

Association of *MMP7* –181A→G Promoter Polymorphism with Gastric Cancer Risk

INFLUENCE OF NICOTINE IN DIFFERENTIAL ALLELE-SPECIFIC TRANSCRIPTION VIA INCREASED PHOSPHORYLATION OF cAMP-RESPONSE ELEMENT-BINDING PROTEIN (CREB)*

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Background: Effect of tobacco carcinogen nicotine on *MMP7* transcription and gastric cancer risk remains unknown.

Results: Preferential binding of pCREB, induced by nicotine, to G-allele promoter enhanced the transcription and thus aggravated gastric cancer risk.

Conclusion: Nicotine shows an additive effect over *MMP7* GG genotype toward gastric cancer risk.

Significance: These findings may help in the clinical management of gastric cancer.

Elevated expression of matrix metalloproteinase7 (*MMP7*) has been demonstrated to play a pivotal role in cancer invasion. The –181A→G (rs11568818) polymorphism in the *MMP7* promoter modulates gene expression and possibly affects cancer progression. Here, we evaluated the impact of –181A→G polymorphism on *MMP7* promoter activity and its association with gastric cancer risk in eastern Indian case-control cohorts ($n = 520$). The GG genotype as compared with the AA genotype was predisposed ($p = 0.02$; odds ratio = 1.9, 95% confidence interval = 1.1–3.3) to gastric cancer risk. Stratification analysis showed that tobacco addiction enhanced gastric cancer risk in GG subjects when compared with AA subjects ($p = 0.03$, odds ratio = 2.46, and 95% confidence interval = 1.07–5.68). Meta-analysis revealed that tobacco enhanced the risk for cancer more markedly in AG and GG carriers. Activity and expression of *MMP7* were significantly higher in GG than in AA carriers. In support, *MMP7* promoter-reporter assays showed greater transcriptional activity toward A to G transition under basal/nicotine-induced/cAMP-response element-binding protein (CREB) overexpressed conditions in gastric adenocarcinoma cells. Moreover, nicotine (a major component of tobacco) treatment significantly up-regulated *MMP7* expression due to enhanced CREB phosphorylation followed by its nuclear translocation in gastric adenocarcinoma cells. Furthermore, chromatin immunoprecipitation experiments revealed higher binding of phosphorylated CREB with the –181G than the –181A allele. Altogether, specific binding of phosphorylated CREB to the G allele-carrying promoter enhances *MMP7* gene expression that

is further augmented by nicotine due to increased CREB phosphorylation and thereby increases the risk for gastric cancer.

Gastric cancer is the second largest cause of global cancer-related mortality estimated at about 800,000 deaths worldwide per year that constitutes ~50% of the world's gastric cancer diagnoses in eastern Asia (1, 2). It is the most common cancer type in the southern and northeastern states of India (3). The pathogenesis of gastric cancer involves a number of events, including *Helicobacter pylori* infection, tobacco addiction, chronic inflammation along with high salt intake and exposure to chemical carcinogen (4, 5). As gastric cancer is a multifactorial disease, the study should consider environmental, genetics, and host-related factors to understand its pathology. Previous epidemiologic studies suggested that tobacco addiction was an independent risk factor for gastric cancer development. In addition to these environmental factors, genetic factors, including polymorphism within the promoter region of several genes, also play an important role in gastric cancer etiology (6). Hence, thorough research is necessary to identify the genetic determinants that predict cancer prognosis and the synergetic effect of different exogenous risk factors on them. Cancer progression takes place through extracellular matrix remodeling via modulation of several susceptible genes, mainly matrix metalloproteinases (MMPs),³ which are attributed to characteristic changes in tissues architecture and function (7, 8). MMPs are a diverse family of enzymes, playing a significant role in the degradation of extracellular matrix and basal membrane (9–11). Most of the MMP family members are associated with the pro-

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³ The abbreviations used are: MMP, matrix metalloproteinase; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; CREB, cAMP-response element-binding protein; AGS, gastric adenocarcinoma cell line; ANOVA, analysis of variance; nAChR, nicotine acetylcholine receptor; RR, relative risk.

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motion of tumor growth, invasion, and metastasis, the major hallmarks of cancer progression (7, 10, 12).

MMPs are broadly classified into five classes (collagenases, gelatinases, stromelysins, matrilysin, and membrane-type) on the basis of their putative substrate specificity and internal homologies (13, 14). MMP7, a matrilysin, is the smallest known member of the MMP family and possesses potent extracellular matrix degradative activity against a variety of components, including elastin, gelatin, type IV collagen, fibronectin, vitronectin, laminin, entactin, aggrecan, and proteoglycans (15, 16). Like other MMPs, the activity of MMP7 is regulated at multiple levels, including gene expression, spatial localization, zymogen activation, and inhibition by its endogenous inhibitor (14, 17). In contrast to other MMPs, which usually are expressed in stromal tissues, MMP7 is expressed mainly in tumor cells (18, 19) and enhances tumor progression by inhibiting apoptosis in cancer cells, by reducing cell adhesion, or by inducing angiogenesis or both (20).

Accumulated evidence showed a positive correlation between MMP7 expression and the invasive potential of cancers (18, 19). Previous study demonstrated that knockdown or overexpression of MMP7 alters tumorigenicity. Wilson *et al.* (21) reported that intestinal tumorigenesis is suppressed in mice lacking *Mmp7*. Moreover, MMP7 overexpression in MCF-7 cells enhances cellular invasiveness (22). However, the molecular basis of MMP7 up-regulation in gastric cancer remains unclear. Single base pair substitutions in the *MMP* promoter may create or abolish the transcription factor-binding site, thereby modifying transcriptional activity (23, 24). Two common functional SNPs ($-181A \rightarrow G$ and $-153C/T$) located in the *MMP7* promoter region known to influence gene expression were studied in several disease conditions, including hypercholesterolemia, idiopathic pulmonary fibrosis, and several cancer types (*e.g.* breast, colon, and esophagus) (10, 13, 15, 25–27). Previous studies demonstrated a correlation between *MMP7* $-181A \rightarrow G$ substitution and gastric cancer susceptibility in Chinese and Caucasian populations (13, 28). So far, the relationship between the *MMP7* $-181A \rightarrow G$ polymorphism and the risk of gastric cancer has not been investigated in any eastern Indian population. Moreover, the genetic variant of *MMP7* and tobacco addiction may play independent or synergistic roles in enhancing gastric cancer risk. Also, the effect of nicotine (the major component of tobacco) on *MMP7* gene expression and its consequences in gastric cancer progression has not been documented yet. We hypothesize that genetic variants of *MMP7* underlie the association with gastric cancer risk and/or that their combined effect with tobacco addiction may modify the risk of gastric cancer.

Herein, we conducted a hospital-based case-control study in an east Indian population to explore the role of *MMP7* $-181A \rightarrow G$ SNP and the influence of tobacco addiction on gastric cancer risk. We also compared *MMP7* activity and expression level between two *MMP7* genetic variant (GG and AA) patient groups to assess the possible alteration in the genotype-specific *MMP7* transcription. In addition, *in vitro* *MMP7* promoter activity assay was performed in cultured gastric adenocarcinoma cell lines in the presence and absence of nicotine to determine the relative affinities of transcription factors to GG

or AA promoter. In this context, studies on the functional polymorphism of the *MMP7* promoter under basal and nicotine-treated conditions are important to understand the risk of gastric cancer.

Experimental Procedures

Study Subjects, Eastern Indian Case-control Cohort—Blood samples were collected from 260 clinically diagnosed gastric cancer patients attending the Department of Gastro-oncology of Saroj Gupta Cancer Centre and Research Institute, Kolkata, India, and the Department of Gastric Surgery, Medical College and Hospital, Kolkata, Institute of Post Graduate Medical Education and Research, Kolkata, India from June 2008 to 2013. Blood samples from 260 subjects, screened at the same hospital and confirmed as noncancer, were collected as the control subject. The case-control cohort includes unrelated Indian nationalities from West Bengal or the surrounding eastern Indian states, which considerably represents the eastern Indian population. The study protocols were approved by the Ethical Review Board of the Saroj Gupta Cancer Centre and Research Institute and the Human Ethics Committee of Council of Scientific and Industrial Research-Indian Institute of Chemical Biology, Kolkata, India, and informed consent was obtained from all participants. The patient's demographics, symptoms, and tumor grading were recorded, and blood samples were collected after taking the subject's history. Biopsy specimens were used to determine histological tumor typing. Individuals who were formerly or currently addicted to tobacco for at least 2 years were defined as tobacco-addicted. The exclusion criteria included previous history of any other cancer and prior chemotherapy. The diagnosis of gastric cancer and tumor, node, metastasis staging were based on accepted clinical, histological, and radiological findings and that of International American Joint Committee on Cancer and International Union against Cancer criteria.

DNA Extraction and Serum Purification from Blood—Venous blood (5 ml) was drawn aseptically from each subject, and 3 ml was collected in Vacutainer tubes (Qiagen GmbH, Feldbachstrasse, Switzerland) containing EDTA and stored at 4 °C prior to genomic DNA extraction. Genomic DNA was extracted within 2 weeks after sampling from whole-blood samples using QIAamp DNA blood midi kit (Qiagen) according to the manufacturer's protocol. The remaining 2 ml of blood was used for serum preparation. Serum sample was mixed with protease inhibitor mixture and stored at -80 °C. Blood vials, serum tubes, DNA tubes, and patient questionnaire forms were carefully labeled with corresponding sample identification numbers.

Genotyping—The *MMP7* $-181A \rightarrow G$ polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism method (13). The PCR primers were used for amplifying the promoter sequence covering *MMP7* -181 nucleotide using forward primer 5'-TGGTACCATAATGTC-CTGAATG-3' and reverse primer 5'-TCGTTATTGGCAGG-AAGCACACAAGAATT-3'. PCR was performed in PCR SP-RINT Thermal Cycler (Thermo Electron Corp., Japan). The target sequence was amplified in a 25- μ l reaction volume containing 10–20 ng of genomic DNA, 0.2 mM dNTP, 10 mM Tris-

HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.3 mM of each primer, and 1.0 unit of Taq DNA polymerase (Fermentas). The reaction conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 56.5 °C for 60 s, extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. Amplified product was digested using restriction enzyme EcoRI (New England Biolabs) followed by a run in 2.5% agarose gel with molecular weight markers and visualized after staining with ethidium bromide. Genotype was determined observing digested band pattern. Ten percent of samples from both patients and controls were re-genotyped to eliminate any discrepancy.

