

T-138C Polymorphism of Matrix Gla Protein Promoter Alters Its Expression but is not Directly Associated with Atherosclerotic Vascular Calcification

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Received 24 December 2004/ Accepted 13 January 2005

Key Words: Matrix-Gla Protein; AP-1; polymorphism; Atherosclerosis; Mineralization

Matrix Gla protein (MGP) is a crucial inhibitor of vessel and cartilage calcification. We investigated the association of T-138C MGP promoter polymorphism with the degree of atherosclerosis, vascular calcification and patients' clinical background including calcification of the trachea and costal cartilage. Analysis of 108 autopsy cases was carried out by polymorphism-specific PCR on formalin-fixed paraffin-embedded samples. Statistical correlations among eight risk factors and five markers related to atherosclerosis and extra-bone tissue calcification were multivariantly analyzed. We found very high canonical correlations between the factors and the markers, and Pearson's correlation analysis revealed six significant correlations between age and the Gore index; age and costal cartilage calcification; sex and costal cartilage calcification; hypertension and the Gore index; hypertension and the calcification factor of the Gore index; and hyperlipidemia and costal cartilage calcification. The promoter activity of the -138T allele was significantly higher than that of the -138C allele; treatment with 12-O-tetradecanonylphorbol 13-acetate (TPA) significantly activated the former, but had almost no effect on the latter. The C genotype was significantly common among Japanese subjects, (TT 45.5%, TC 37.6% and CC 16.8%) compared with that reported in the Netherlands, Northern Ireland and France. No significant correlation was observed, however, between T-138C MGP promoter polymorphism and the markers. Although the C genotype (TC+CC) tended to show a higher calcification factor than the TT genotype, no significant difference was observed among the genotypes in the Gore index or in the calcification factor. Although MGP promoter activity and the binding of the AP-1 transcription factor were clearly different between T-138 and C-138 MGP promoter polymorphism *in vitro*, T-138C polymorphism was, statistically, not an independent factor of atherosclerosis or atherosclerotic vascular calcification in the abdominal aorta.

There is growing evidence that extracellular matrix mineralization or calcification in the vascular wall is not merely the end-stage of a passive crystallization process, in which mineral is precipitated in extracellular fluids that are nearly saturated with calcium and phosphate, but a biologically regulated phenomenon, and as such, may be subject to prevention and reversal (1, 3, 7, 9, 20). Furthermore, common and well-documented histopathological findings of ectopic bone and cartilage formation in the calcified vascular wall suggest that transdifferentiation of smooth muscle cells in the media into osteogenic and

chondrogenic lineages plays a pivotal role in the calcification process (2, 11). Indeed, a number of studies have demonstrated the up-regulation of bone-associated proteins, such as osteopontin (2, 12, 27), osteocalcin (15), bone morphogenetic protein (BMP)-2a (4), osteonectin (23) at the site of calcified atherosclerotic plaques. At the same time, under normal circumstances, the arterial wall is protected from mineral deposits by a special matrix protein, matrix Gla protein (MGP), which mechanism is also shared by permanent cartilage to prevent mineral deposits (18). Therefore, mineral deposits or calcification in the arteries can be the consequence of both activated process and functional deficit leading to impaired inhibition of mineral deposits (19).

In atherosclerotic arteries, Gla-containing proteins play a crucial role in clearing calcium phosphate (hydroxyapatite) as a consequence of the strong affinity of Gla residues for this compound (24). Gla is formed posttranslationally from glutamic acid through gamma-carboxylation by the vitamin K-dependent γ -glutamate carboxylase. MGP is a 10-kDa circulating protein that contains five Gla residues (5), and has been shown to be present in association with smooth muscle cells and elastic laminae of the tunica media and with the extracellular matrix of the adventitia (8). The fact that integrated colocalization of MGP with the elastic laminae is lost, especially at sites of medial calcification, clearly demonstrates the importance of MGP in the formation of atherosclerotic vascular calcification. The function of MGP in preventing calcification in soft tissues *in vivo* is also well illustrated in the MGP knockout mouse model (19), which exhibits intense arterial calcification leading to vessel wall rupture and premature death.

