A Common Tag Nucleotide Variant in *MMP7* Promoter Increases Risk for Hypertension via Enhanced Interactions With CREB (Cyclic AMP Response Element-Binding Protein) Transcription Factor

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Abstract—MMP (matrix metalloproteinase)-7—a potent extracellular matrix degrading enzyme—is emerging as a new regulator of cardiovascular diseases. However, potential contributions of *MMP7* genetic variations to hypertension remain unknown. In this study, we probed for the association of a tag single-nucleotide polymorphism in the *MMP7* promoter (−181A/G; rs11568818) with hypertension in an urban South Indian population (n=1501). The heterozygous AG genotype significantly increased risk for hypertension as compared with the wild-type AA genotype (odds ratio, 1.60 [95% CI, 1.25–2.06]; $P=2.4\times10^{-4}$); AG genotype carriers also displayed significantly higher diastolic blood pressure and mean arterial pressure than wild-type AA individuals. The study was replicated in a North Indian population (n=949) (odds ratio, 1.52 [95% CI, 1.11–2.09]; $P=0.01$). Transient transfection experiments using *MMP7* promoter-luciferase reporter constructs revealed that the variant −181G allele conferred greater promoter activity than the −181A allele. Computational prediction and structure-based conformational and molecular dynamics simulation studies suggested higher binding affinity for the CREB (cyclic AMP response element-binding protein) to the −181G promoter. In corroboration, overexpression/downregulation of CREB and chromatin immunoprecipitation experiments provided convincing evidence for stronger binding of CREB with the −181G promoter. The −181G promoter also displayed enhanced responses to hypoxia and epinephrine treatment. The higher promoter activity of −181G allele translated to increased MMP7 protein level, and *MMP7* −181AG heterozygous individuals displayed elevated plasma MMP7 levels, which positively correlated with blood pressure. In conclusion, the *MMP7* A-181G promoter polymorphism increased MMP7 expression under pathophysiological conditions (hypoxic stress and catecholamine excess) via increased interactions with CREB and enhanced the risk for hypertension in its carriers. **(*Hypertension*. 2019;74:1448-1459. DOI: 10.1161/HYPERTENSIONAHA.119.12960.)** ● **Online Data Supplement**

Key Words: catecholamine ■ hypertension ■ hypoxia ■ MMP7 ■ single nucleotide polymorphism ■ transcriptional regulation

MMPs (matrix metalloproteinases) degrade the components of extracellular matrix (ECM) and thereby contribute to tissue remodeling and developmental processes. MMPs cleave bioactive molecules, chemokines, cytokines, growth factors involved in physiological processes including inflammation, angiogenesis, and wound healing.[1] To date, 24 different MMPs have been identified and classified into collagenases, stromelysins, gelatinases, matrilysins, and membrane-type MMPs based on their domain and substrate specificities. Expressions of MMP genes are primarily regulated at the transcriptional level, and activities of MMPs are inhibited by tissue inhibitors of MMPs.[2]

MMP7—the smallest MMP (molecular weight, 28 kDa)—is a secreted matrilysin with specificity for a broad range of substrates (*viz.* fibronectin, elastin, type IV collagen, and proteoglycans). MMP7 also cleaves non-matrix substrates (eg, proheparin-binding epidermal growth factor, Fas ligand, E-cadherin, β2 adrenergic receptor, and TNF-α [tumor necrosis factor-α]) and activates other MMPs (*viz.* pro-MMP2 and pro-MMP9).[3] Serum/plasma MMP7 levels are elevated in several types of cancers, atherosclerosis, hypertension, chronic kidney disease, and diabetes mellitus.[4–7] Since uncontrolled proteolytic processes leading to vascular remodeling act as an important determinant of

Received March 6, 2019; first decision March 23, 2019; revision accepted September 26, 2019.

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The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.119.12960.

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cardiovascular complications, MMPs are expected to serve as potential mediators in cardiovascular diseases (CVDs).[8] Indeed, knockdown of *MMP7* in the SHR (spontaneous hypertensive rat) model attenuates hypertension.[9] MMP7 transcriptionally activates MMP2, and knockdown of *MMP7* prevents the progression of Ang II (angiotensin II)–induced hypertension and cardiac hypertrophy.[10] Genetic polymorphisms in *MMP7* have been associated with coronary artery disease, acute myocardial infarction, multiple sclerosis, rheumatoid arthritis, and several cancers.[7,11] Among the most widely studied single nucleotide polymorphisms (SNPs) of *MMP7*, the promoter SNPs A-181G (rs11568818) and C-153T (rs11568819) displayed allele-specific effects and modulated gene expression via differential interaction with nuclear-binding proteins.[11,12] However, potential association of regulatory variants in *MMP7* gene with hypertension has not been reported to date. Here, we aimed to study the association of the common tag SNP A-181G in the *MMP7* gene promoter with hypertension in 2 geographically distinct Indian populations and to elucidate the mechanism governing the allele-specific effects of the polymorphism using systematic computational and experimental analyses.

Materials and Methods

The detailed Materials and Methods are provided in the online-only Data Supplement. The authors declare that all supporting data are available within the article and the online-only Data Supplement.

Human Subjects

The study population comprised of 1354 hypertensive and 1096 normotensive individuals attending the outpatient departments of The Madras Medical Mission, Chennai, and the Postgraduate Institute of Medical Education and Research, Chandigarh, during 2012 to 2018. The study was approved by the Institutional Ethics Committee at Indian Institute of Technology Madras. Demographic, physiological, and biochemical parameters of both the study populations are listed in Tables S1 and S2 in the online-only Data Supplement.

Genotyping of *MMP7* −181A/G Polymorphism

Human genomic DNA samples were polymerase chain reaction amplified using specific primers for the *MMP7* promoter region (−302 to −153 bp), purified, and genotyped (Figure S1).

Cloning and Mutagenesis

MMP7 −181A/G promoter-reporter constructs (harboring −230bp to +22 bp region of *MMP7* gene) were generated using pGL3-Basic vector. *MMP7* −181A/G promoter-cDNA constructs were generated by replacing the firefly luciferase cDNA in *MMP7* promoter-reporter constructs with *MMP7* cDNA.

Cell Lines, Transfection, and Reporter Assays

MMP7 promoter-reporter constructs and β-gal (β-galactosidase) expression plasmid were transfected into IMR-32, SH-SY5Y, H9c2, and N2a cell lines. Cotransfection experiments with CREB (cyclic AMP response element-binding protein) and KCREB (dominant-negative CREB) expression plasmids,[13] CREB siRNA oligos, and treatments (epinephrine and hypoxia) were performed in IMR-32 and H9c2 cells. Luciferase, β-gal, and total protein assays were performed,[14] and promoter activities were expressed as luciferase/β-gal or luciferase/μg of protein.

Experiments were also performed to mimic the homozygous and heterozygous conditions *in cella* with *MMP7* promoter-reporter constructs and by transfecting *MMP7* promoter-cDNA constructs to estimate MMP7 levels *in vitro*.

Western Blotting

Immunoblotting experiments were performed to detect overexpression or downregulation of CREB, phospho-CREB, and HIF-1α (hypoxia-inducible factor 1α) after transfection experiments/epinephrine treatment/hypoxia and to measure the influence of *MMP7* −181G and −181A promoter variants on the MMP7 levels *in vitro* using specific antibodies.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays were performed in N2a and H9c2 cells transfected with *MMP7* −181G and *MMP7* −181A promoter-reporter constructs with/without treatment with epinephrine (5 μM) or hypoxia.[5] Immunoprecipitated DNA in case of −181G and −181A alleles due to CREB/phospho-CREB binding were quantified by quantitative polymerase chain reaction using fold enrichment method relative to IgG signal.