Meta-analysis of MMP7 -181A→G Genotype—We did a comprehensive literature search through the electronic databases of PubMed, ISI Web of Knowledge, Medline, Embase, and Google Scholar Search using the search terms *MMP7* or matrix metalloproteinase 7 polymorphism, cancer, and tobacco addiction to identify all case-control studies that evaluated potential modification of association between *MMP7* polymorphism and cancer risk by tobacco addiction. The eligible studies were selected by inclusion and exclusion criteria. We confined the search studies to those published in English to evaluate the association of cancer risk with *MMP7* polymorphism and the modifying effect of tobacco. Studies that only investigated tobacco addiction or *MMP7* polymorphism in relation to cancer risk or studies without adequate information on addiction status and *MMP7* -181 genotypes were not included. Information such as ethnicity of the study population, cancer type, *MMP7* -181 genotype status, number of tobacco-addicted/nonaddicted cases and controls were collected from each study. Meta-analysis was conducted using Metafor package of R Statistical Software version 3.0.1. We evaluated the effect of tobacco addiction on genotype with respect to cancer risk, assuming tobacco-nonaddicted groups as reference. The strength of the association between *MMP7* genotype and cancer risk in terms of tobacco addiction was measured by relative risk (RR) and 95% confidence interval (CI). Relative risk was computed assuming random effect models; two-tailed *p* value of less than 0.05 was considered as significant. Forest plot was used for graphical representation.

Casein Zymography—For assay of *MMP7* activity, serum samples were mixed with 1× nonreducing Laemmli sample loading buffer and 4 mg/ml heparin and were electrophoresed in SDS-12% polyacrylamide gel containing 1.5 mg/ml casein under nonreducing conditions (29). The gels were given a pre-run at 40 mA with sample loading buffer until the dye front touched the edge of the gel. The gels were run at 20 mA in nonreducing conditions at 4 °C. The gels were washed twice in 2.5% Triton X-100 and incubated in stromelysin assay buffer for 40–46 h at 37 °C. Gels were then stained with 0.1% Coomassie Brilliant Blue stain followed by destaining. The zone of casolytic activities appeared as negative staining. Quantification of zymographic bands was performed by densitometric analysis using Lab Image software (Kapelan GmbH, Germany).

Western Blotting—Equal volumes of serum samples from patients having AA and GG genotypes were taken for detection of *MMP7* expression by Western blotting using anti-*MMP7* polyclonal antibody (Santa Cruz Biotechnology). Serum sam-

ples were resolved on 10% reducing SDS-PAGE and transferred to nitrocellulose membranes. In other experiments, AGS cells were cultured to 70% confluence and were treated with 50, 100, and 200 μM doses of nicotine bitartrate (Sigma) for varied time periods (*viz.* 10, 20, and 30 h). The untreated and treated cells were scraped out and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 1 mM PMSF, and protease inhibitor mixture (Sigma) followed by sonication. The protein content in the cell lysates and supernatant were estimated using Bradford's assay reagent. Cell lysates and supernatant containing 100 μg of protein were subjected to SDS-PAGE on a 10% resolving gel and transferred to PVDF membrane (Pall Corp.). The membrane was blocked in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) and 3% BSA for 1 h and then probed overnight with 1:500 polyclonal anti-CREB (06-863, Millipore), pCREB-1 (06-519, Millipore), *MMP7* (sc-8832, Santa Cruz Biotechnology), and β-tubulin (sc-9104, Santa Cruz Biotechnology) antibodies. The blots were then washed and incubated with rabbit anti-goat IgG alkaline phosphatase conjugate (catalog no. 172-1034, Bio-Rad) and were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (Sigma).

Immunofluorescence—For the immunofluorescence study, the biopsy tissue sample was fixed in 4% paraformaldehyde solution and dehydrated in ascending alcohol series. The tissue was paraffinized and sectioned at 5-μm thickness using a microtome. The sections were deparaffinized with xylene followed by rehydration with descending alcohol series (30). Antigen retrieval was performed by trypsin (0.05% trypsin, 0.1% CaCl₂) followed by blocking using 5% BSA in TBS and incubated overnight at 4 °C in primary antibody solution (1:200 dilutions in TBS with 1% BSA) in a humid chamber. After washing with TBST, the tissue sections were incubated with fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology) solution. The sections were counterstained with propidium iodide. An equal number of AGS cells (~70% confluency) were transferred into each well of a 24-well tissue culture plate (Nunc, Roskilde, Denmark) previously coated with poly-L-lysine, and they were treated with 200 μM doses of nicotine bitartrate (Sigma) for 10-, 20-, and 30-h time periods. The medium was removed, and the cells adhering to the bottom of the plate were fixed in 3–4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100 (31). Blocking was performed using 1% BSA in TBST followed by incubation overnight at 4 °C in primary antibody solution (1:200 dilutions in TBS with 1% BSA) in a humid chamber. The culture wells were washed with PBS followed by incubation with fluorescein isothiocyanate and Texas Red-conjugated secondary antibody (Santa Cruz Biotechnology) solution. Nuclear staining was performed with DAPI. The images were observed in confocal microscopy. Images at ×10 and ×40 magnification were captured using Andor iQ 2.7 software (Andor spinning disc confocal microscope, Belfast, Ireland) and processed under Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA).

Generation of Human *MMP7* Promoter-Reporter Constructs—The ~250-bp upstream promoter region of human *MMP7* was amplified by PCR using genomic DNA of known

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homozygotes for the GG and AA genotypes and the following primers: forward, 5'-CGGGGTACCTGGAGTCAATTTATGCAGCAGACAG-3', and reverse, 5'-CCGCTCGAGTTGGACCTATGGTTGATTTGGTG-3' (restriction sites inserted for KpnI and XhoI at the 5'-end of forward and reverse primers, respectively, are indicated in boldface). The purified promoter fragments were inserted between KpnI and XhoI sites in the promoterless firefly luciferase reporter vector pGL3-Basic (Promega). The generated promoter-reporter constructs consisted of the -230- to +22-bp region of *MMP7*, numbered upstream (-) or downstream (+) with respect to the cap site. The presence of appropriate alleles at the SNP position and accurate cloning of the inserts were confirmed by DNA sequencing. The plasmids were purified on columns using an endotoxin-free plasmid DNA purification kit (Hi Media, India) for transfection experiments.

Cell Culture, Transfection, and Reporter Assays—The human gastric adenocarcinoma cell line AGS and colon adenocarcinoma cell line SW480 were obtained from the laboratory of Dr. Kumaresan Ganesan, Madurai Kamaraj University, India. The human embryonic kidney cell line HEK-293 and mouse neuroblastoma N2a cells were obtained from the National Center for Cell Sciences, Pune, India. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose with L-glutamine and sodium pyruvate (HyClone), supplemented with 10% fetal bovine serum (Invitrogen), penicillin G (100 units/ml), and streptomycin sulfate (100 μ g/ml) (Invitrogen) at 37 °C with 5% CO₂. The AGS and HEK-293 cells were grown to 60% confluence and transfected with 1.0 μ g/well of the promoter-reporter plasmids by the calcium phosphate method. SW480 cells were grown to 75% confluence and transfected using Lipofectamine (Invitrogen). A β -galactosidase expression plasmid was used at a concentration of 400 ng/well as an internal control for transfection across various conditions. Luciferase and β -galactosidase assays were carried out 24 h after transfection as described previously (32). The results were expressed as a ratio of luciferase activity to β -galactosidase activity. In one set of experiments, AGS cells transfected with the promoter-reporter plasmids were treated with 200 and 500 μ M and 1 mM doses of nicotine bitartrate (Sigma) 6 h after transfection. The luciferase activities were assayed after 18–20 h of treatment and normalized with protein levels estimated by the Bradford assay, and the results were expressed as relative light units/ μ g of protein.

In another set of experiments, AGS cells were transfected with promoter-reporter plasmids (1.0 μ g/well) and different amounts of CREB expression plasmid (100, 250, and 500 ng) (33). To ensure that equal amounts of DNA were transfected across all the cells, the pCDNA 3.1 plasmid was used as the balancing plasmid. The luciferase activity was assayed 24–30 h after transfection, and the results were expressed as relative light units/ μ g of protein.

In another set of experiments, CREB siRNA (10 nM) was co-transfected with 1.0 μ g of the respective G and A allele harboring promoter-reporter constructs in AGS cells. A nonspecific siRNA was used as control. Luciferase and Bradford assays were carried out after 48 h of transfection. The protein from cell lysates were blotted and probed for CREB expression. GAPDH was used for normalization.

Nicotine-mediated CREB activation of the *MMP7* promoter was probed by co-transfecting *MMP7* G and A promoter-reporter constructs separately with KCREB followed by treatment with 200 μ M of nicotine after 6–8 h of transfection. Luciferase and Bradford assays were performed 24–30 h after transfection. KCREB is a dominant repressor of CREB, which, due to mutations in the DNA binding domain, forms an inactive dimer with CREB, thereby blocking its ability to bind to cyclic AMP-response element (33). In another set of experiments, AGS cells were transfected with *MMP7* G and A promoter-reporter constructs separately and treated with 25 μ M tubocurarine hydrochloride (Sigma), an inhibitor of the nicotinic acetylcholine receptor and 200 μ M nicotine 12 h post-transfection. Luciferase and Bradford assays were carried out after 3 h of treatment.

Chromatin Immunoprecipitation—N2a cells were transfected with the G and A allele-containing *MMP7* promoter-reporter constructs. Our CHIP protocol was based on the Kroll laboratory method (34, 35). Cells ($\sim 5 \times 10^6$) were cross-linked in fixation solution containing 37% formaldehyde in PBS to a final concentration of 1%, and after 12 min of incubation at room temperature, the unreacted formaldehyde was quenched with 0.125 M glycine in PBS for 5 min. Cells were scraped in PBS containing 1 mM PMSF, pelleted, and lysed in lysis buffer (50 mM Tris (pH 8.0), 1% Triton X-100, 0.1% sodium deoxycholate, 5 mM EDTA, 150 mM NaCl, 1% SDS) with protease inhibitors for 20 min on ice. The nuclei pellets were washed with Tris-EDTA buffer and resuspended in Nuclei buffer (50 mM Tris (pH 8.0), 10 mM EDTA, 0.01% SDS, 1 mM PMSF) for sonication. The lysates were sonicated for 17 cycles of 30 s (with 30 s rest) (LAB-SONIC M, Sartorius) and then spun at maximal setting at 4 °C for 15 min to retrieve the clear supernatant. The samples were pre-cleared with *Escherichia coli* genomic DNA/BSA/Sepharose 4B slurry (30 μ l per immunoprecipitation with 5 μ g of DNA, 30 μ g of BSA) to reduce nonspecific background. Immunoprecipitation of chromatin was performed at 4 °C overnight by incubation with 5 μ g each of CREB-1 antibody (sc-186x, Santa Cruz Biotechnology), and p-CREB-1 antibody (sc-7978x, Santa Cruz Biotechnology). Rabbit IgG (I5006, Sigma) was used as a control, and the 1% sample was frozen as input. The immune complex was captured with *E. coli* genomic DNA/BSA/Sepharose 4B slurry (60 μ l per immunoprecipitation) after incubation at 4 °C for 4 h. The immune complex was pelleted and washed thoroughly with low salt, high salt, LiCl wash buffers, TE buffer followed by elution in buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, 1% SDS, and 50 mM NaHCO₃. The cross-links were reversed by heating with 5 M NaCl at 65 °C overnight followed by RNase digestion at 37 °C for 30 min and proteinase K digestion at 37 °C for 90 min. The DNA was extracted and purified using spin columns (HiMedia, India).