The single-nucleotide polymorphisms (SNPs) are, on the other hand, single-base changes that can be found at any site in the DNA, either in the 5' regulatory sequences (promoter) or after the coding region (3' untranslated region), or within the DNA which codes for the gene product. The promoter polymorphism, when laid in transcription factor binding sites, leads to alteration of the binding of the transcription factors and affects transcriptional regulation. The promoter region of MGP contains nucleotide variations, especially T-138C that can alter the expression level of MGP (10), that are related to the occurrence of acute myocardial infarction as demonstrated by population-based extensive clinical studies (14). Nevertheless, the actual impact of T-138C polymorphism on the degree of atherosclerosis, vascular calcification, and other clinical features, possibly related to altered MGP expression such as Mönckeberg medial calcific sclerosis and calcification of the ribs, has not yet been determined.

The purpose of this study was to investigate the association of T-138C polymorphism with the degree of atherosclerosis, vascular calcification and other data on patients' clinical background. Also the molecular mechanism by which this polymorphism affects MGP gene expression was evaluated.

SUBJECTS AND METHODS

Subjects

One hundred and eight autopsy records (70 men and 38 women ranging in age from 0 to 102 years, mean \pm SD 63.8 \pm 16.7 years) were investigated, and their T-138C polymorphism was identified from their formalin-fixed, paraffin-embedded tissue sections. Autopsies were carried out between November 1999 and July 2002 in the Division of Molecular Pathology, Department of Biomedical Informatics, Kobe University Graduate School of Medicine, Kobe, Japan. The study protocol was approved by Kobe University Ethics Committee. The degree of the aortic atherosclerosis was evaluated by the quantitative atherosclerotic index (Gore index) (13). Risk factors underlying atherosclerosis including arterial hypertension, smoking,

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diabetes, hyperlipidemia were evaluated from hospital records. Calcification of the aortic arch and the costal cartilage was confirmed from chest and abdominal X-ray films.

Genotyping

The DNAs for genotyping were extracted from 108 paraffin sections (10- μ m thick) with DNA isolater PS-Rapid Reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The T-138C polymorphism was genotyped by nested polymorphism-specific PCR amplification. First, DNA fragments were amplified with the forward primer (5'-AAGCATACGATGGCCAAAACCTTCTGCA-3') and the reverse primer (5'-GAACTAGCATTGGAACCTTTCCCAACC-3'). The PCR product was then amplified with the forward primer (5'-AAACTTCTGCACCAGAGCAG-3') and the reverse primer (5'-ATTGGAACCTTTCCCAACCA-3') for the T genotype, and with the same forward primer and the reverse primer (5'-ATTGGAACCTTTCCCAACCG-3') for the C genotype (Fig. 1). The PCR was conducted in a total volume of 25 μ l of a buffer solution containing 2.0 mM of MgCl₂, 0.16 mM of dNTP, 2.5 units of Taq DNA polymerase, and 0.4 μ M of the

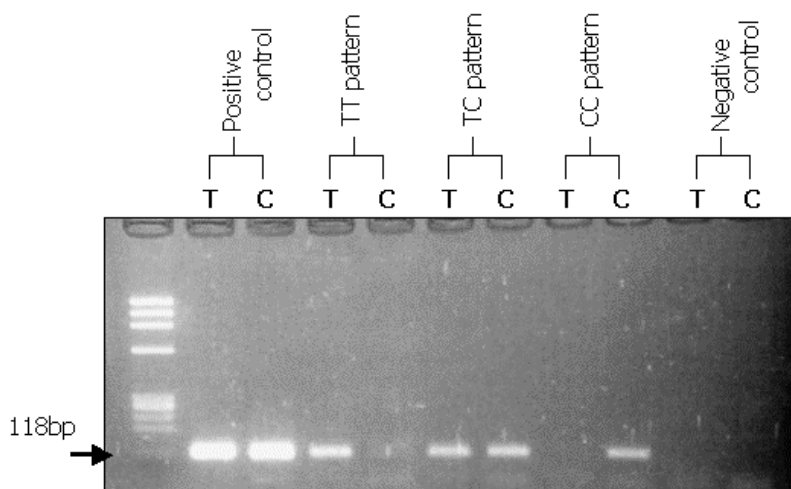
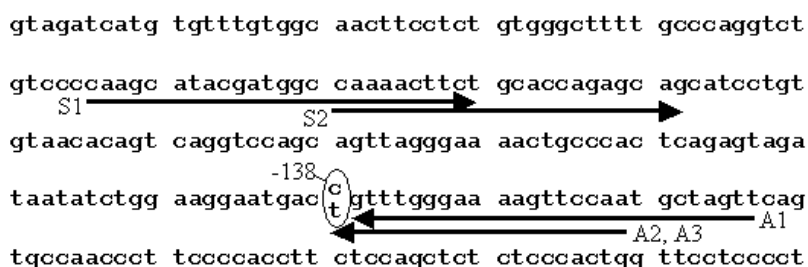