Computational Analysis

The homology model of CREB1 was built using MODELLER v11.9[15] based on the template 1DH3 (*Mus musculus* mCREB1). The *MMP7* −181G and *MMP7* −181A promoter DNA models were built using 3DDART[16] and SCFBio (http://www.scfbio-iitd.res.in/research/drugdna.html). Protein-DNA docking was performed with HDOCK[17] and HADDOCK[18] tools. Molecular dynamics simulation study for all CREB1-*MMP7* (protein-DNA) complexes/models was performed using Desmond[19,20] tool in Maestro.

Data Presentation and Statistical Analysis

Genotype-phenotype associations were tested by 1-way ANOVA with post hoc tests using Statistical Package for Social Sciences. Promoter-reporter transfection results were expressed as mean±SEM from representative experiments. Statistical significance was calculated by Student *t* test, 1-way ANOVA, or 2-way ANOVA with post hoc tests, as applicable, using Prism 5 program.

Results

Identification and Linkage Disequilibrium Analysis of SNPs in the *MMP7* Promoter

SNPs occurring at a frequency ≥1% in the 5-kb region of *MMP7* promoter in the South Asian population of the 1000 Genomes project were identified from dbSNP database (Figure S2; Table S3). Of the 10 SNPs identified, 7 were common polymorphisms (occurring at a frequency of ≥5%). Pairwise linkage disequilibrium analysis was performed to predict nonrandom association of the alleles of these 10 SNPs and to shortlist tag SNPs. Eight of the 10 SNPs constituted a haplotype block suggesting that these alleles could be inherited together (Figure S2B). Additionally, linkage disequilibrium tag SNP selection tool from SNPinfo[21] web server predicted the rs11568818 (*MMP7* A-181G) polymorphism as a tag SNP; it occurred at a minor allele frequency (MAF) of 0.36 in the overall 1000 Genomes population and 0.43 in the South Asian superpopulation. This tag SNP was in linkage disequilibrium with SNPs at −1378 bp (rs17098318) and −1773 bp (rs17881620) (Figure S2B).

Occurrence of *MMP7* −181 SNP in Indian Populations

The *MMP7* A-181G SNP occurred at an MAF of 0.47 (≈72.4% of the subjects) in the Chennai population (n=1501). MAF of this SNP in a geographically distinct Chandigarh population (n=949) was 0.41 (≈64.3% of the subjects). The genotypic

frequencies in both populations were in Hardy-Weinberg equilibrium (Chennai population: X^2 P=0.94 and Chandigarh population: X^2 P=0.44) (Table S4).

MMP7 −181AG Genotype Is Associated With Hypertension in Indian Populations

Logistic regression analysis was performed to estimate the relative risk contributed by the variant G allele toward hypertension by both genotypic (AA versus AG and AA versus GG) and dominant (AA versus AG+GG) models. The −181AG heterozygous genotype displayed association with hypertension risk as compared with the −181AA genotype with odds ratios (ORs) of 1.60 ([95% CI, 1.25–2.04] P=1.6×10^{-4}) in Chennai population and 1.49 ([95% CI, 1.12–1.97]; P=0.006) in Chandigarh population (Table). These associations remained significant even after adjusting for age/sex/body mass index (Chennai population: OR, 1.60 [95% CI, 1.25–2.06]; P=2.4×10^{-4} and Chandigarh population: OR, 1.52 [95% CI, 1.11–2.09]; P=0.01). Although the homozygous variant GG genotype did not show a statistically significant OR, the dominant model (AG+GG) exhibited a significantly strong association with hypertension in both Chennai (adjusted OR, 1.37 [95% CI, 1.09–1.73]; P=0.006) and Chandigarh (adjusted OR, 1.46 [95% CI, 1.09–1.98]; P=0.013) populations (Table).

Differential Activities of *MMP7* −181A/G Promoter-Reporter Constructs in Cultured Cardiomyoblast and Neuroblastoma Cells

To test the functional role of −181A and −181G alleles, *MMP7* promoter-reporter constructs harboring A/G allele were transfected into cardiomyoblast (H9c2) and neuroblastoma (IMR-32, SH-SY5Y, and N2a) cell lines (Figure 1). *MMP7* −181G construct consistently displayed higher promoter activity than the −181A construct in all these cell lines (H9c2: ≈1.7-fold, P<0.01; IMR-32: ≈1.4-fold, P<0.001; SH-SY5Y: ≈1.3-fold, P<0.05; N2a: ≈1.5-fold, P<0.05) (Figure 1).

Computational Analyses Reveal Differential Interactions of CREB With *MMP7* −181A/G Promoters

To probe for potential differential interactions of transcription factors at the −181 bp position that could contribute to the higher *MMP7* promoter activity in case of the −181G allele, computational analysis was performed using ConSite/MatInspector/P-Match programs. The transcription factor CREB was predicted to bind to the −181G allele with higher affinity by both ConSite and P-Match. The Transfac position-weight matrix for CREB with binding scores for −181A and −181G alleles is shown in the Figure S3. Structure-based conformational and molecular dynamics simulation studies were next performed using models of CREB and *MMP7* promoters.

The CREB1 homology model was generated for the DNA binding region corresponding to residues 280-341 of CREB1 based on DP-Bind and PredictProtein tool predictions. Models of *MMP7* −181A and *MMP7* −181G promoters were also generated, and docking of the CREB1 structure to the promoter DNA structures followed by molecular dynamics simulations was performed (Figure 2A; Figure S4; Table S5).

The average structure was extracted from the first 40 ns simulation to generate the comparable stable interaction map between *MMP7*-promoter DNA and CREB1. In case of CREB1-chain A:*MMP7* −181A complex, N293 and R301 were the 2 main amino acids that participated in the interactions with the promoter DNA segment. The residue N293 formed total 4 hydrogen bonds (*viz.* one with A24 nucleotide at DNA-chain C, 2 with A25 nucleotide at DNA-chain C, and 1 with T23 nucleotide at DNA-chain D) (Figure 2B and 2D); the residue R301 formed 1 hydrogen bond with T22 nucleotide at DNA-chain D. On the contrary, the CREB1-chain A:*MMP7* −181G complex, apart from having interactions similar to that of *MMP7* −181A complex, also established 3 additional hydrogen bonds with residue R289 (2 hydrogen bonds with G23 nucleotide at DNA-chain C [i.e., the −181G variant nucleotide] and 1 hydrogen bond with T25 nucleotide at DNA-chain D) (Figure 2C and 2E).

The 2-dimensional interaction map of the *MMP7* −181A and *MMP7* −181G systems reflects the same interaction pattern (Figure S5). The interaction map displays 2 regions of interactions for CREB1-chain A, between 22-26 nucleotide on *MMP7* DNA:chain C (i.e., −180 to −184 bp of *MMP7* promoter) and 21-22 nucleotide on *MMP7* DNA:chain D (i.e., −185 to −186 bp of *MMP7* promoter). Similarly, CREB1-chain B interacts with 29-31 nucleotide on *MMP7* DNA:chain C (i.e., −187 to −189 bp of *MMP7* promoter) and 15-18 nucleotide on *MMP7* DNA:chain D (i.e., −189 to −192 bp of *MMP7* promoter). Despite differences in the van der Waals contacts made by the amino acid residues of CREB1-chain A and CREB1-chain B with the *MMP7* wild-type and mutated promoters, the direct hydrogen bonds between the *MMP7* −181G nucleotide and a hot-spot residue R289 could facilitate the favorable binding of CREB to the *MMP7* −181G promoter with higher affinity over *MMP7* −181A promoter.