Following the purification PCR was carried out to amplify the DNA sequence encompassing the G-181A site in the human *MMP7* promoter (-230 to +22 bp; forward primer 5'-TGGAGTCAATTTATGCAGCAGACAG-3' and reverse primer 5'-TTGGACCTATGGTTGATTTGGTG-3') followed by agarose gel electrophoresis of the PCR products. CHIP quantitative PCR was performed using DyNAmo ColorFlash SYBR Green

quantitative PCR kit (Thermo Scientific) using the same primers. The amounts of DNA immunoprecipitated by CREB in the case of G and A alleles were quantified by the fold enrichment method relative to IgG signal.

Statistical Analysis—GraphPad InStat3 software (GraphPad Software, Inc., San Diego) was used to perform statistical analysis. Significant differences between age of controls and cases were assessed using the Student's *t* test for comparison of means. Genotype frequencies of *MMP7* –181A→G were compared between cancer patients and controls using two-sided 2-by-2 contingency tables according to the genotype by the Pearson χ^2 test. The odds ratio (OR) and 95% confidence interval (CI) of the genotypes were calculated from a binary logistic regression model to measure the directional strength of genotype-phenotype association. In this study, the A allele of the 181-A/G SNP was used as a reference allele and was used to analyze the results using the binary logistic regression model. To evaluate the relationship between the polymorphic genotypes and disease status of gastric cancer, the disease parameter-like stage of cancer, depth of tumor invasion, and others were transformed to binary data (stage I plus II *versus* stage III plus IV). The relationships between genotype distributions and clinicopathological parameters were also examined using a multivariate logistic regression model adjusted for age (continuous variable), sex, and tobacco addiction as a potential confounding factor. Data for the activity and expression of *MMP7* were fitted using SigmaPlot and represented as the mean values. Comparison between groups was carried out using one-way analysis of variance (ANOVA), followed by Student's-Newman-Keul test. All results were considered statistically significant if the *p* value was <0.05.

Results

Study Population Characterization—The gastric cancer patients consisted of 193 males (74.23%) and 67 females (25.77%) with a mean age of 54.38 years (± 11.30 years), and control subjects with a mean age of 47.51 years (± 12.72 years) consisted of 180 males (69.23%) and 80 females (30.77%). There were statistically significant differences in the distribution of sex and age between patients and controls. Also, tobacco-addicted individuals were significantly more among patients (64.61%) compared with controls (33.46%). The adjusted OR for risk estimation was calculated considering age, sex, and addiction parameter.

***MMP7* –181A→G Polymorphism Detection and Gastric Cancer Risk Assessment with Genotypes and Alleles**—*MMP7* –181A→G genotyping was performed by PCR-restriction fragment length polymorphism analysis using EcoRI restriction enzyme. The *MMP7* –181G alleles were assessed by DNA bands of 120 and 30 bp, and the –181A alleles by a size 150 bp. The heterozygotes displayed a combination of both alleles having band size of 150, 120, and 30 bp. The genotype frequencies of *MMP7* –181A→G polymorphism in noncancer controls and patients with gastric cancer are shown in Table 1. Subjects with the *MMP7* –181GG genotype were at a higher risk of gastric cancer (*p* = 0.02; OR = 1.9, 95% CI = 1.10–3.30) as compared with that of the AA genotype. The contribution of the G allele was also reflected in cancer manifestation, because

TABLE 1

***MMP7* –181A→G SNP and the risk of gastric cancer**

OR was calculated by binary logistic model by using GraphPad InStat. *p* value is for χ^2 test showing the significance of difference in the distribution of the genotype between patients and controls. Significantly different values are shown in bold. CI = confidence interval, Ref = reference genotype for calculation of OR.

Genotype/allele	GC patients		Controls		OR	95% CI	<i>p</i> value
	<i>n</i>	%	<i>n</i>	%			
<i>MMP7</i> (–181A→G)	260		260				
AA	107	41.1	118	45.3	1 (Ref)		
AG	108	41.5	116	44.6	1.027	(0.70–1.48)	0.92
GG	45	17.3	26	10	1.9	(1.10–3.30)	0.02
AG+GG	153	58.8	142	54.6	1.18	(0.83–1.68)	0.37

individuals with AG plus GG genotypes were at modest risk as compared with the AA genotype.

Association between *MMP7* –181A→G Polymorphism and Clinicopathology of Gastric Cancer Patients—Considering *MMP7* –181A→G polymorphism, individuals below the 50-year age group with GG genotypes in comparison with AA genotypes (*p* = 0.02, OR = 2.5, CI = 1.13–5.54) were susceptible to higher gastric cancer risk (Table 2). In addition, males with GG genotypes (*p* = 0.007, OR = 2.47, CI = 1.28–4.76) displayed a significantly higher risk for gastric cancer. Moreover, males with a combination of GG and AG *versus* AA genotypes showed higher OR for cancer predisposition (*p* = 0.11), although the *p* value is not statistically significant. Furthermore, tobacco-addicted individuals having GG genotype as compared with AA carriers displayed quite significantly an enhanced risk for gastric cancer (*p* = 0.003, OR = 2.46, and CI = 1.07–5.68). However, tobacco-addicted individuals carrying the G allele showed predisposition toward gastric cancer development (*p* = 0.13, OR = 1.66, and CI = 0.88–3.15), although they were statistically insignificant. No correlation between patient genotype and site of cancer development, tumor size, or depth of invasion was noticed. Although *MMP7* overexpressed at a late stage of cancer progression, we did not detect any positive correlation between lymph node metastasis and susceptible genotype. Thus, the results suggested that the tobacco-addicted male population below 50 years of age having a GG genotype as compared with the AA genotype carriers have a higher risk of gastric cancer.

Up-regulation of *MMP7* in Gastric Cancer Patients—To understand the involvement of *MMP7* in gastric cancer progression, we compared the abundance of *MMP7* protein between gastric cancer patients and noncancer control subjects. Immunofluorescence detection of *MMP7* levels in the stomach tissue of gastric cancer patients *versus* controls showed a higher abundance of *MMP7* protein in the cancer patients (Fig. 1, A and B).

Higher Activity and Abundance of *MMP7* in the Serum of GG as Compared with AA Genotype Patients—The activity of *MMP7* protein in the serum of gastric cancer patients having a GG genotype was significantly higher (~2.4-fold, *p* = 0.001) as compared with that of the AA genotype (Fig. 1, C and D). In support, the serum of GG patients also exhibited higher *MMP7* expression as compared with the AA patients (~1.5-fold; *p* = 0.0006) (Fig. 1, E and F). Comparable loading of serum proteins was visualized by Ponceau S staining of the blot. The significant

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TABLE 2

Genotypes of MMP7 SNP and clinicopathological characteristics of gastric cancer patients

p value, ORs, and 95% CIs were calculated by unconditional multivariate logistic regression (using GraphPad InStat) with respective reference genotypes. Cont, control; Ref, reference.

	MMP7 –181A→G			
	A/A	A/G	G/G	A/G+G/G
Age (years)				
≥50 (Case/Cont)	72/45 (40.6/45.9)	78/42 (44/42.8)	27/11 (15.2/11.2)	105/53 (59.3/54)
OR (95% CI) ^a	1(Ref)	1.16 (0.68–1.97)	1.53 (0.69–3.39)	1.23 (0.75–2.03)
<50 (Case/Cont)	35/73 (42.1/45)	30/74 (36.1/45.6)	18/15 (21.6/9.25)	48/89 (57.8/54.9)
OR (95% CI) ^a	1(Ref)	0.84 (0.47–1.5)	2.5 (1.13–5.54)	1.12 (0.65–1.92)
Gender				
Male (Case/Cont)	73/83 (37.8/46.1)	83/80 (43/44.4)	37/17 (19.1/9.4)	120/97 (62.1/53.8)
OR (95% CI) ^b	1(Ref)	1.18 (0.76–1.83)	2.47 (1.28–4.76)	1.407 (0.93–2.12)
Female (Case/Cont)	34/35 (50.7/43.7)	25/36 (37.3/45)	8/9 (11.9/11.2)	33/45 (52.3/56.2)
OR (95% CI) ^b	1(Ref)	0.71 (0.35–1.43)	0.91 (0.31–2.6)	0.75 (0.39–1.44)
Addiction to tobacco				
Yes (Case/Cont)	63/40 (37.5/45.9)	70/38 (41.6/43.6)	35/9 (20.8/10.3)	105/47 (62.5/54.02)
OR (95% CI) ^c	1(Ref)	1.17 (0.66–2.04)	2.46 (1.07–5.68)	1.418 (0.83–2.39)
No (Case/Cont)	44/78 (47.8/45.08)	38/78 (41.3/45.08)	10/17 (10.8/9.8)	48/95 (52.1/54.91)
OR (95% CI) ^c	1(Ref)	0.86 (0.50–1.47)	1.04 (0.43–2.47)	0.89 (0.53–1.48)
Location of cancer				
Lower stomach/upper stomach	62/36 (41/42.8)	61/34 (40.3/40.4)	28/14 (18.5/16.6)	89/48 (58.9/57.1)
OR (95% CI) ^d	1(Ref)	1.04 (0.57–1.87)	1.16 (0.54–2.48)	1.07 (0.62–1.84)
Tumor size				
≥5 cm/<5 cm	54/53 (42.8/42)	53/55 (42.8/41)	19/26 (15/19.4)	72/81 (57.1/60.4)
OR (95% CI) ^d	1(Ref)	0.94 (0.55–1.61)	0.71 (0.35–1.44)	0.87 (0.53–1.43)
TNM classification				
Stage III and IV/stage I and II	76/31 (45.2/33.6)	64/44 (38/47.8)	28/17 (16.6/18.47)	92/61 (54.7/66.3)
OR (95% CI) ^d	1(Ref)	0.59 (0.33–1.04)	0.67 (0.32–1.39)	0.61 (0.36–1.04)
Depth of invasion^e				
T3+T4/T1+T2	52/55 (46.8/36.9)	39/69 (35.1/46.3)	20/25 (18/16.7)	59/94 (53.1/63.0)
OR (95% CI) ^d	1(Ref)	0.59 (0.34–1.03)	0.84 (0.42–1.70)	0.66 (0.40–1.09)
Regional lymph node metastasis				
N+ve/N–ve	86/21 (41.5/39.6)	87/21 (42/39)	34/11 (16.4/20.7)	121/32 (58.4/60.3)
OR (95% CI) ^d	1(Ref)	1.01 (0.51–1.98)	0.75 (0.32–1.73)	0.95 (0.51–1.77)
Distant metastasis				
Yes/no	41/66 (39/42.5)	44/64 (41.9/41.2)	20/25 (19/16.1)	64/89 (60.9/57.4)
OR (95% CI) ^d	1(Ref)	1.10 (0.64–1.91)	1.28 (0.63–2.60)	1.15 (0.69–1.91)

^a Data adjusted by sex and addiction.