Fig. 1. Nested polymorphism specific PCR for genotyping T-138C polymorphism. DNA fragments were first amplified with the forward primer S1 and the reverse primer A1. The PCR product was then amplified with the forward primer S2 and the reverse primer A2 for the T genotype, and with the forward primer S2 and the reverse primer A3 for the C genotype. The 5' end of each polymorphism-specific primer was set to either T (A2) or C (A3) so that each primer specifically amplified genomic DNA in a genotype-specific manner. Agarose gel electrophoresis of the PCR products showed the specificity of the polymorphism-specific PCR with DNA samples of the genotype that was already confirmed by direct DNA sequencing.

forward and reverse primers. Both the first and second PCR were run for 2 min of denaturation at 94 °C followed by 20 cycles at 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s and for 2 min of additional extension at 72 °C. PCR products were electrophoresed on 3 % agarose gel, and analyzed under an ultraviolet lamp. All samples were subjected to PCR together with positive (samples of known T and C genotypes) and negative (without template) controls.

Immunohistochemical Staining

The sections (4- μ m thick) were deparaffinized and washed in phosphate buffered saline. After microwave treatment, the internal peroxidase and endogenous non-specific antibody binding sites of the section were blocked with 1% sodium periodate and the blocking solution (the MaxiTags immunoperoxidase system; Thermo Shandon, Pittsburgh, PA, USA). Serial sections were incubated with mouse monoclonal antibodies against MGP (1:3) and rabbit polyclonal antibodies against c-Fos (1:20, Oncogene Research Products) at 4 °C overnight. Biotinylated goat anti-mouse and anti-rabbit IgG (Dako LSAB 2 Kit /HRP) was used as secondary antibody for each primary antibody. The antibodies were visualized with a DAB substrate kit (Dako ENVISION+ Kit /HRP). The sections were mounted with coverslips without counterstaining. For negative controls, non-immunized serum was used as the primary antibody, instead of the specific primary antibody.

Plasmid Construction

The MGP promoter region was amplified by PCR from genomic DNA of T-138C heterozygous subjects using forward primer (5'-ACAGAACACTAGGGCTCTGTCTG-3') and reverse primer (5'-GCTGCTACACAAGACCCTGAGA-3'). The PCR was conducted in a total volume of 25 μ l of a buffer solution containing 2.0 mM of MgCl₂, 0.16 mM of dNTP, 2.5 units of Taq DNA polymerase, and 0.4 μ M of forward and reverse primers. The reaction was run for 2 min of denaturation at 94 °C followed by 30 cycles at 94 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s and for 2 min of additional extension at 72 °C. The product was blunt-ended and subcloned into the pCR2.1 vector (Invitrogen). The products containing the -138T and -138C alleles were cut with BsrSI (Promega) and sequenced by the dideoxy nucleotide termination method with an ABI PRISM 310 automated sequence analyzer (Applied Biosystems). The -138T allele product was cut with KpnI and XhoI and the -138C product was cut with XhoI and HindIII for replacing the corresponding fragment in the full-length promoter. For functional analysis, each promoter was isolated and subcloned upstream of the luciferase reporter gene in the pGL3-Basic vector (Promega).

Cell Culture and Transient Transfection Study

Human breast cancer cell line MCF-7 was cultured in RPMI1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma) and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. For standardization each of the plasmid constructs was cotransfected together with the pRL-TK vector (Promega) into MCF-7 cells using Effectene Transfection Reagent, a liposome-mediated technique (Qiagen K.K., Tokyo, Japan). The transfected MCF-7 cells were cultured in RPMI 1640 supplemented with 2% charcoal-stripped fetal bovine serum, at which point luciferase activity of Firefly and Renilla from cell lysates was determined with an ATP-300 luminometer (Advantec, Tokyo, Japan). The transcription efficiency of the constructs containing the products of alleles -138T (pGL3-MGP-T) and -138C (pGL3-MGP-C) was evaluated by determining the activity of Renilla luciferase. The transfection study was repeated three times, and representative data are expressed as the mean \pm SD of relative luciferase activity standardized by TK promoter activity obtained from four culture wells. To assess the effect of the PKC agonist on

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promoter activity, transfected cells were treated with 10^{-6} M of TPA, a protein kinase C activator, 24 hours before harvesting, and luciferase activity from cell lysates was determined with a luminometer (Model ATP-3010, Advantec, Japan).