Table. Association of *MMP7* −181A/G Polymorphism With Hypertension Risk in Indian Populations

MMP7 Promoter Genotype	Chennai Population				Chandigarh Population			
	Logistic Regression (Unadjusted), n=1501		Logistic Regression (Age, Sex, and BMI Adjusted), n=1453		Logistic Regression (Unadjusted), n=949		Logistic Regression (Age and Sex Adjusted), n=949	
	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
AA	1 (ref)	…	1 (ref)	…	1 (ref)	…	1 (ref)	…
AG	1.60 (1.25–2.04)	1.6×10^{-4}	1.60 (1.25–2.06)	2.4×10^{-4}	1.49 (1.12–1.97)	0.006	1.52 (1.11–2.09)	0.010
GG	0.99 (0.75–1.33)	0.98	1.01 (0.75–1.36)	0.94	1.14 (0.78–1.66)	0.49	1.31 (0.86–2.01)	0.210
AG+GG	1.37 (1.09–1.73)	0.006	1.39 (1.09–1.75)	0.007	1.38 (1.06–1.80)	0.017	1.46 (1.09–1.98)	0.013

BMI indicates body mass index; CI, confidence interval; MMP7, matrix metalloproteinase-7; and OR, odds ratio.

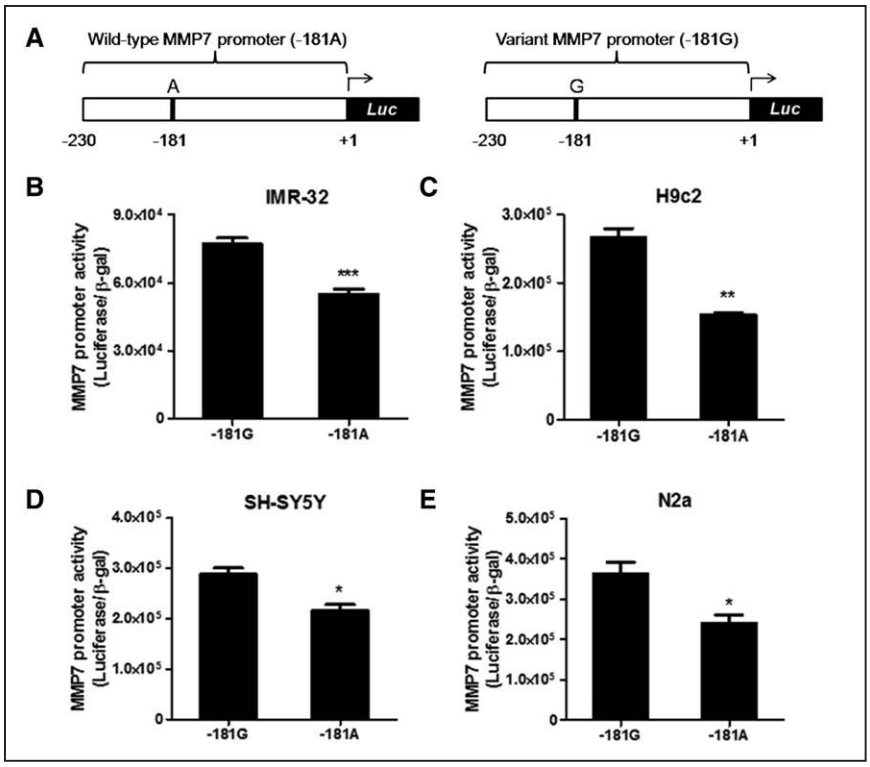

Figure 1. Differential promoter activities of *MMP7* promoter-reporter constructs. **A**, Schematic representation of the *MMP7* promoter-reporter constructs. **B–E**, *MMP7* −181G and −181A promoter-reporter constructs were transfected into rat cardiomyoblast H9c2 (**B**) neuroblastoma cell lines IMR-32 (**C**), SH-SY5Y (**D**), and N2a (**E**) along with β-gal (β-galactosidase) expression plasmid. Results are expressed as mean±SEM of triplicate values of the ratio of luciferase/β-gal activity. **P*<0.05, ***P*<0.01, and ****P*<0.001 with respect to −181G construct.

Experimental Evidence for Interactions of CREB With *MMP7* −181A/G Promoters

In view of the prediction of enhanced interactions of CREB with the *MMP7* −181G allele (Figure 2; Figure S5), experimental validation of differential activation of *MMP7* −181A/G promoters by CREB was performed. Overexpression of CREB increased activities of *MMP7* promoter-reporter constructs in a concentration-dependent manner; the −181G construct

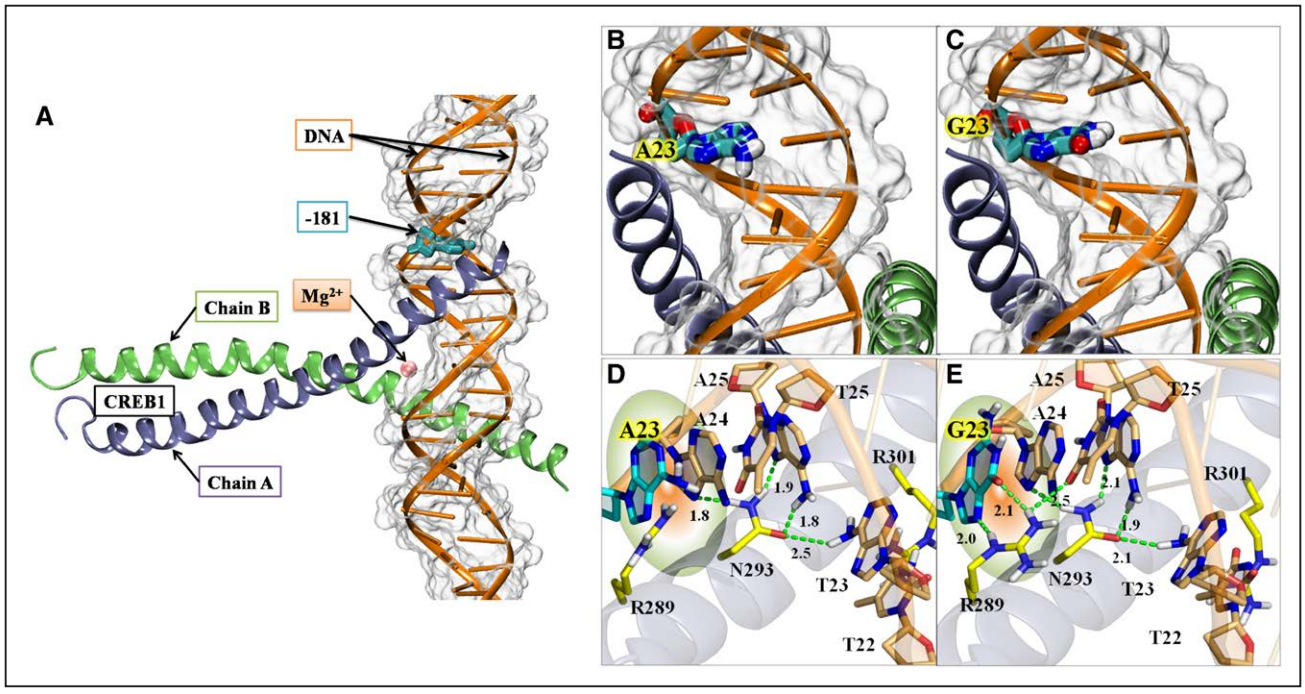

Figure 2. Schematic diagram of interactions of *MMP7*-promoter DNA with CREB (cyclic AMP response element-binding protein)-1 transcription factor. **A**, Representative energy minimized model of CREB1:wild-type *MMP7* complex rendered in new cartoon representation. DNA is shown in orange color; CREB1-chain A and CREB1-chain B are shown in ice-blue and green colors, respectively. DNA is shown in transparent white surface view as well. Position of the −181 bp nucleotide and Mg^{2+} has been indicated. **B**, Positioning of the nucleotide −181A (labeled as A23) in wild-type promoter. **C**, Positioning of the nucleotide −181G (labeled as G23) in the mutant promoter. Comparison of wild-type (**D**) and mutant (**E**) *MMP7*-promoter DNA interactions with CREB1 in an enlarged view. Amino acids are indicated by single-letter codes and based on their positions in the CREB1-chain A. Amino acids and nucleotides are rendered in licorice and colored atom-wise; C, light orange; N, blue; O, red; H, white. Hydrogen bonds are shown in green dotted lines.