^b Data adjusted by age and addiction.

^c Data adjusted by age and sex.

^d Data adjusted by age, sex, and addiction. Values in bold indicate positive significance (*p*).

^e Depth of tumor was defined according to the criterion of American Joint Committee on Cancer. TNM (tumor, node, metastasis) staging were classified according to the criterion of the American Joint Committee on Cancer and the International Union against Cancer TNM stage grouping. Cont means control.

differences in the abundance and activity of serum MMP7 suggested possible alteration of its transcriptional activity between GG and AA genotype carriers.

Basal Expression Pattern of MMP7 Promoter-Reporter Constructs in Cultured Cells—The functional implication of the –181G→A polymorphism was tested by transfecting the promoter-reporter constructs harboring the G and A allele, at the site of polymorphism, into AGS, HEK-293, and SW480 cells in culture. In AGS cells, the promoter activity of the plasmid containing the G allele was ~1.8-fold higher (*p* < 0.0001) than the plasmid containing the A allele (Fig. 2A). In addition, SW480 and HEK-293 cells displayed similar patterns in promoter reporter gene activity. The promoter containing the G allele showed ~1.5- and ~2.2-fold more activity in SW480 and HEK-293 cells (*p* < 0.01 in each case), respectively, than the one containing the A allele (Fig. 2, B and C). Thus, the higher activity of the MMP7 promoter containing the G allele over A allele was clearly observed across different epithelial cell lines. This finding was consistent with the systemic expression of MMP7 (both protein level as well as enzyme activity) in subjects carrying the GG genotype (Fig. 1).

Induction of MMP7 Promoter Activity and Gene Expression by Nicotine—Because tobacco is a widely accepted risk factor for gastric cancer, we determined MMP7 promoter activity and gene expression upon nicotine induction. The effect of nicotine on MMP7 gene expression and activity was determined in AGS cells using casein zymography, Western blotting, and immunofluorescence techniques. AGS cells exhibited significantly higher levels of MMP7 activity and expression upon nicotine treatment as compared with the untreated control group in a time- and dose-dependent manner (Fig. 3, A–D). The highest activity and expression were achieved with a 200 μM dose in 30 h of incubation (Fig. 3, A, B, and D). We further tested the possible differential activity of the G/A allele-containing promoters by treatment with nicotine. This was performed by transfecting the promoter reporter constructs into AGS cells followed by treatment with 200 and 500 μM and 1 mM doses of nicotine. Nicotine dose-dependently induced the promoter activities of both the G and A allele-containing promoter constructs; the extent of activation of the G allele-containing construct was more prominent than the A allele (Fig. 3F). Interestingly, although the promoter containing the G allele displayed

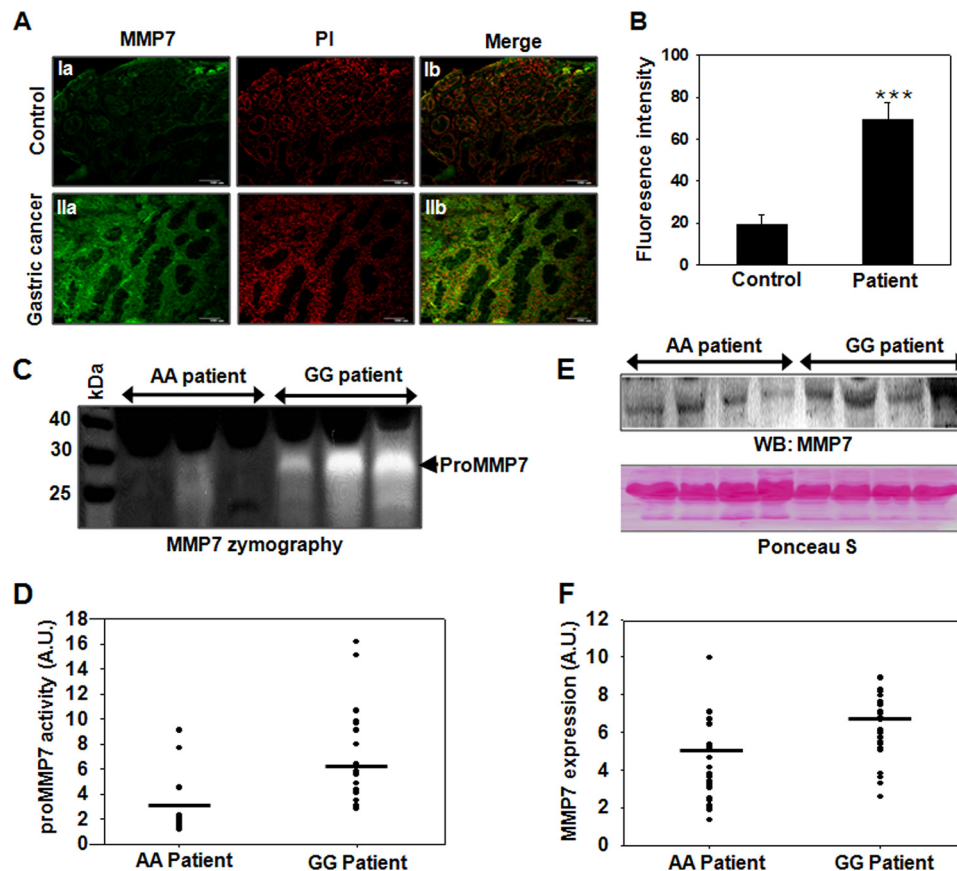


FIGURE 1. Localization of MMP7 in the gastric carcinoma tissues and correlation of MMP7 levels in serum of gastric cancer patients with MMP7 promoter variants. Tissues were collected, fixed in 4% paraformaldehyde, and processed for MMP7 immunofluorescence staining with fluorescein isothiocyanate-conjugated anti-goat IgG, and nuclei were stained with propidium iodide (PI) as described under "Experimental Procedures." Immunostaining of MMP7 for control (A, panel Ia) and gastric cancer patient (A, panel IIa) their PI merge picture (A, panels Ib and IIb). B, MMP7 protein expression was quantified by image analysis using ImageJ software. ***, significantly higher MMP7 expression was observed in cancer patient as compared with control ($p < 0.001$). Serum MMP7 activity and expression levels were analyzed in a subset of AA patients ($n = 22$) and GG patients ($n = 22$) by casein zymography (C) and Western blotting (WB) (E), respectively. Correlation of serum MMP7 activity and expression with respective genotype were analyzed using linear regression analysis. GG patient exhibited higher serum MMP7 activity (~ 2.4 -fold, $p = 0.001$) (D) and expression (~ 1.5 -fold; $p = 0.0006$) (F) when compared with the AA patient as shown in the scatterplot. Loading of serum protein was visualized by Ponceau S staining of the gel.

~ 1.2 -fold activation at the lowest tested dose of nicotine (200 μM), the A allele-containing promoter did not show any effect at that dose (Fig. 3F).

Alteration in the Transcription Factor Binding at the $-181\text{A} \rightarrow \text{G}$ Polymorphic Site—The differential activities of the promoters harboring G and A alleles could be attributed to the preferential binding of transcription factors to one allele over the other. This was computationally predicted using ConSite for identification of *cis*-regulatory elements that bound differentially across the G and A alleles. The program predicted the binding of the CREB transcription factor with higher affinity to the promoter motif containing the G allele over the A allele (score 3.605 versus -0.482 at 61% cutoff) (Fig. 4A). This prediction was validated experimentally by co-transfecting the G and A allele-containing promoter-reporter plasmids with different amounts of CREB expression plasmid. The activity of the G allele-containing *MMP7* promoter construct was increased in a CREB dose-dependent manner (Fig. 4B); it was significantly higher than the activity of the A allele-containing *MMP7* promoter-reporter constructs across all doses of the transcription factor. For example, the activity of the A allele-containing promoter was ~ 2 -fold lower than G-allele-contain-

ing promoter at the highest tested dose (500 ng) of the transcription factor expression plasmid (Fig. 4B).

Allele-specific *In Vivo* Interaction of CREB at the $-181\text{A} \rightarrow \text{G}$ Polymorphic Site—Chromatin immunoprecipitation was carried out to confirm the interaction and differential binding of CREB with the *MMP7* promoter harboring G and A alleles *in vivo* in the context of chromatin. The sonicated chromatin from N2a cells transfected separately with the G and A allele-containing *MMP7* promoter domain was immunoprecipitated with phospho-CREB, CREB, and rabbit IgG (mock) antibodies. ChIP quantitative PCR of the purified DNA revealed that the signal enrichment (representing the fold increase in signal relative to the corresponding IgG control) with the G allele in comparison with the A allele was ~ 9 -fold higher ($p < 0.001$) for immunoprecipitation with the p-CREB-1 antibody and ~ 1.5 -fold higher ($p < 0.05$) for immunoprecipitation with the CREB-1 antibody (Fig. 4C). This evidence suggests a stronger interaction of the transcription factors with the G allele compared with the A allele.

Nicotine Induces CREB Phosphorylation in Gastric Cancer Cells—We investigated the mechanisms underlying nicotine-mediated *MMP7* induction through phosphorylation of CREB,

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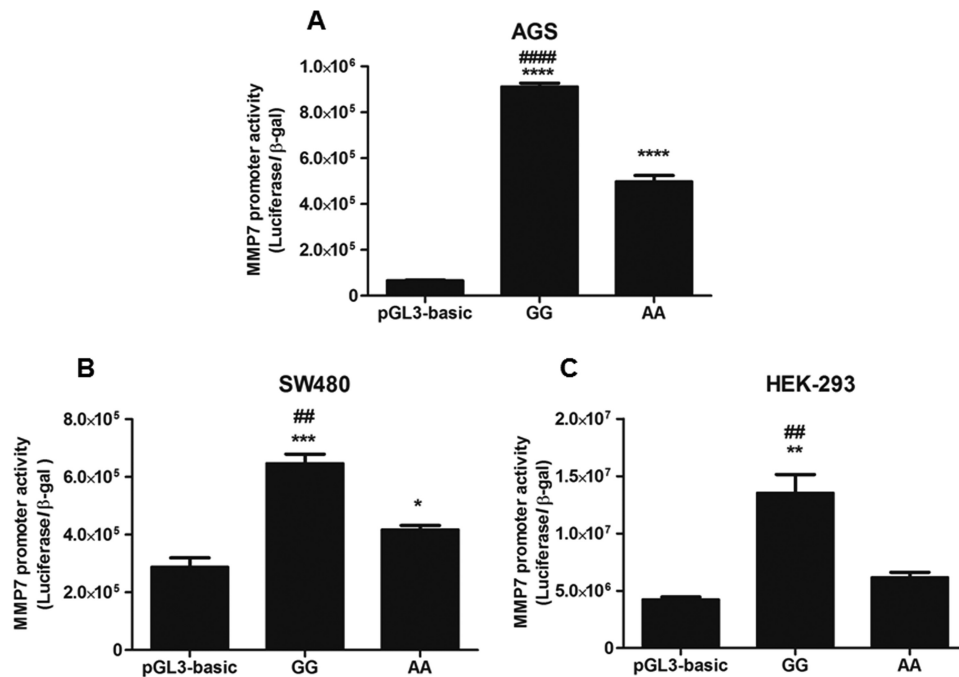