Gelshift Assay

The following pairs of complementary oligonucleotides containing the T-138C polymorphism site (-150 to -126) were synthesized for annealing:
MGPT (sense): 5'-GGAAGGAATGACTGTTTGGGAAAAG-3'
MGPT (antisense): 5'-CTTTTCCCAAACAGTCATTCCTTCC-3'
MGPC (sense): 5'-GGAAGGAATGACCGTTTGGGAAAAG-3'
MGPC (antisense): 5'-CTTTTCCCAAACGGTCATTCCTTCC-3'.
Double stranded DNA fragments were 5'-end-labeled with [γ - 32 P] ATP (3000 Ci/mmol) by T4 polynucleotide kinase (Promega). The binding reaction was carried out by preincubating labeled nucleotides with nuclear extract protein (Hela nuclear extract) and specific antibodies against c-Jun, JunB, JunD, c-Fos, FosB, Fra1 (Santa Cruz Biotechnology, Santa Cruz, CA) at 25°C for 30 minutes. The samples were loaded onto a 5% polyacrylamide gel and electrophoresed at room temperature for 2 hours at 100 V. The gel was dried and visualized with a BAS 2000 image analyzer.

Estimation of Aortic Atherosclerosis and Calcification

The degree of the aortic atherosclerosis was evaluated by Gore index, a method for quantitating atherosclerosis observed at autopsy for a better comparison of the extent and severity of the disease. The area of the abdominal aorta between the branches of renal and iliac arteries was evaluated in 87 of the 108 autopsy cases; a representative calculation of the Gore index is shown.

Statistical Analysis

The SPSS program (version 12.0J) for Windows (SPSS, Chicago, IL, USA) was used for statistical analyses. The relation between the eight risk factors and the five markers related to atherosclerosis and extra-bone tissue calcification was assessed by Pearson's correlation analysis. For those categorical data, nonlinear canonical correlation analysis was also used. The mean values among the three groups were compared by the Bonferroni test.

RESULTS

MGP Promoter Activity and Inhibition by TPA

In the steady-state control group (CNT), the promoter activity of pGL3-MGP-C (2.83 ± 0.32) was significantly lower than that of pGL3-MGP-T (3.75 ± 0.57 , $P < 0.05$); furthermore, TPA treatment significantly enhanced the promoter activity of the latter (5.72 ± 0.76 , $P <$

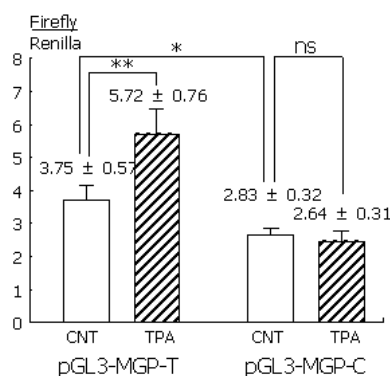


Fig. 2. MGP promoter activity and inhibition by TPA. In the steady-state control group (CNT), the promoter activity of pGL3-MGP-C was significantly lower than that of pGL3-MGP-T. Furthermore, TPA treatment significantly increased the promoter activity of pGL3-MGP-T, while that of pGL3-MGP-C remained almost constant.
* $P < 0.05$; ** $P < 0.01$

0.01), while that of the former remained almost constant (Fig. 2).

Gelshift Assay

Electrophoretic mobility shift assays (EMSA) was carried out to examine the binding of regulatory proteins to the AP-1-like sequence of either the T or the C genotype at -138. Oligonucleotides containing the AP-1-like sequence TGACTGT (T genotype) formed a specific DNA-protein complex that was mainly supershifted by anti-c-Jun and c-Fos antibodies (arrows), and partly shifted by anti-FosB (Fig. 3, left panel). On the other hand, oligonucleotides containing TGACCGT (C genotype) did not form any protein complex that was supershifted or blockshifted by the antibodies (Fig. 3, right panel). These data indicated that SNP at -138 of the MGP promoter altered the binding affinity of the AP-1 complex for DNA, and mainly the T genotype served as the AP-1 binding element.