exhibited significantly higher promoter activity than the −181A construct in IMR-32 (1-way ANOVA F=162.9, $P<0.0001$) and H9c2 cells (1-way ANOVA F=43.3, $P<0.0001$) (Figure S6A and S6B). Overexpression of CREB was confirmed by Western blotting. In corroboration, cotransfection of KCREB plasmid lead to a highly significant concentration-dependent decrease (at least 60%) in the promoter activity of *MMP7* −181G construct in both cell types (IMR-32: 1-way ANOVA F=13.72, $P<0.0001$; H9c2: 1-way ANOVA F=11.45, $P<0.001$) (Figure S6C and S6D). The −181A construct did not show any change in promoter activity in response to KCREB cotransfection. Downregulation of CREB using siRNA also reduced the promoter activity of −181G construct as compared with the negative control oligo in IMR-32 (by ≈45%; 1-way ANOVA F=7.13, $P<0.05$) and H9c2 (by ≈60%; 1-way ANOVA F=79.02, $P<0.0001$) cells; −181A construct did not show consistent reduction in promoter activity under similar conditions (Figure 3A and 3B). Downregulation of CREB was confirmed by Western blotting.

Further, chromatin immunoprecipitation assays were performed to study the interaction and preferential binding of CREB with the *MMP7* −181G promoter in the context of chromatin. Quantitative polymerase chain reaction of purified, CREB-immunoprecipitated chromatin from N2a and H9c2 cells transfected with *MMP7* −181G and −181A promoter constructs revealed that the enrichment of the *MMP7* −181G allele was ≈1.7- and ≈1.8-fold higher, respectively, as compared with the −181A allele ($P<0.05$; Figure 3C and 3D).

Enhanced Response of *MMP7* −181G Promoter to Epinephrine: Crucial Role for CREB

Since elevated levels of catecholamines are associated with hypertension, we evaluated the effect of epinephrine and the concomitant role of CREB, if any, on *MMP7* −181G and −181A promoter activities. Indeed, epinephrine treatment augmented the promoter activity of *MMP7* −181G construct in a concentration-dependent manner in IMR-32 (≤2.5-fold; 1-way ANOVA F=22.03, $P<0.0001$) and in H9c2 (≈2.2-fold; 1-way ANOVA F=17.10, $P<0.0001$) cells; on the contrary, *MMP7* −181A construct did not show significant increase in promoter activity (Figure S7A and S7B). Consistently, epinephrine treatment enhanced phospho-CREB levels in a concentration-dependent manner in these cell lines (Figure S7C and S7D). Further, to test whether CREB mediated the activation of *MMP7* −181G promoter in response to epinephrine, IMR-32 and H9c2 cells were cotransfected with KCREB and *MMP7* −181G or −181A promoter-reporter constructs followed by epinephrine treatment. Epinephrine, which augmented the promoter activity of *MMP7* −181G construct significantly, failed to evoke a similar response in the KCREB cotransfected condition in IMR-32 (2-way ANOVA genotype effect: F=56.05, $P<0.0001$; treatment effect: F=15.30, $P<0.0001$) and H9c2 (2-way ANOVA genotype effect: F=16.50, $P<0.001$; treatment effect: F=17.58, $P<0.0001$) cells. The −181A construct did not show any significant difference in promoter activity upon epinephrine treatment either in the presence or absence of KCREB (Figure S7E and S7F).

In chromatin immunoprecipitation assays using *MMP7* −181G/A promoter-transfected and epinephrine-treated cells, the −181G allele displayed significantly higher fold enrichment with CREB (N2a: ≈5.0-fold; H9c2: ≈1.8-fold) and phospho-CREB (N2a: ≈4.5-fold; H9c2: ≈3.5-fold) as compared with the −181A allele (Figure S7G and S7H). Thus, the epinephrine induced activation of *MMP7* −181G promoter activity appears to be strongly mediated by CREB.

Hypoxic Stress Activates *MMP7* −181G Promoter via CREB

Since hypoxia is known to increase blood pressure (BP) and is a major contributor to cardiac pathophysiology, the effect of hypoxia on *MMP7* −181G and −181A promoter-reporter constructs was studied. IMR-32 and H9c2 cells were subjected to hypoxia after transfection with −181G/A promoter constructs for 12 hours with/without CREB and KCREB cotransfection. The *MMP7* −181G construct displayed significantly enhanced promoter activity under hypoxia in both IMR-32 (≈1.7-fold) and H9c2 (≈2.3-fold) cells; however, no significant increase was observed with the −181A construct (Figure 4A and 4B). Upon CREB overexpression, hypoxia treatment further increased the *MMP7* −181G promoter activity (≈5.7-fold in IMR-32 and ≈7.5-fold in H9c2) as compared with *MMP7* −181A construct in IMR-32 (2-way ANOVA genotype effect: F=39.7, $P<0.0001$; treatment effect: F=267.8, $P<0.0001$) and H9c2 (2-way ANOVA genotype effect: F=30.97, $P<0.0001$; treatment effect: F=73.01, $P<0.0001$) cells. On the contrary, hypoxia resulted in only a modest increase in promoter activity in the KCREB cotransfected cells. Under hypoxia, concomitant with HIF-1α (IMR-32: ≈6.6-fold; H9c2: ≈7.9-fold), CREB (IMR-32: ≈1.4-fold; H9c2: ≈1.8-fold), and phospho-CREB (IMR-32: ≈1.5-fold; H9c2: ≈1.3-fold) levels were also found to be elevated (Figure 4C and 4D).

Further, interactions of CREB/phospho-CREB with −181G/A alleles under hypoxia were probed in N2a and H9c2 cells transfected with *MMP7* promoter-reporter constructs. The fold enrichment for CREB (N2a: ≈1.5-fold; H9c2: ≈1.4-fold) and phospho-CREB (N2a: ≈2.3-fold; H9c2: ≈1.9-fold) in case of the −181G allele under hypoxia was significantly higher (Figure 4E and 4F) than the −181A allele (N2a: 1-way ANOVA F=63.7, $P<0.0001$; H9c2: 1-way ANOVA F=28.0, $P<0.0001$).

MMP7 −181G Promoter Results in Higher MMP7 Levels *In Vitro* and *In Vivo*

By generating recombinant plasmids wherein hMMP7 cDNA was placed under the control of *MMP7* −181G and *MMP7* −181A promoters, we probed whether the differential activities of G/A alleles result in altered MMP7 levels *in vitro* (Figure S8A). Expression of these plasmids in N2a (Figure S8B and S8C) and H9c2 (Figure S8D and S8E) cells revealed significantly elevated levels of MMP7 in case of −181G promoter-driven *MMP7* cDNA (N2a: ≈1.6-fold, $P<0.0001$; H9c2: ≈1.4-fold, $P<0.05$) as compared with −181A promoter-driven *MMP7* cDNA suggesting higher activity of −181G promoter in the genomic context.