FIGURE 2. Expression of *MMP7* promoter-luciferase reporter plasmids in cultured cells. *MMP7* promoter reporter constructs (1 μ g/well) harboring GG and AA genotypes at the -181 -bp position in -230 to $+22$ -bp region of *MMP7* gene were separately transfected to AGS, SW480, and HEK-293 cells (A–C) along with control plasmid pCMV- β -gal (β -galactosidase driven by CMV promoter). The cells were assayed for luciferase and β -galactosidase activities 24–30 h after transfection. Values were plotted as means \pm S.E. of normalized luciferase activity to the β -galactosidase activity from at least three independent experiments. The GG and AA promoters displayed significantly different activities in AGS (ANOVA $F = 604.3$, $p < 0.0001$), SW480 (ANOVA $F = 47.31$, $p = 0.0002$) and HEK-293 (ANOVA $F = 26.59$, $p = 0.001$) cells as determined by one-way ANOVA with Bonferroni's, Tukey's, and Dunnett's multiple comparisons post hoc tests. The GG genotype-containing promoter was more active than the AA genotype promoter. ****, ***, **, and * indicate $p < 0.0001$, $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively, when compared with the control pGL3-basic plasmid; #### and ## indicate $p < 0.0001$ and $p < 0.01$, respectively, when compared with the AA genotype.

and CREB activation was assessed following exposure of AGS cells to nicotine. Nicotine exposure (200 μ M) led to a rapid transient increase in CREB phosphorylation within a 30-min short time span (Fig. 5A), and then the phosphorylation level decreased below basal levels at 1 h. Prolonged nicotine treatment further stimulated a second round significant increase in the phosphorylation of CREB after 5 h and attained a peak at 10 h of exposure as detected by Western blot. In addition, increased phosphorylation of CREB and nuclear localization of pCREB were detected by immunofluorescence (Fig. 5, B and C). This evidence suggests the exposure of AGS to nicotine induced the phosphorylation of CREB at a maximum of 10 h that then persisted up to 30 h (Fig. 5, B and C).

Down-regulation of CREB Function Displays *MMP7* Allele-specific Transcriptional Effect as Well as Diminished Nicotine-induced Promoter Activation—Down-regulation of CREB by siRNA transfection diminished the promoter activity of the G allele harboring *MMP7* promoter construct significantly (~ 1.5 -fold, $p < 0.05$), although no marked change was observed in the case of the A allele (Fig. 6A). The endogenous levels of CREB showed an $\sim 30\%$ decrease after siRNA transfection (Fig. 6B).

The crucial role of CREB in differential transcriptional regulation of the *MMP7* promoter containing the G and A alleles was further corroborated by expressing a CREB dominant negative mutant plasmid (KCREB) along with the respective promoter-reporter constructs in AGS cells. A profound reduction ($\sim 50\%$; $p < 0.01$) in the promoter activity was observed in the

case of the G allele, whereas the A allele-containing construct did not show a significant decrease (Fig. 6C). Co-transfection of KCREB plasmid also diminished nicotine-evoked *MMP7* promoter activity almost to the basal/uninduced level. The decline in promoter activity exhibited in case of the G allele was far more pronounced than that of the A allele. Thus, blockade of endogenous CREB function caused reduction of both basal as well as nicotine-stimulated activation of the *MMP7* promoter carrying -181 G allele.

Furthermore, the nicotine-induced augmentation of the *MMP7* promoter activity was blunted in response to tubocurarine hydrochloride (a well known competitive inhibitor of the nicotinic acetylcholine receptor). Co-application of tubocurarine with nicotine decreased the activity of the *MMP7* G promoter-reporter construct significantly (~ 2 -fold, $p < 0.01$) when compared with the corresponding nicotine-treated condition, and the A allele did not show significant reduction (Fig. 6D) thus substantiating that nicotine-stimulated *MMP7* promoter activity is mediated via nAChR.

Tobacco Addiction, *MMP7* -181 Genotypes, and Cancer Risk: Meta-analysis—To evaluate whether the association of the *MMP7* -181 G \rightarrow A polymorphism with cancer risk also holds true in other populations, we carried out a meta-analysis considering tobacco addiction as a modifying parameter. Based on the inclusion and exclusion criteria (as mentioned under "Experimental Procedures"), seven studies (consisting of a total 2240 subjects including 1160 patients and 1080 controls) were finally selected (13, 27, 36–38) for our meta-analysis. Stratifi-

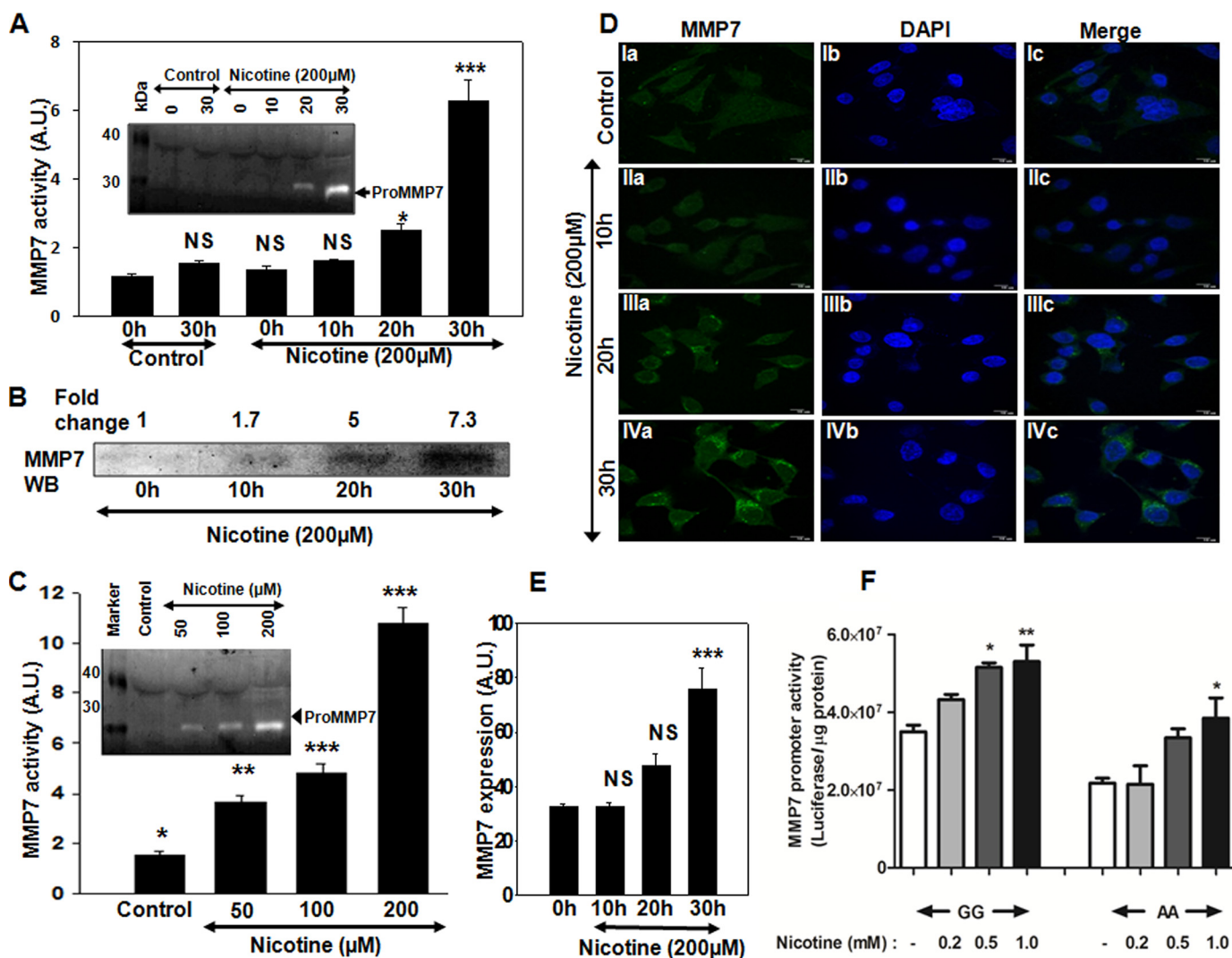


FIGURE 3. Effect of nicotine on MMP7 gene expression and promoter activity. AGS cells were treated with 200 μM dose of nicotine and were incubated up to 30 h in serum-free media. MMP7 activity and expression in cell supernatant (100 μg of protein) were assayed by casein zymography and Western blotting, respectively. Nicotine time-dependently up-regulated MMP7 activity (A, inset) and expression (B). AGS cells were treated with increasing doses of nicotine (50, 100, and 200 μM) and were incubated up to 30 h in serum-free media. MMP7 activity of cell supernatant protein (100 μg) was assayed by casein zymography. Nicotine dose-dependently up-regulated MMP7 activity (C). Arbitrary fold changes of MMP7 activity at different time intervals (A) were measured by Lab Image software design densitometry from three independent set of experiments. Results were reported as the means \pm S.E.; *, $p < 0.05$ and ***, $p < 0.001$. The data of arbitrary activity of MMP7 were compared with 0-h control groups. Nicotine treatment induced the expression of MMP7 with increasing time as seen in immunofluorescence assay (D). Treatment with 200 μM nicotine in serum-starved AGS cells up-regulated MMP7 at 20 and 30 h. MMP7 was detected by fluorescence isothiocyanate-conjugated anti-goat IgG, and the nucleus was stained by DAPI. Right panel represents their merged picture. MMP7 protein expression was quantified by image analysis using ImageJ software (E). AGS cells were transfected with either GG or AA MMP7 promoter (–230 to +22 bp)-firefly luciferase constructs (F). Transfected cells were treated with increasing doses of nicotine (200 and 500 μM and 1 mM) and incubated for 16–18 h. Cells were lysed and assayed for luciferase activity and protein concentration. Results were expressed as mean \pm S.E. of the ratio of firefly luciferase activity/ μg protein (F). Each experiment was performed in triplicate and repeated at least three times. Nicotine significantly induced the expression of both the MMP7 promoters but to a greater extent in the case of GG-containing promoter than AA. ***, **, and * indicate $p < 0.001$, $p < 0.01$, and $p < 0.05$ with respect to the corresponding mock (without stimulation). WB, Western blot; NS, not significant.

cation of subjects on the basis of their MMP7 –181 genotype status showed that tobacco addiction significantly increased the cancer risk in the AA smokers (RR = 1.92, 95% CI = 1.28–2.88, $p = 0.0001$) as compared with AA nonsmoker subjects, and there was an increase in cancer risk for addicted AG (RR = 2.14, 95% CI = 1.51–3.03, $p = 0.0009$) or AG+GG (RR = 2.15, 95% CI = 1.61–2.87, $p = 0.0007$) genotype with respect to non-addicted subjects (Fig. 7). It appears that tobacco addiction significantly enhances cancer risk in the presence of the –181G allele.