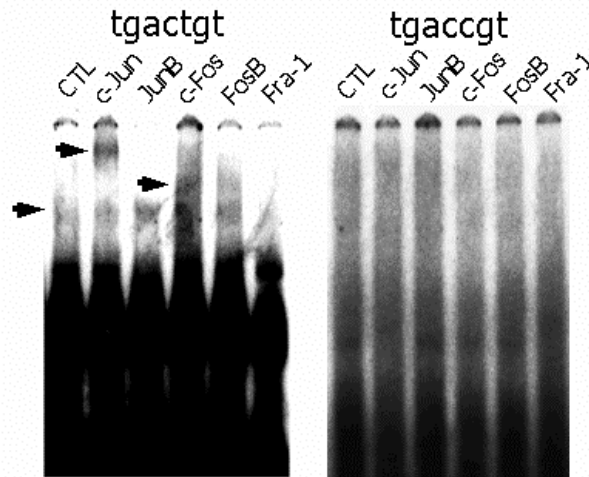


Fig. 3. Gelshift assay to examine the binding of regulatory proteins to the AP-1-like sequence of either the T or the C genotype at -138. The binding reaction was carried out by preincubating labeled nucleotides with nuclear extract protein and specific antibodies against c-Jun, JunB, c-Fos, FosB, Fra1. Oligonucleotides containing the AP-1-like sequence TGACTGT for the T genotype formed a specific DNA-protein complex that was mainly supershifted by anti-c-Jun and c-Fos antibodies (arrows), and partly shifted by anti-c-Fos (left panel). On the other hand, oligonucleotides containing TGACCGT for the C genotype did not form any protein complex with and were neither supershifted nor blockshifted by the antibodies (right panel).

Immunohistochemical Staining

Serial sections of the aorta with severe atherosclerosis and mineral deposits were selected for morphological evaluation by H.E. and immunohistochemistry to examine the localization of MGP and c-Fos. H.E. staining showed the arterial wall structure with mineral deposits (girdled by dotted lines) mainly in the media of the aorta (Fig. 4A). Positive MGP immunoreactivities (arrows in Fig. 4B) were found in almost intact or non-mineralized smooth muscle cells, elastic laminae of the tunica media and the extracellular matrix of the adventitia in the aortic wall but not in smooth muscle cells with severe atherosclerotic change or mineralized medial wall (girdled by dotted lines, Fig. 4B). The immunohistochemical staining with anti-c-Fos antibody showed diffuse and intense staining in smooth muscle and mesenchymal cells in the intima at both the severe atherosclerotic change with mineral deposits and the almost intact or mild atherosclerotic change (Fig. 4C). Negative controls prepared by non-immunized animal serum showed no significant staining

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(Fig. 4D).

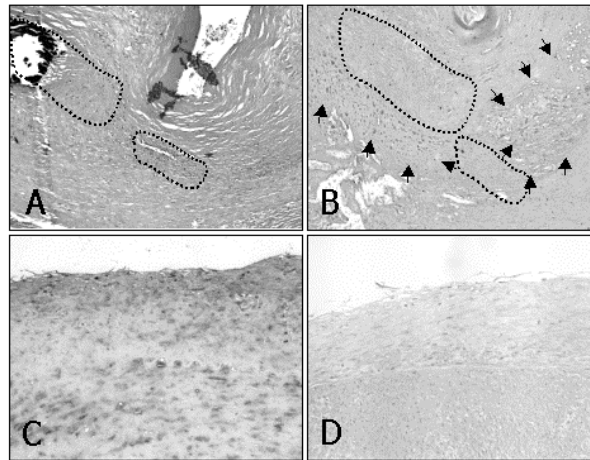


Fig. 4. Serial sections of the aorta with severe atherosclerosis and mineral deposits. A section stained by H.E. showed the arterial wall structure with mineral deposits (girdled by dotted lines) mainly in the media of the aorta (A). Positive MGP immunoreactivities (arrows in B) were found in almost intact or non-mineralizing smooth muscle cells, elastic laminae of the tunica media and the extracellular matrix of the adventitia of the aortic wall but not in smooth muscle cells with severe atherosclerotic change or mineralized medial wall (girdled by dotted lines). Immunohistochemical staining by anti-c-Fos antibody showed diffuse and intense staining in smooth muscle and mesenchymal cells in the intima (C). Negative controls prepared by non-immunized animal serum showed no significant staining (D).