Furthermore, we analyzed plasma MMP7 levels in a section of normotensive/untreated subjects from the study population after stratification into *MMP7* promoter genotypes; *MMP7* −181AG individuals displayed higher plasma MMP7 levels (Figure 5A) than −181AA and −181GG individuals (1-way ANOVA F=3.06, $P<0.05$). Although plasma MMP7

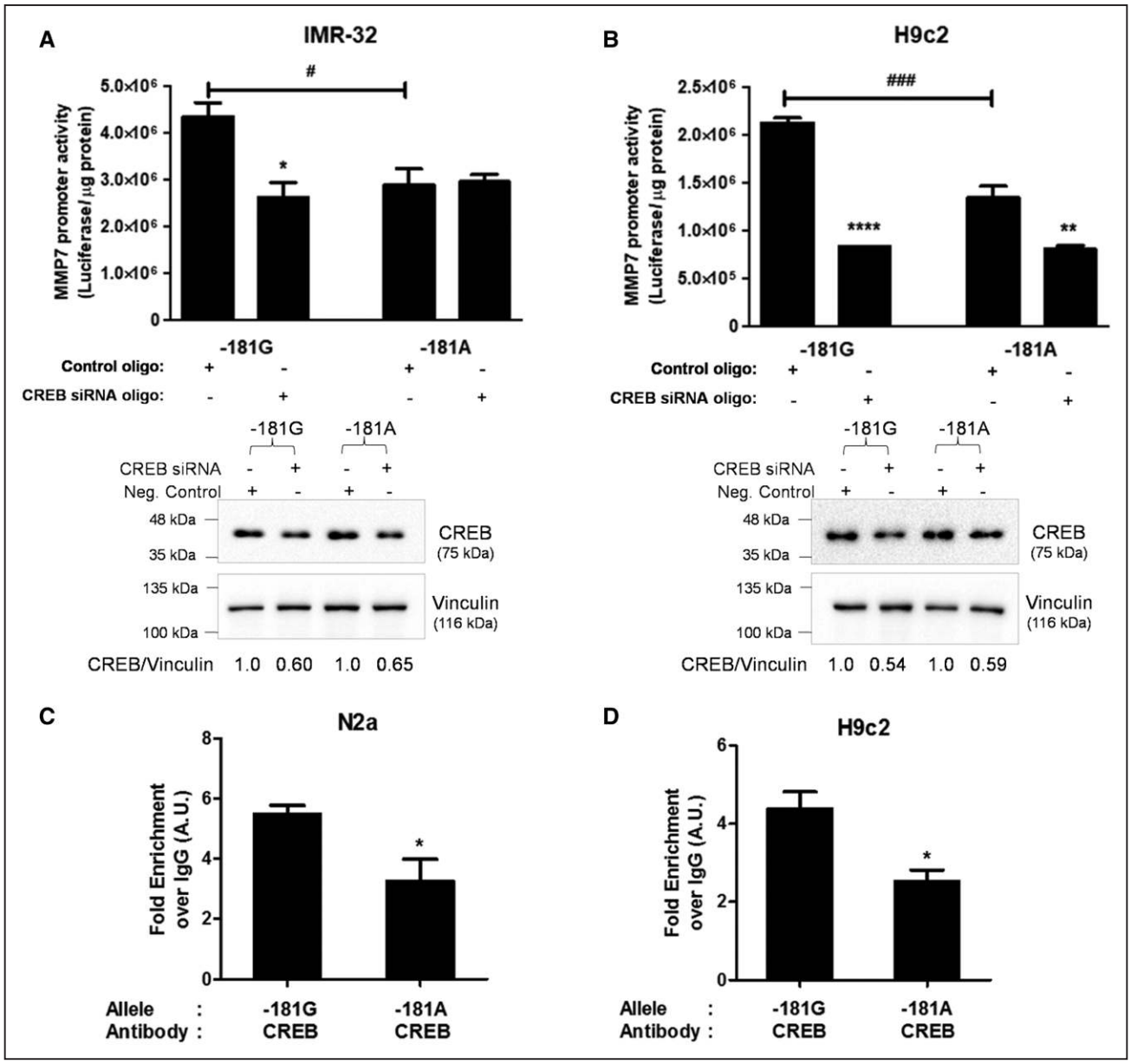

Figure 3. Enhanced interactions of CREB (cyclic AMP response element-binding protein) with *MMP7* −181G allele. **A** and **B**, siRNA-mediated knockdown of CREB decreases the *MMP7* −181G promoter activity. IMR-32 (**A**) and H9c2 (**B**) cells were transfected with *MMP7* −181G or −181A constructs along with control siRNA oligo or CREB siRNA oligo. Assays were performed after 48 h of transfection. Results are expressed as mean±SEM of triplicate values of the ratio of luciferase activity/μg protein. **P*<0.05, ***P*<0.01, and *****P*<0.0001 as compared with the basal activity of the corresponding construct. #*P*<0.05 and ###*P*<0.001 as compared with the basal activity of *MMP7* −181G construct. Downregulation of CREB was confirmed by Western blotting. **C** and **D**, Binding of CREB to −181G and −181A alleles of *MMP7* promoter. Chromatin immunoprecipitation of N2a (**C**) and H9c2 (**D**) cells transfected with −181G or −181A construct was performed using antibody against CREB/control IgG. Immunoprecipitated chromatin was subjected to quantitative polymerase chain reaction. Fold enrichment in case of CREB antibody over IgG control is shown. **P*<0.05.

levels were modestly higher in individuals of −181GG genotype than those with −181AA genotype, the difference was not statistically significant. Thus, the plasma levels of MMP7 follow the same trend of association displayed by the −181AG individuals in the logistic regression analysis toward hypertension risk (Table).

Activities of *MMP7* Promoter Constructs in Diploid Combinations *in Cella*

In view of the association of *MMP7* −181AG genotype with hypertension risk, as well as higher MMP7 levels *in vivo*, we sought to test whether this observation could also be demonstrated in transfected cultured cells. We transfected IMR-32 and H9c2 cells with −181G or −181A promoter-reporter plasmids in 3 diploid combinations: −181G/G (ie, only −181G construct), −181G/A (ie, equimolar amounts of −181G and −181A constructs), and −181A/A (ie, only −181A construct) to mimic the homozygous variant, heterozygous and wild-type conditions, respectively. Interestingly, both −181G and −181G/A transfected conditions showed similar extent of elevated promoter activity (≈1.8-fold and ≈1.75-fold, respectively, in IMR-32 cells; ≈1.4-fold and ≈1.3-fold, respectively, in H9c2 cells) as compared with the −181A construct alone (IMR-32: 1-way ANOVA F=63.74, *P*<0.0001;

