interview for assessment of demographic data, cancer stage and tobacco usage among histopathologically confirmed patients of cervical cancer.

Our study had some limitations as well and these included limited sample size that might have lead to a relatively lower statistical power in the subgroups. The HPV status of the subjects enrolled in the study was unknown. As we did not have access to tumor tissue samples, we were not able to perform a detailed cancer stage-specific molecular and cellular expression studies at the mRNA and/or protein level in tissue biopsy samples of cervical cancer. Another study limitation was the lack of availability of established cervical cancer cell-lines that would have further helped in arriving at more definitive conclusions regarding the role of *MMP-2*/*TIMP-2* gene polymorphisms and cervical cancer in Indian women.

Acknowledgments This study was supported by Uttar Pradesh Council of Science and Technology (UPCST) (Grant No. CST/SERPD/

D-1112), Lucknow, India. We are grateful to Departments of Radiotherapy, CSMMU and SGPGIMS, Lucknow for clinical support for sample collection.

Conflict of interest There is no conflict of interest among the authors.

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