T-138C Polymorphism in Japan

Of the 108 autopsy cases, 7 demonstrating polymorphism-specific PCR were not informative, probably due to the complete degradation of the genomic DNA in the postmortem process. The number of TT, TC and CC variants were 46 (45.5%), 38 (37.6%) and 17 (16.8%) respectively. The CC variant was significantly more common in Japan than has been reported in the Netherlands, Northern Ireland and France (10, 14) ($P < 0.05$).

Calcification Risk Factors and Their Correlation with Calcification

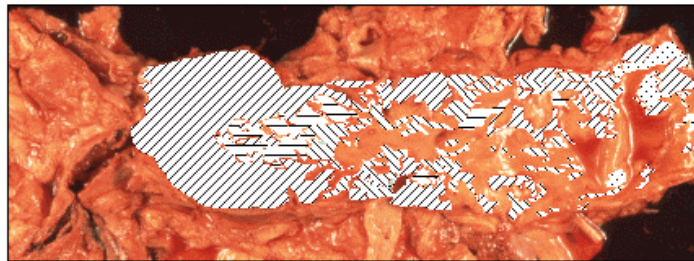
Statistical analysis of the correlations among the eight risk factors (age, sex, diabetes, arterial hypertension, hyperlipidemia, body mass index (BMI), smoking and MGP polymorphism) and the five markers (Gore index, calcification factor, trachea calcification, costal cartilage calcification and Mönckeberg medial calcific sclerosis) related to atherosclerosis and extra-bone tissue calcification (Table I) showed very high coefficients for the first and second canonical correlation of the factor variables and the marker variables (0.96, 0.94). Pearson's correlation analysis revealed six significant correlations between age and the Gore index; age and costal cartilage calcification; sex and costal cartilage calcification; hypertension and the Gore index; hypertension and calcification factor of the Gore index; and hyperlipidemia and costal cartilage calcification. No significant correlation was observed, however, between T-138C MGP promoter polymorphism and the markers. Although the C genotype (TC+CC) tended to show a higher calcification factor than the TT genotype, no significant difference was observed among the genotypes in the Gore index (**Fig. 6A**) or in the calcification factor (**Fig. 6B**).





Table I. Correlation coefficients among eight risk factors and five markers related to atherosclerosis and extra-bone tissue calcification.

	Gore Index	Calcification Factor	Trachea Calcification	Costal Cartilage Calcification	Mönckeberg's Calcification
Age	0.336*	0.242	0.172	0.346*	0.164
p value	0.026	0.114	0.265	0.021	0.287
Sex	-0.146	-0.155	0.284	0.319*	0.128
p value	0.346	0.316	0.062	0.035	0.406
Diabetes	0.183	0.112	0.052	0.135	-0.293
p value	0.235	0.470	0.737	0.383	0.054
Hypertension	0.599**	0.517**	0.170	0.115	0.085
p value	<0.001	<0.001	0.269	0.459	0.582
Hyperlipidemia	0.239	0.198	-0.042	0.375*	-0.307
p value	0.118	0.197	0.788	0.012	0.042
BMI	-0.225	-0.149	-0.227	-0.136	0.019
p value	0.200	0.400	0.197	0.442	0.915
Smoking	0.101	0.123	-0.115	0.026	-0.103
p value	0.513	0.428	0.456	0.869	0.506
Polymorphism	-0.026	0.080	-0.107	0.043	-0.037
p value	0.865	0.607	0.487	0.780	0.810

* $P < 0.05$; ** $P < 0.01$

BMI, body mass index



	Grade 1 area (1)	: 1/10
	Grade 2 area (10)	: 2/10
	Grade 3 area (100)	: 4/10
	Grade 4 area (100)	: 1/10

Total Area of Atherosclerosis : Group D - weight 30

Total Atherosclerotic Index
 $(1 \times 1/10 + 10 \times 2/10 + 100 \times 4/10 + 100 \times 1/10) \times 30 = 1563$