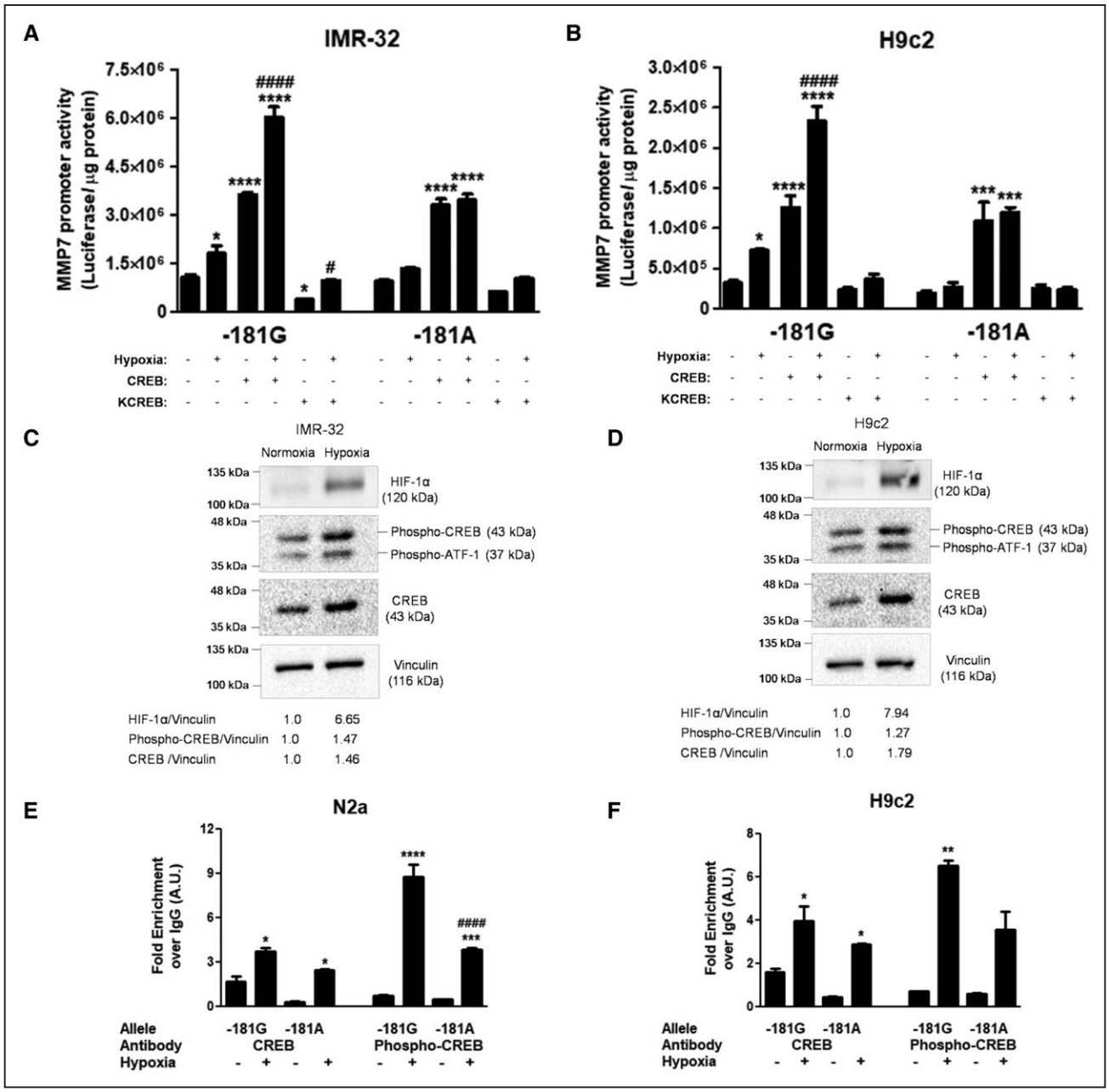

Figure 4. Effect of hypoxia on *MMP7* promoter: crucial role for CREB (cyclic AMP response element-binding protein). **A** and **B**, *MMP7* −181G and −181A promoter-reporter constructs were transfected into IMR-32 (**A**) and H9c2 (**B**) cells with/without CREB and KCREB (dominant-negative CREB) expression plasmid and subjected to 12 h of hypoxia. Results are expressed as mean±SEM of triplicate values of luciferase activity/μg protein. **P*<0.05, ****P*<0.001, and *****P*<0.0001 when compared with basal activity of the respective construct and #*P*<0.05 and ####*P*<0.0001 as compared with the corresponding normoxic condition. IMR-32 (**C**) and H9c2 (**D**) cells were subjected to hypoxia for 12 h, and Western blotting of the total proteins was performed probing for HIF-1α (hypoxia-inducible factor 1α), CREB, phospho-CREB, and vinculin. **E** and **F**, Chromatin immunoprecipitation of N2a and H9c2 cells transfected with *MMP7* −181G or −181A promoter construct, with/without exposure to hypoxia was performed using antibody against CREB, phospho-CREB, and preimmune IgG. Fold enrichment in case of CREB/phospho-CREB antibody over preimmune IgG is shown. **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001 when compared with the respective untreated condition and ####*P*<0.0001 as compared with the corresponding treatment in case of *MMP7* −181G promoter construct.

and H9c2: 1-way ANOVA F=51.37, *P*<0.001) (Figure 5B). These results suggest that the *MMP7* −181G allele may act in a dominant manner while impacting the promoter activity.

Association of *MMP7* −181AG Genotype With BP

To evaluate potential associations of the *MMP7* promoter SNP with cardiovascular traits, inferential statistics were performed using demographic/physiological/biochemical parameters of the study subjects after stratifying them based on their genotypes. The BP data were adjusted for antihypertensive drugs according to Cui et al.[22] We observed higher mean arterial pressure (MAP) (≈2–3 mm Hg) and diastolic BP (DBP) (≈2–3 mm Hg) in individuals of *MMP7* −181AG genotype than −181AA or −181GG individuals in both Chennai (MAP: 1-way ANOVA F=3.15, *P*<0.05; DBP: 1-way ANOVA F=3.69, *P*<0.05) and Chandigarh (MAP: 1-way ANOVA F=3.02, *P*<0.05; DBP: 1-way ANOVA F=4.21, *P*<0.05) populations (Figure 5C and 5D). The MAP and DBP levels in