Discussion

Cancer is a polygenic disease, and multiple signaling pathways as well as exogenous risk factors govern the disease progression. Recent genetic studies indicate that certain genetic traits or a particular ethnicity may be predisposing for certain cancers (3, 6, 39). The effect of the predisposed genetic traits may accelerate by major exogenous risk factors, including tobacco addiction and environmental pollution, which serve as an important parameter for the individual in heightening the risk of cancer (39). Genetic factors, including functionally

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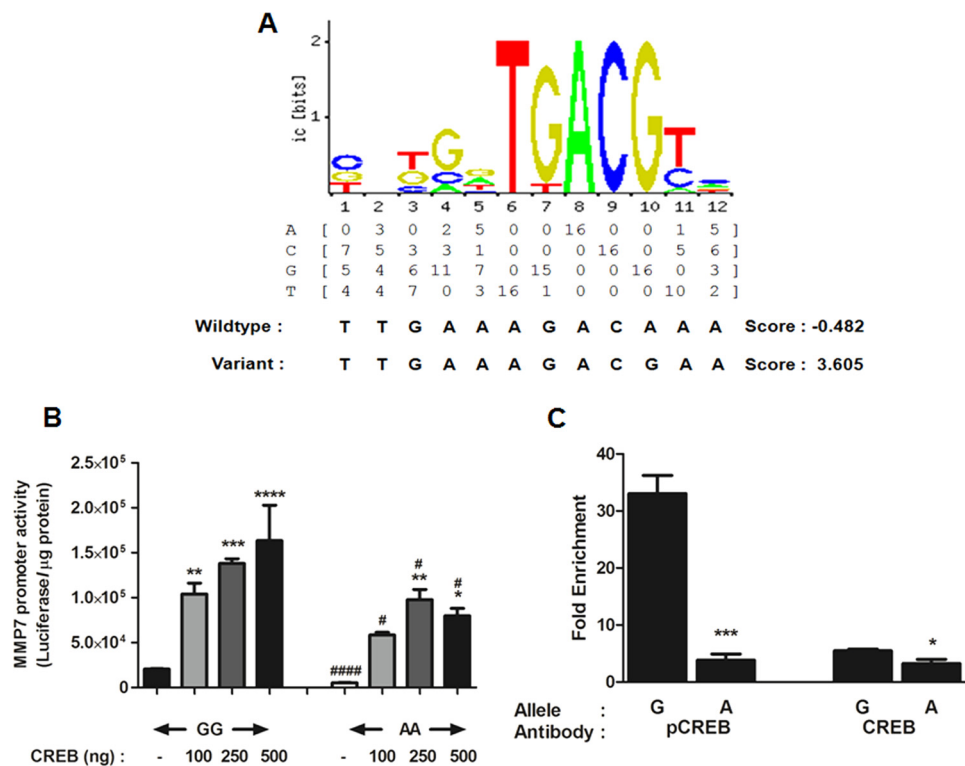


FIGURE 4. Differential activation of *MMP7* promoter harboring G and A alleles by CREB. *A*, *MMP7* promoter-SNP alters potential binding affinities of promoter motifs with putative transcription factors. Pictorial presentation as well as numerical nucleotide matrix for CREB-binding motif according to ConSite is shown. The -192 - to -181 -bp region of the human *MMP7* promoter contains a putative binding site for CREB. The A/G SNP at -181 bp altered the potential binding affinity of CREB to the promoter motif in GG versus AA, with the ConSite scores being 3.605 versus -0.482 at 61% cutoff, respectively. *B*, activation of *MMP7* promoter activity was observed by overexpression of CREB. AGS cells were transiently transfected with increasing quantities (0–500 ng/well) of CREB expression plasmid and 1.0 μ g/well of GG and AA *MMP7* promoter (-230 to $+22$ bp)-firefly luciferase construct. The total amount of plasmid DNA transfected to each well was made equal by using balancing amounts of pcDNA 3.1. Cells were lysed 24–30 h after transfection and assayed for luciferase activity. The results were expressed as ratios of firefly luciferase activity/ μ g of protein and the mean \pm S.E. ($n = 3$ transfections for each construct). Although both GG and AA promoters displayed, in general, activation by the transcription factor in a dose-dependent manner with respect to the control, the extent of activations was greater in the case of GG genotype promoter plasmid. ****, ***, **, and * indicate $p < 0.0001$, $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively, when compared with the corresponding mock (i.e. without a co-transfected transcription factor expression plasmid). #### and # indicate $p < 0.0001$ and $p < 0.05$ when compared with the corresponding dose in case of G allele. *C*, N2a cells were transfected with the G and A promoter constructs, and the chromatin was cross-linked with 1% formaldehyde. Cells were lysed, and the sonicated chromatin was immunoprecipitated with p-CREB-1, CREB-1 antibodies with rabbit IgG as control. The DNA obtained after reverse cross-linking was subjected to real-time PCR using primers flanking the 230-bp promoter region. Fold enrichments of DNA precipitated using p-CREB and CREB antibodies were calculated with respect to the corresponding IgG controls. Representative data are shown as mean \pm S.E. The G-allele-containing chromatin fragments were ~ 9 - and ~ 1.5 -fold higher with p-CREB and CREB, respectively, as compared with the A-allele chromatin fragments, indicating a higher affinity of CREB for G allele over A allele of *MMP7* $-181G \rightarrow A$ polymorphism. *** and * indicate $p < 0.001$ and $p < 0.05$, respectively when compared to G allele.

important SNPs within the promoter region of certain genes, contribute to cancer susceptibility. *MMP7* genetic variation and cancer susceptibility have been studied mainly in two promoter polymorphisms ($-181A \rightarrow G$ and $-153C \rightarrow T$) that have been found to affect protein expression (10, 23). Most of the studies showed positive associations of $-181GG$ genotype with the risk of cancer development among different populations (13). However, the *MMP7* promoter polymorphism and risk of gastric cancer in any Indian population have not been investigated.

We performed a case-control study to evaluate the association of *MMP7* $-181A \rightarrow G$ polymorphism and gastric cancer risk and the dependence on age, sex, as well as tobacco addiction in an eastern Indian population. Our study reports for the first time that *MMP7* $-181GG$ genotype is associated with a significant risk of gastric cancer in the eastern Indian population. The frequency of the GG genotype in the eastern Indian gastric cancer patients was much higher (17.3%) compared with control (10%) and strongly associated with disease susceptibil-

ity ($p = 0.02$; OR = 1.9). The occurrence of the $-181GG$ genotype in our patients (as compared with healthy individuals) was consistent with previous reports on other cancer types (cervical, esophagus, ovarian, and endometrial) in different populations. For example, individuals with *MMP7* $-181GG$ genotype were at a significantly higher risk of endometrial cancer in a Taiwanese population (40). Likewise, *MMP7* $-181GG$ genotype was predisposed to a higher risk of esophageal squamous cell carcinoma, gastric cardiac carcinoma, and non-small cell lung carcinoma in the Han Chinese population (13). In contrast, in the Caucasian population, the frequency of GG genotype was lower in gastric cancer (28). In the Indian population, the *MMP7* $-181GG$ genotype was at a significantly higher risk of cervical cancer (41) and contributed to squamous cell gastric cancer susceptibility in the Kashmir Valley (36).

We addressed the effect of promoter variants of *MMP7* $-181A \rightarrow G$ on expression of *MMP7* protein. Significant contributions of *MMP7* in proliferation, invasion, and metastasis in various types of cancer have been documented (16, 18, 20).

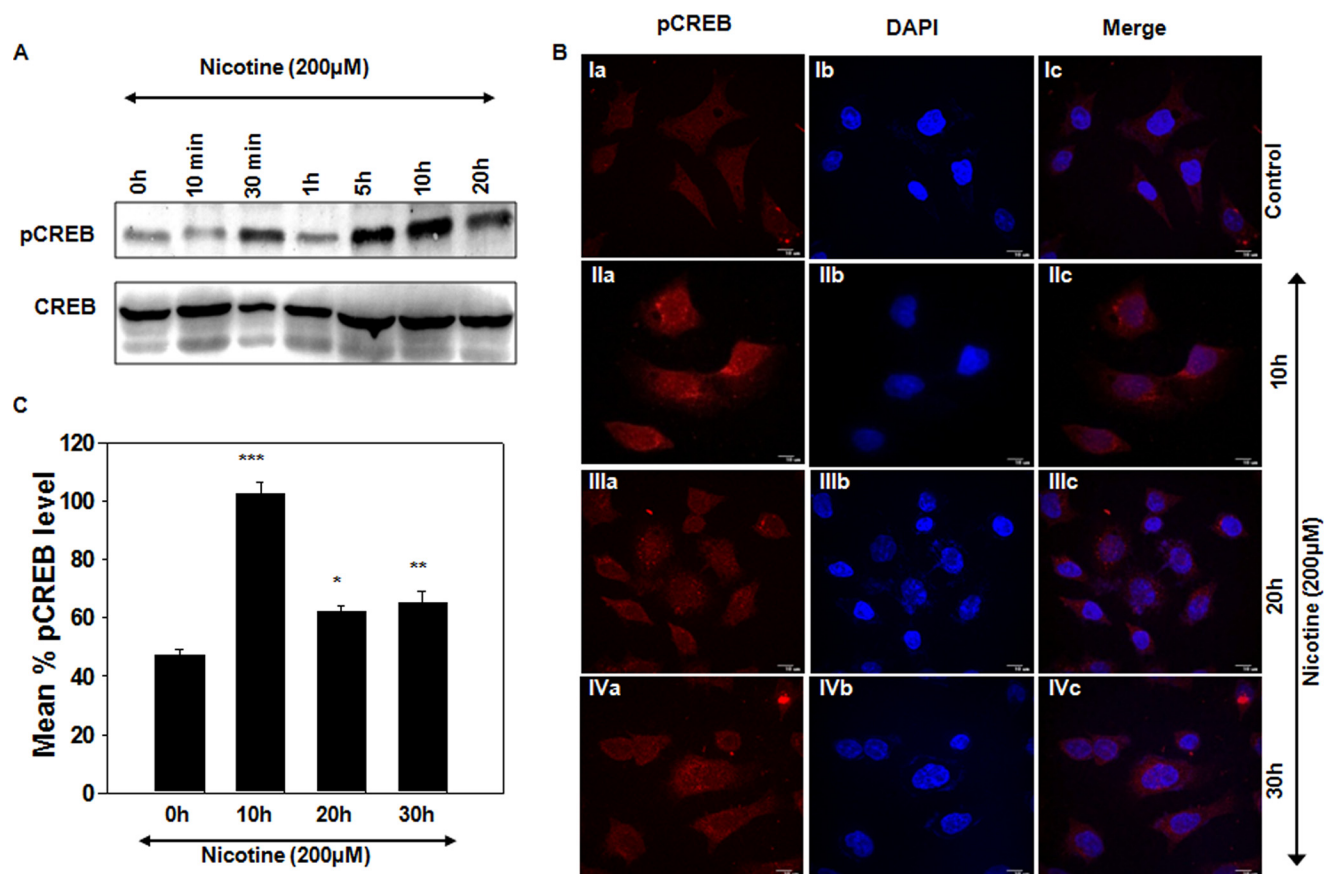


FIGURE 5. Nicotine induces phosphorylation of CREB. AGS cells were treated with 200 μM nicotine for indicated time points in serum-starved condition. Western blotting performed using (100 μg) protein of total cell extract indicates enhanced phosphorylation of CREB molecule up to 20 h (A). Nicotine treatment (200 μM) induces the phosphorylation and nuclear translocation of CREB with increasing times as seen in immunofluorescence assay (B). Phospho-CREB was detected by Texas Red-conjugated anti-rabbit IgG, and the nucleus was stained with DAPI. Right panel (merged picture) indicates nuclear localization of pCREB. Phospho-CREB level was quantified by image analysis using ImageJ software (C). ***, **, and * indicate $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively, when compared with 0-h nicotine-treated condition.