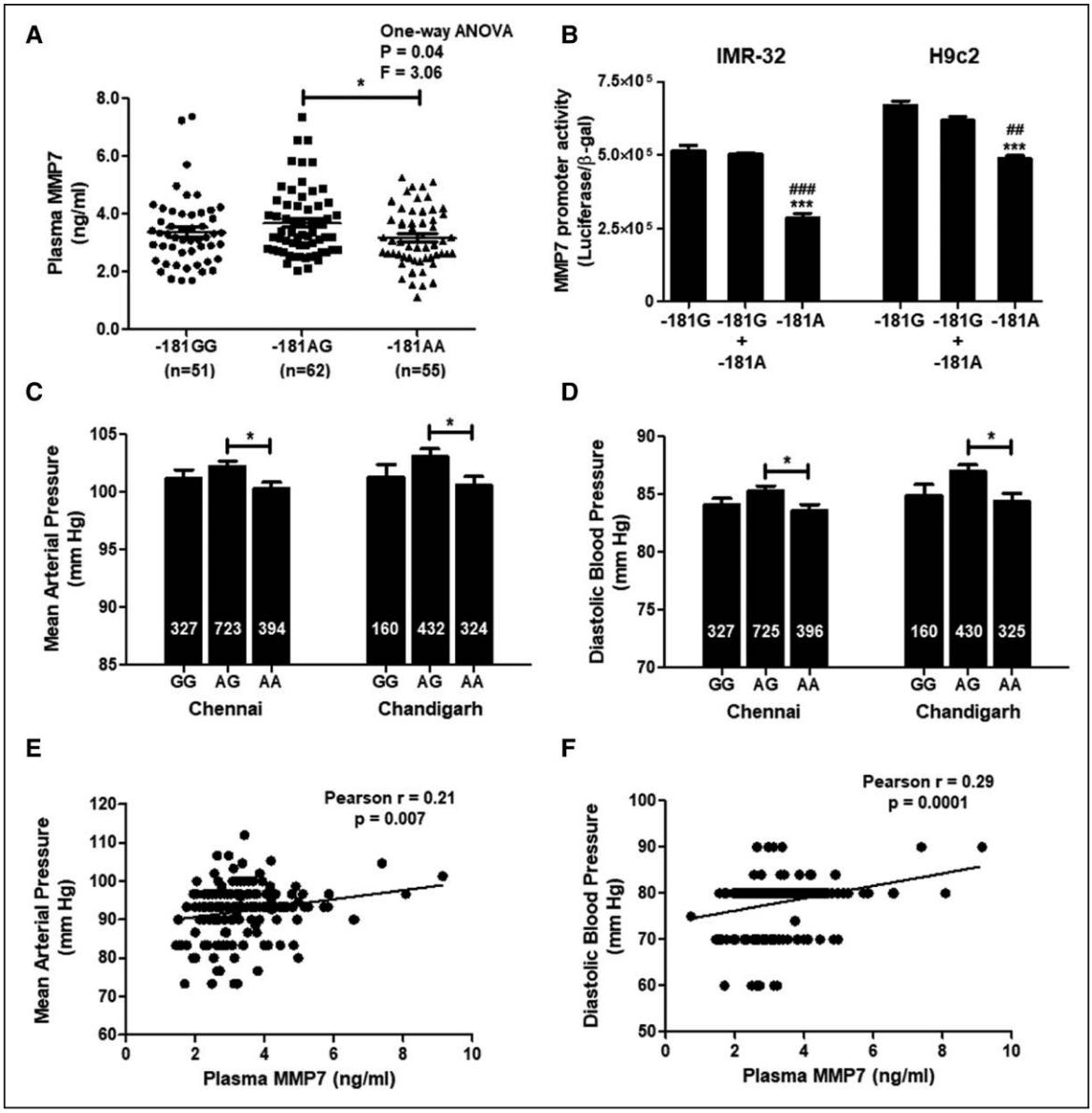

Figure 5. Allele-specific association of *MMP* (matrix metalloproteinase)-*7*-181 promoter genotypes with plasma MMP7 levels and blood pressure in Indian populations. **A**, Plasma MMP7 levels in −181GG, −181AG, and −181AA individuals as measured by ELISA. **B**, IMR-32 and H9c2 cells were transfected with 1 μg each of *MMP7* −181G or −181A or 500 ng each of −181G and −181A constructs. Results are mean±SEM of triplicate values of luciferase/β-gal activity. ***P<0.001 as compared with the −181G and ##P<0.01, ###P<0.001 as compared with the −181G+−181A conditions. **C** and **D**, *MMP7* −181AG individuals display higher mean arterial pressure (**C**) and diastolic blood pressure (**D**) in Chennai and Chandigarh populations. *P<0.05 **E** and **F**, Correlation of diastolic blood pressure (**E**) and mean arterial pressure (**F**) with MMP7 levels. MMP7 levels showed significant positive correlation with diastolic blood pressure and mean arterial pressure. Pearson r and P for the correlations are indicated.

these subjects positively correlated with plasma MMP7 levels (MAP: Pearson r=0.21, P<0.01; DBP: Pearson r=0.29, P<0.001) (Figure 5E and 5F). *MMP7* −181AG individuals also displayed a trend toward higher systolic BP (≈3 mm Hg), although the difference was not statistically significant (Figure S9). However, a modest positive correlation was observed with plasma MMP7 levels in these individuals (Pearson r=0.17, P<0.05) (Figure S9).

Discussion

Overview

Hypertension—a major risk factor for CVD—is characterized by several complex pathophysiological mechanisms including cardiac remodeling.[23] The remodeling that occurs initially as an adaptive response results in an altered ECM content ultimately leading to cardiovascular dysfunction.[24] MMPs play a crucial role in cardiac remodeling by degrading ECM components, which provide structural and mechanical support to the vasculature.[25] MMP7 cleaves a wide range of substrates including ECM components, vasoactive ligands, growth factor receptors, proinflammatory molecules, and other MMPs.[26] Knockdown of *MMP7* in SHR model attenuated hypertension and stopped the development of cardiac hypertrophy.[9] MMP7 is involved in early stages of agonist-induced hypertension (serving as a transcriptional regulator of MMP2) wherein knockdown of MMP7 and TACE (TNF-α

converting enzyme) prevented hypertension, as well as the development of cardiac hypertrophy.[10] Since expression of MMP7 is known to be tightly regulated at transcriptional level, polymorphisms in the promoter region may affect the gene expression by altering the binding affinities of transcription factors. Such functional polymorphisms could potentially alter susceptibility to pathophysiological phenotypes.[11] Two polymorphisms in the *MMP7* promoter (A-181G and C-153T) have been associated with the risk of coronary artery disease[11] but association of these variants with hypertension risk has not been investigated.

Occurrence and Association of *MMP7*A−181G Polymorphism With Hypertension in Human Populations

Pairwise linkage disequilibrium analysis of common SNPs in the *MMP7* upstream promoter region (Figure S2) identified *MMP7* A-181G as a tag SNP, which was further analyzed. Occurrence of this polymorphism in Indian populations (MAFs, 0.47 and 0.41; Table S4) was similar to that in European (MAF, 0.44), South Asian (MAF, 0.43), and African (MAF, 0.45) superpopulations of the 1000 Genomes project. However, frequency of this polymorphism was lesser in American superpopulation (MAF, 0.34) and the least in East Asian superpopulation (MAF, 0.085) suggesting its ethnicity-dependent occurrence in different World populations.

Next, we probed for association of the *MMP7* A-181G polymorphism with hypertension risk. The −181AG genotype conferred at least ≈1.5-fold higher risk of hypertension in both North Indian (Chandigarh) and South Indian (Chennai) populations; the dominant model also displayed a highly significant association with hypertension risk (Table). One limitation of the present study is that the −181GG genotype did not show a statistically significant association with hypertension. However, to the best of our knowledge, this is the first report on association of *MMP7* A-181G SNP with hypertension although many studies demonstrated its association with several types of cancers.[5,7,27–30] Of note, a previous study (using high-order gene-gene interaction analysis) in a North Indian population predicted that individuals carrying the combination of *MMP7* −181AG+GG, MMP9 668RQ+QQ, *AT1* (*Ang II type 1*) 1166 AC+CC and *NFKB1* -94 ATTG Ins/Ins genotypes had a significantly higher risk for left ventricular dysfunction (adjusted OR, 8.14; P=0.003).[31] It would be interesting to perform large-scale studies on the potential association of *MMP7* A-181G SNP with CVD states in different human populations.

Allele-Specific Effect of CREB in Activation of *MMP7* −181G Promoter in Basal and Pathophysiological Conditions

Transient transfections of *MMP7* promoter-reporter constructs displayed consistently higher promoter activity of the −181G promoter than the −181A promoter across different cell lines (Figure 1). Detailed computational analyses, CREB overexpression/downregulation studies, and chromatin immunoprecipitation assays affirmed stronger interaction of CREB transcription factor with the −181G promoter in neuronal/cardiomyoblast cells (Figures 2 and 3; Figures S5 and S6). This is consistent with our previous finding that CREB interacts with the −181G allele with higher affinity in adenocarcinoma cells.[5] The higher promoter activity of *MMP7* −181G allele has also been attributed to the generation of a putative binding site (NGAAN) for a heat-shock transcription factor in U937 macrophage cells.[11]

CREB is a ubiquitously expressed leucine-zipper transcription factor with well-known roles in cell proliferation, differentiation, and survival. The role of CREB in the development of CVDs has been recently recognized.[32] Several kinases (including protein kinase A/B/C) activate CREB by phosphorylating the Ser-133 residue which in turn recruits the CREB-binding protein to activate gene expression.[33] Transgenic mice overexpressing cardiomyocyte-specific dominant-negative CREB developed dilated cardiomyopathy and displayed reduced cardiac contractility upon isoproterenol treatment.[34] Phospho-CREB levels were also elevated in cerebral arteries of hypertensive rats.[35] Involvement of CREB in Ang II–induced IL (interleukin)-6 expression in vascular smooth muscle cells was attributed to a crucial cyclic AMP response element site. Ang II activated several kinases via AT1 receptor to result in CREB phosphorylation suggesting that CREB may have a role to play in the vascular remodeling associated with cardiac hypertrophy, heart failure, and atherosclerosis.[36] In view of these reports, our findings on the transcriptional regulation of MMP7 by CREB provide new insights into molecular mechanisms of CVDs.

Since stress elevates catecholamine levels through the hypothalamic-pituitary-adrenal axis[37] and hypertension is characterized by elevated levels of vasoconstrictive agonists such as catecholamines, we checked whether epinephrine (a catecholamine and known activator of CREB) exhibited any allele-specific effect with respect to the *MMP7* −181A/G promoters. Indeed, the *MMP7* −181G construct displayed a concentration-dependent increase in promoter activity in response to increasing doses of epinephrine with a concomitant increase in phospho-CREB levels (Figure S7). Epinephrine treatment also enhanced promoter occupancy of CREB/phospho-CREB for the −181G allele in the context of chromatin (Figure S7). Thus, −181G allele-carrying hypertensive individuals may have higher expression of *MMP7* due to catecholamine excess. Interestingly, isoproterenol activated MMP7 in gastric cancer cells and increased expression of MMP7 in gastric cancer tissue was observed at the sites where β2-adrenergic receptor was overexpressed.[38] Thus, epinephrine may play a key role in activation of *MMP7* −181G promoter in hypertension and other stress-induced cardiac complications.

Similarly, hypoxia is a common pathophysiological condition in CVDs including atherosclerosis and heart failure. Normotensive Sprague-Dawley rats developed sustained arterial hypertension when subjected to hypobaric hypoxia.[39] The hypoxic environment in the brain stem of SHR animals resulted in elevated sympathetic activity and a corresponding increase in arterial BP.[40] Moreover, HIF-1α signaling plays an important role in macrophage activation, which in turn contributes to the tissue remodeling processes and influences the severity of CVDs.[41] In addition to HIF-1α, hypoxic response

may also be mediated by NF-κB (nuclear factor-κB) and CREB. Interestingly, CREB/ATF-1 may also bind to the HIF-1 DNA recognition site.[42] In adrenal medullary PC12 cells, hypoxia resulted in Ser-133 phosphorylation of CREB, which persisted up to 24 hours.[43] In line with these reports, hypoxia significantly enhanced the promoter activity of *MMP7* −181G construct under basal as well as CREB cotransfected conditions via increased promoter occupancy of CREB (Figure 4). In corroboration, primary human monocyte-derived macrophages displayed elevated *MMP7* mRNA levels under hypoxia.[44] Thus, the A-181G polymorphism governs *MMP7* gene expression under basal, as well as pathophysiological, conditions.

Genotype-Phenotype Correlations of *MMP7* A-181G Polymorphism

Consistent with the higher transcription/translation of *MMP7* in the presence of the −181G allele *in vitro* (Figure S8), plasma MMP7 level was significantly higher in heterozygous AG carriers as compared with wild-type AA individuals (Figure 5A). Interestingly, the −181AG heterozygous combination also yielded significantly higher promoter activity than the −181A wild-type upon expression of diploid combinations of −181A/−181G constructs in cultured cells (mimicking the homozygous and heterozygous conditions), indicating possible enhancement of *MMP7* gene expression in the heterozygous individuals (Figure 5B). Indeed, previous reports suggested that altered expression of MMPs in different individuals could result due to the polymorphisms in the regulatory regions (eg, promoter) of MMP genes.[2]

Of note, in a preliminary study, hypercholesterolemic patients with coronary artery disease possessing the −181G allele presented with smaller reference luminal diameters before percutaneous transluminal coronary angioplasty than patients with the wild-type allele suggesting a functional role for the *MMP7* A-181G polymorphism in the matrix remodeling associated with coronary artery disease.[11] We also probed for association of the *MMP7* A-181G polymorphism with biochemical parameters (*viz.* total cholesterol, triglycerides, LDL [low-density lipoprotein], and HDL [high-density lipoprotein] cholesterol and blood glucose levels) that may act as comorbidities to CVD; however, no significant correlations were observed. Nonetheless, the significantly higher DBP and MAP observed in individuals of AG genotype (Figure 5C and 5D) corroborated with their increased hypertension risk (Table). The higher BP in *MMP7* −181AG individuals might result from enhanced proteolytic cleavage of the β2-adrenergic receptor by MMP7 as MMP7 level is elevated in these individuals because of higher promoter activity associated with the −181G allele. The cleavage of β2-adrenergic receptor may suppress the normal vasodilatory stimulus provided by this receptor upon agonist binding resulting in a lack of vasodilatory input to the arterial/arteriolar tone; this signaling cascade may lead to elevation of arterial BP.[45] Further studies are required to establish the mechanisms modulating BP homeostasis in these individuals.

In summary, we genotyped a common naturally occurring polymorphism (A-181G) in the upstream regulatory region of *MMP7* gene in 2 geographically distinct Indian populations. The *MMP7* A-181G polymorphism showed strong association with increased hypertension risk in our study populations. The −181G allele-containing promoter displayed higher promoter activity than the −181A promoter in basal/pathophysiological conditions (hypoxia, catecholamine excess) due to preferential binding of CREB, translating to increased MMP7 levels *in vitro*. The risk genotype (−181AG) also had a correlative association with increased plasma MMP7 levels and BP suggesting that this functional regulatory polymorphism may contribute to cardiovascular risk.

Perspectives

Dysregulated proteolytic processes modulated by MMPs result in cardiac remodeling and play a major role in CVDs. MMP7—a potent metalloproteinase with a wide range of ECM/non-ECM substrates—is implicated in several cancers and atherosclerosis. This study sheds light on the association of a highly frequent tag SNP in the promoter region of *MMP7* (A-181G; rs11568818) with increased BP and higher risk for hypertension in Indian population. This work also provides a plausible transcription regulatory mechanism behind the observed elevated levels of MMP7 in the carriers of the variation. Thus, this study paves way toward better understanding of the role of interindividual variations and functional regulatory polymorphisms in conferring disease risk, which may help to develop preventive strategies for individuals predisposed to hypertension and the related cardiovascular complications.

Acknowledgments

We acknowledge all the volunteers who participated in this study. We thank David Ginty (Harvard Medical School) for the CREB (cyclic AMP response element-binding protein) expression plasmid (VP16–CREB+bZIP) and Richard H. Goodman (Vollum Institute, Oregon Health Sciences University) for the CREB dominant-negative plasmid (KCREB). L. Subramanian acknowledges the clinical research team at Madras Medical Mission, Chennai, for helping with the sample collection and Abrar Ali Khan, Vikas Arige, and Amrita Anand for their support during the study.

Sources of Funding

This study was supported, in part, by grants from the Department of Biotechnology, Department of Science and Technology, and Ministry of Human Resource Development, Government of India.

Disclosures

None.

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Novelty and Significance

What Is New?

- This is the first study, to the best of our knowledge, demonstrating the association of *MMP* (*matrix metalloproteinase*)*7* A-181G (rs11568818) polymorphism with hypertension risk.
- This study provides evidence for the role of CREB (cyclic AMP response element-binding protein) in allele-specific transcriptional activation of *MMP7* –181G promoter resulting in higher MMP7 protein levels.

What Is Relevant?

- The study identified a highly prevalent tag SNP that conferred hypertension risk in Indian populations. This finding may be useful for developing preventive strategies for individuals predisposed to hypertension and its complications.

Summary

We identified a common genetic variation (A-181G) in the promoter region of *MMP7* gene that shows strong association with increased hypertension risk in 2 geographically distinct populations consisting a total of ≈2450 hypertensive/normotensive subjects. The –181G allele-containing promoter displayed higher activity than the –181A promoter under basal/pathophysiological conditions due to preferential binding with transcription factor CREB. Carriers of the risk genotype AG also had increased plasma MMP7 levels and blood pressure suggesting that this functional regulatory polymorphism may act as a predictor for cardiovascular risk.