However, very little is known about the molecular basis of the MMP7 up-regulation in gastric cancer and elevated protein expression in body fluids. We found a significant elevation of MMP7 expression in the biopsy specimens from gastric cancer patients which is consistent with a previous report (16). A higher level of MMP7 in serum was also reported in several cancers. Sarkissian *et al.* (42) suggested abundant plasma pro-MMP7 may serve as a disease diagnosis marker in renal cancer. Earlier studies revealed higher serum MMP7 expression in *H. pylori*-related gastric, colorectal, and urinary bladder cancer patients as compared with control subjects (43–45). Moreover, to decipher the functional importance of this promoter polymorphism on MMP7 transcriptional regulation, we compared serum MMP7 expression as well as activity in gastric cancer patients with the two promoter variants. Our result revealed that serum MMP7 expression and activity level were higher in the GG genotype than in the AA genotype patients, suggesting the influence of this promoter polymorphism on MMP7 gene expression (Fig. 1, C and E). However, a significant association between MMP7 – 181AA genotype and elevated plasma levels of MMP7 was reported in idiopathic pulmonary fibrosis patients (26). Thus, increased sensitivity of the polymorphic MMP7 promoter to certain transcription factors in turn provides a molecular mechanism for elevated expression of MMP7, which is reflected in peripheral blood expression level.

Moreover, we addressed which transcription factors bind with specific allele-containing MMP7 promoter and the importance of MMP7 – 181A \rightarrow G polymorphism in regulating transcription and functional activity in relevance to the development of gastric cancer. We evaluated the regulation of MMP7 gene expression by the common promoter SNP (*viz.* – 181A \rightarrow G) in cultured gastric adenocarcinoma cells. Interestingly, the promoter-reporter construct containing the G allele showed higher activity than that containing the A allele under basal conditions (Fig. 2), suggesting a regulatory role for this SNP. We asked what might be the molecular basis for higher promoter activity in the case of the G allele? Computational analysis predicted that the presence of A at – 181 bp of MMP7 caused disruption of a cyclic AMP-response element domain, leading to a drastically reduced binding affinity for CREB (Fig. 4A). Indeed, co-transfection of a CREB expression plasmid evoked much stronger MMP7 promoter transcription in the G allele instead of the A allele (Fig. 4B). Moreover, ChIP assays also revealed stronger interaction of CREB with the G allele-containing human MMP7 promoter than the A allele in the context of chromatin, providing a plausible molecular mechanism for allele-specific alteration in the gene expression and the pathogenetic risk for gastric cancer. In support, inhibition of endogenous CREB function by overexpression of a dominant negative mutant CREB or siRNA against CREB led to the down-

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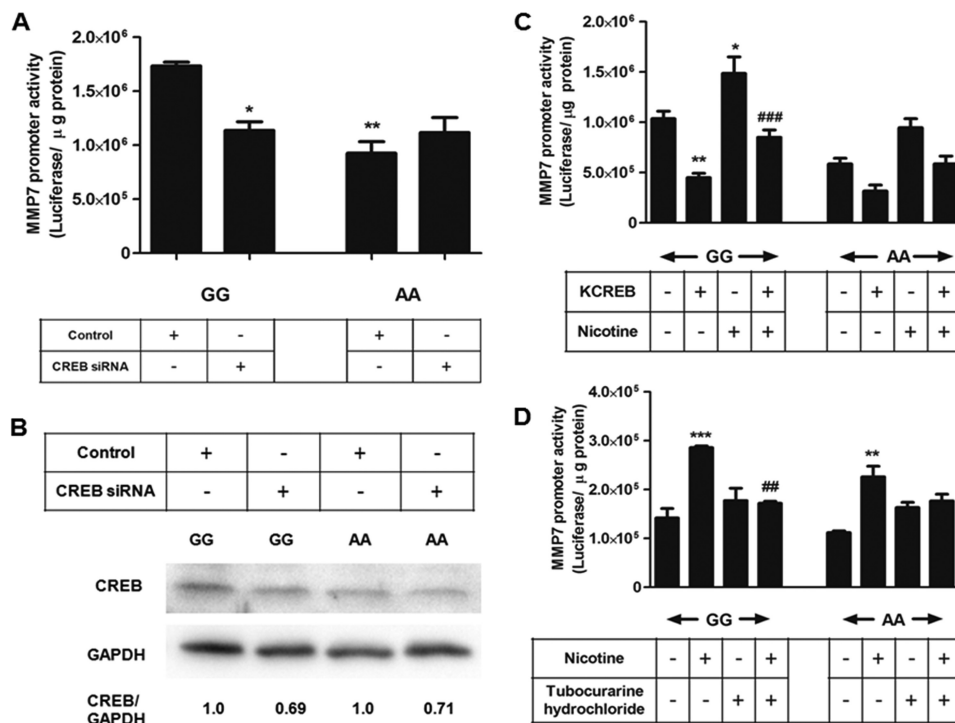


FIGURE 6. CREB mediates the nicotine-induced activation of MMP7 promoter. *A*, effect of siRNA-mediated down-regulation of CREB on MMP7 promoter activity. AGS cells were transfected with 1.0 μg of GG and AA MMP7 promoter (-230 to $+22$ bp)-firefly luciferase constructs and co-transfected with CREB siRNA. Cells were lysed and assayed for luciferase activity. Results are expressed as mean \pm S.E. of luciferase activity/ μg of protein. One-way ANOVA followed by Bonferroni post hoc tests were carried out. ** and * indicate $p < 0.01$ and $p < 0.05$ when compared with the corresponding G-allele activity. *B*, endogenous CREB levels after siRNA-mediated down-regulation. AGS cells were lysed following siRNA treatment and processed for Western blotting to detect protein level. GAPDH was used as normalization control. *C*, blockade of endogenous CREB and effect on MMP7 promoter activity. AGS cells were transfected with 1.0 μg of GG and AA MMP7 promoter-firefly luciferase constructs and co-transfected with 1.0 μg of KCREB (a dominant negative mutant of CREB) plasmid. Cells were treated with a 200 μM dose of nicotine wherever indicated and incubated for 18–24 h. Cells were lysed and assayed for luciferase activity and protein concentration. Results are expressed as mean \pm S.E. of luciferase activity/ μg of protein. One-way ANOVA followed by Bonferroni post hoc tests were carried out. * and ** indicate $p < 0.05$ and $p < 0.01$ when compared with the corresponding basal promoter activity, and ### indicates $p < 0.001$ when compared with the respective nicotine-treated condition. *D*, MMP7 promoter activity is activated via nicotinic acetylcholine receptors upon nicotine treatment. AGS cells were transfected with 1.0 μg of GG and AA MMP7 promoter/reporter constructs and treated with 25 μM tubocurarine hydrochloride and 200 μM nicotine for 3 h after 12 h of transfection. Cells were lysed and assayed for luciferase activity and protein concentration. Results are expressed as mean \pm S.E. of luciferase activity/ μg of protein. One-way ANOVA followed by Bonferroni post hoc tests were carried out. ** and *** indicate $p < 0.01$ and $p < 0.001$ when compared with the corresponding basal promoter activity, and ## indicates $p < 0.01$ when compared with the respective nicotine-treated condition.

regulation of the basal MMP7 promoter activity (Fig. 6), further strengthening the role of CREB in the MMP7 expression. In this context, it has been reported that the expression and promoter activity of the MMP7 -181G allele was 2–3-fold higher than the -181A allele in U937 cell type and was attributed to the formation of a putative binding site (NGAAN) for a heat-shock transcription factor in the case of the G-allele although not for the A-allele (25). On the contrary, elevated plasma MMP7 levels in AA genotype of idiopathic pulmonary fibrosis patients were governed by the G to A transition in the -181 site of the MMP7 promoter resulting in a novel binding site for the forkhead box A2 (FOXA2) transcription factor, which led to higher promoter activity in the case of the A allele (26). The above divergence prompted us to speculate that this promoter discrimination mechanism by transcription factor might be a tissue-specific phenomenon.

We found that tobacco addiction significantly enhanced the risk of gastric cancer in GG ($p = 0.03$, OR = 2.46, and 95% CI = 1.07–5.68) genotypes in the eastern Indian population. In line with our observation, an association of functional polymorphism of the MMP7 gene and elevated cancer risk among smokers was documented, but it remained inconclusive (13). To better evaluate the contribution of tobacco addiction in can-

cer susceptibility, we included all the studies in various ethnic groups. Meta-analysis, including a total of 2240 subjects, extended our finding of association of the MMP7 $-181\text{A} \rightarrow \text{G}$ SNP with various cancers in different populations (Fig. 7). Consistently, our meta-analysis data also suggested that the relationship of tobacco and cancer risk is stronger among subjects having AG or GG than AA genotype carriers (Fig. 7). As tobacco is one of the exogenous risk factors and MMP7 is over-expressed in gastric cancer (Figs. 1 and 3), we then evaluated the contribution of nicotine (the major component of tobacco) on MMP7 gene induction in AGS cells. A significant up-regulation of MMP7 in both expression and activity level upon nicotine treatment suggested that the MMP7 gene expression could be regulated by nicotine signaling pathways (Fig. 3). In line with our observation, nicotine has been reported to induce epithelial mesenchymal transition in gastric cancer cells through up-regulation of MMP7 gene expression (46).

Now the following question arises: What could be the effect of nicotine on two different MMP7 promoter variants in terms of gene expression as GG carriers having tobacco addiction are more susceptible to gastric cancer? Our data that we believed to be novel because the promoter-reporter construct containing the G allele showed higher promoter activity compared with A

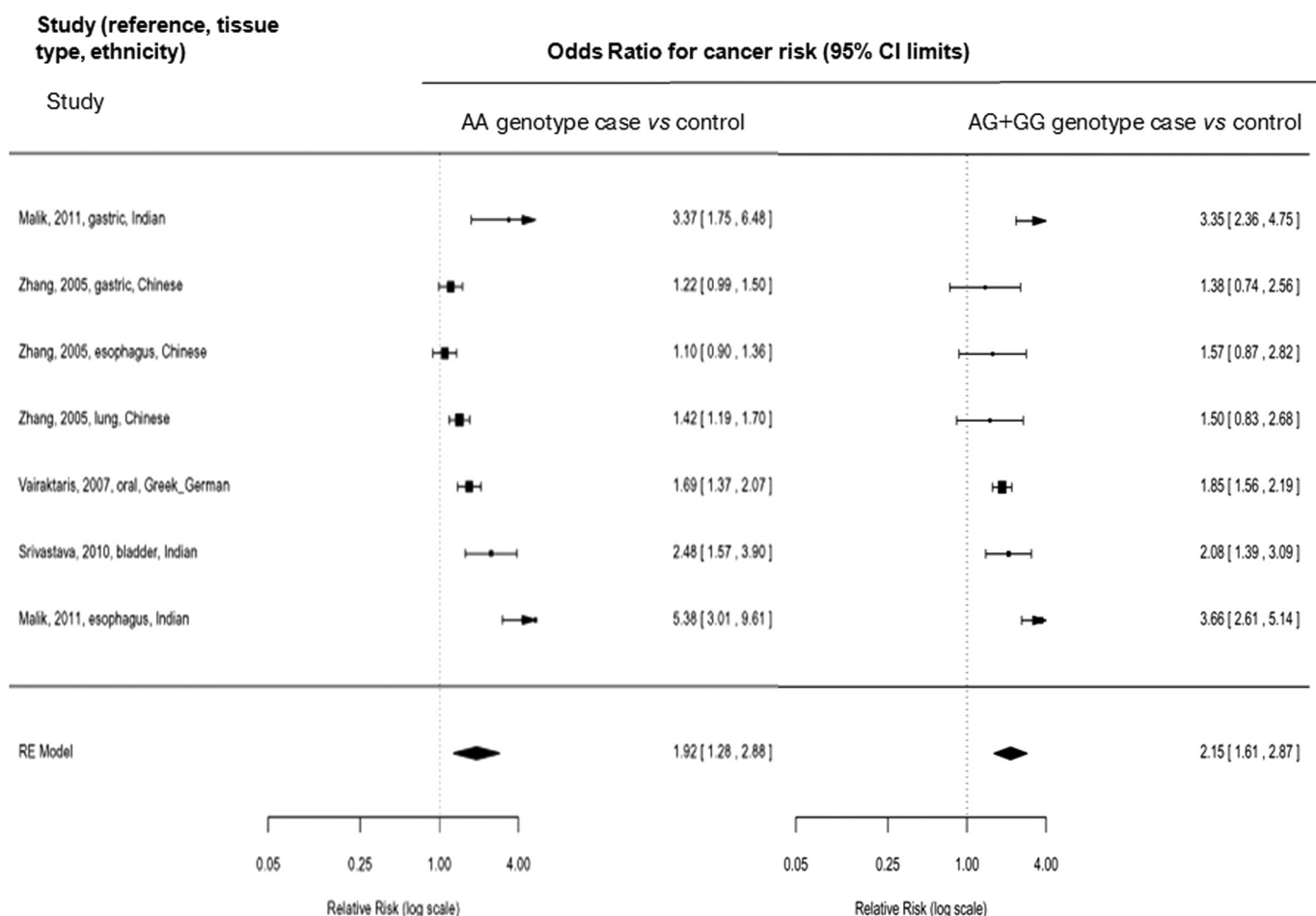


FIGURE 7. Forest plot of cancer risk associated with tobacco addiction for subjects having *MMP7* –181 AA and AG+GG genotypes. A random effect model was used. The squares and horizontal lines correspond to the study-specific RR and 95% CI, respectively. The area of the squares reflects the study-specific weight. The diamond represents the pooled results of RR and 95% CI. Nonaddicted groups were considered as reference.

allele under nicotine-stimulated conditions (Fig. 3F). As nicotine showed allele-specific induction of *MMP7* promoter activity, and CREB exerted preferential binding with the G-allele under basal/nicotine-stimulated conditions (Fig. 3), we speculated that additive activity of the G-allele-carrying promoter under nicotine-stimulated conditions may be facilitated by the activation of CREB through phosphorylation. Consistent with this notion, the abundance of phosphorylated CREB (pCREB) in the nicotine-treated AGS cells was observed, which persisted for 30 h (Fig. 5). It is noteworthy that prolonged activation of CREB phosphorylation was observed in other cell types as well. For example, stimulation of CREB phosphorylation by ethanol in lung fibroblast showed elevated pCREB levels up to at least 24 h (47). Likewise, treatment of PC12 cells with nicotine triggered rapid but transient elevation of pCREB followed by a second sustained rise after 5 h of continuous nicotine treatment (48). However, nicotine induced CREB phosphorylation has not been investigated yet in AGS cells. Herein, we found a biphasic activation of CREB in AGS cells, although the second rise in CREB phosphorylation for a much longer period might be associated with *MMP7* gene transcription. Moreover, our results established that nicotine-mediated *MMP7* promoter activity was substantially reduced under endogenous CREB-depleted conditions (Fig. 6). The additive effect of nicotine in

lung, pancreatic, and colon cancer development has been investigated in several *in vivo*, *in vitro*, and case-control studies (49–51). A classical research in this line showed that nicotine exerts its biological function through its receptor nicotine acetylcholine receptor (nAChR). In neuronal cells, binding of nicotine with its receptor led to activation of the MAPK pathway resulting in phosphorylation and subsequent activation of CREB (52). Nicotine-induced proliferation and migration of gastric cancer cells were mediated by activation of ERK pathways (51, 53). The first etiological link between the nAChR-mediated regulation of β -adrenergic signaling and the development of smoking-associated cancer was documented by Schuller *et al.* (54, 55). Very recently, Al-Wadei *et al.* (56) demonstrated that chronic nicotine treatment in the pancreatic cell line up-regulated catecholamine production with significant phosphorylation of signaling proteins ERK and CREB. In addition, Shi *et al.* (16) reported overexpression of β 2-adrenergic receptor along with *MMP7* in gastric cancer patients. Moreover, catecholamine treatment resulted in up-regulation of *MMP7* in the gastric cancer cell line suggesting the regulatory role of the β -adrenergic receptor pathway (16). Furthermore, West *et al.* (57) showed that rapid Akt activation by nicotine via $\alpha(3)$ -/ $\alpha(4)$ -containing or $\alpha(7)$ -containing nicotinic acetylcholine receptors modulate the normal human airway epithelial cells to acquire

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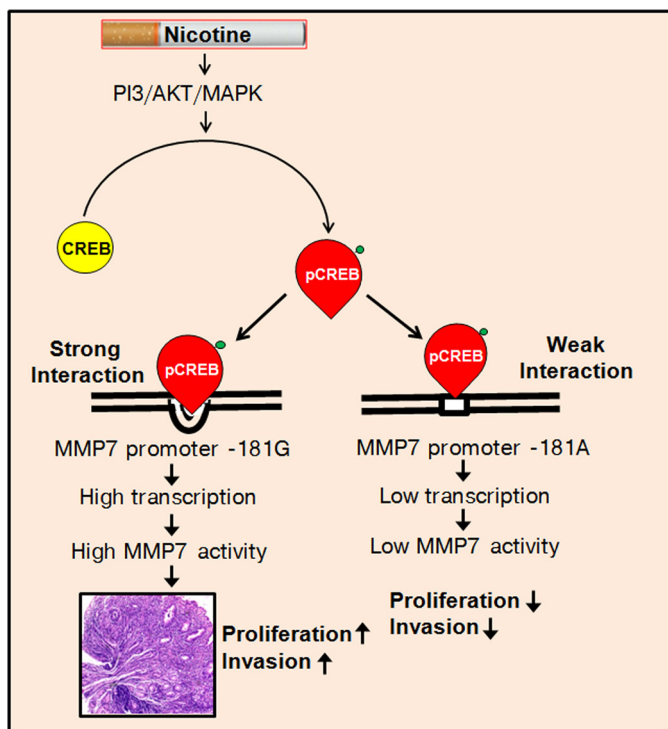


FIGURE 8. Proposed mechanisms of the regulatory role of CREB in nicotine-induced MMP7 promoter activation. nAChR indirectly stimulates MMP7 up-regulation as well as gastric carcinogenesis through PI3/AKT, MAPK, or adenylyl cyclase-mediated induction of CREB phosphorylation pathways. MMP7 promoter shows two promoter variants (–181A→G) at the binding site of CREB transcription factor. Preferential binding of pCREB with MMP7 –181G promoter up-regulated MMP7. Nicotine augments this interaction by enhancing CREB phosphorylation.

cancer cell-like property. Nicotine caused up-regulation of VCAM-1, MMP-2, and MMP-9 through the $\alpha 7$ -nAChR-JNK pathway (58), and stimulation of cell migration through $\alpha 7$ nicotinic acetylcholine receptor in gastric cancer cells (59). Similarly, in our recent finding (Fig. 6D), the up-regulation of MMP7 is mediated by nAChR upon nicotine treatment in AGS cells. However, a direct link between nicotine-induced phosphorylation of CREB and subsequent enhancement of the MMP7 promoter activity was not investigated. Herein, we provide the first molecular evidence that nicotine enhanced phosphorylation of CREB in the gastric cancer cell line and subsequently trans-activated the MMP7 promoter (Fig. 8). These data thus define a critical link between a human predisposition marker, exogenous risk factor, and gastric cancer susceptibility that is likely to extend to many other types of cancer as well and possibly could guide personalized approaches to cancer prevention.

In summary, we uncovered the molecular basis for higher MMP7 expression in gastric cancer patients. GG genotype at the –181 site of the MMP7 promoter significantly increased susceptibility to gastric cancer in eastern Indian population. Higher MMP7 expression is accomplished by an allele-specific differential binding of pCREB on the MMP7 promoter with G allele carriers. Our result also showed that pCREB-mediated MMP7 gene transcription is robust for the G allele promoter under the influence of nicotine. Nicotine intake significantly heightened G allele-containing MMP7 promoter activity by

enhancing CREB phosphorylation. Future research on the evaluation of multiple gene interactions along with the effect of other environmental factors may provide an effective measure for screening gastric cancer-susceptible populations. Hence, this research provides an optimistic message for gastric cancer prevention by controlling tobacco addiction and addresses the importance of the susceptible genetic variants for pharmacogenomics.

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Association of *MMP7* -181A→G Promoter Polymorphism with Gastric Cancer Risk: INFLUENCE OF NICOTINE IN DIFFERENTIAL ALLELE-SPECIFIC TRANSCRIPTION VIA INCREASED PHOSPHORYLATION OF cAMP-RESPONSE ELEMENT-BINDING PROTEIN (CREB)

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