Calcification Factor
 $100 \times 1/10 \times 30 = 300$

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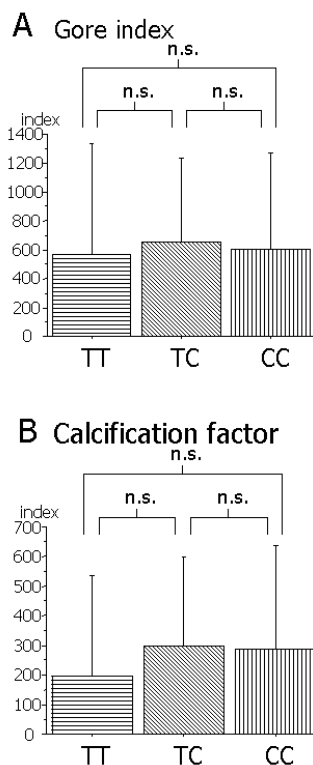


Fig. 6. Correlation between T-138C MGP promoter polymorphism and the Gore index and the Calcification factor. There was no significant correlation between T-138C polymorphism and the degree of atherosclerotic change quantitated by the Gore index in the abdominal aorta (A). Although the C genotypes (TC+CC) tended to show a higher calcification factor than the TT genotype, no statistically significant difference was observed among the genotypes in the calcification factor (B).

DISCUSSION

Extracellular matrix mineralization or calcification is strictly regulated under physiological conditions and usually confined to the skeletal bone tissue (orthotopic calcification). On the other hand, ectopic calcification occurs 1) when concentrations of calcium and phosphate in extracellular fluid exceed the saturation point (metastatic calcification), 2) as a consequence of replacement or transition of injured, degenerated and necrotic tissue by mineral deposits (dystrophic calcification), or 3) by transdifferentiation of mesenchymal cells into bone tissue (ectopic ossification) (11, 17). Among various forms of ectopic calcification, vascular wall calcification is by far the most common, and at least two mechanisms are known to be involved in the process: passive calcification by the breakdown of the protection system (a form of dystrophic calcification) and active calcification by transdifferentiation of mesenchymal cells in the vascular wall into the bone (ectopic ossification). These two mechanisms, especially dystrophic calcification, albeit some mutual relation may exist and differently contribute to the formation of vascular wall calcification in various vascular lesions, play central roles in the development of atherosclerotic plaque calcification in aged populations, the most common form of vascular wall calcification.

Several matrix proteins such as MGP, osteopontin and fetuin-A ($\alpha 2$ Heremans-Schmid

glycoprotein) (21, 25), although some contradictory reports exist, have been identified as protective factors in non-osseous tissues against dystrophic calcification. Since inactivation of MGP in knockout animals shows heavy and diffuse vascular calcification, MGP plays a central role in protecting the vascular wall from dystrophic calcification (19). Furthermore, that mutation in the coding regions of the MGP genes causes Keutel syndrome, a rare human recessive disorder characterized by diffuse cartilage calcifications, also indicates the importance of MGP in the prevention of dystrophic calcification (22).

MGP gene expression in vascular endothelial and smooth muscle cells is regulated locally by various growth factors and hormones, at the same time, because the functioning vitamin K-dependent γ -carboxylation system is present in the arterial wall, MGP precursors are assumed to be processed into the general circulation in an active or secretory form (26). Therefore the physiologic functions of MGP can be both local and systemic, and although how differently these functions affect bone and extrabone tissues remains to be clarified, the function of MGP in preventing vascular calcification per se is now considered to be regulated locally, especially that vascular calcification in the MGP-knockout mouse is not prevented by raising the serum level of MGP per se but by restoring MGP expression in the arteries (6). In the present study albeit TC- and CC genotypes tended to show higher calcification indices (Fig. 6B) than did the TT genotype, we did not find any statistical significance between polymorphism at -138 and the degree of atherosclerotic change or calcification of the abdominal aorta (Fig. 6 A, B). Furthermore, as shown in Table I, T-138C polymorphism did not show any statistically significant correlation with trachea calcification, costal cartilage calcification or Mönckeberg medial calcific sclerosis. Serum MGP levels assessed by enzyme-linked immunosorbent assay (in 115 Japanese patients with suspected coronary artery disease) have shown inverse correlation with the severity of coronary artery calcification, suggesting that circulating systemic, rather than locally regulated, MGP is more important in specific coronary artery calcification (16). Taken together with our results, these data indicate that T-138C polymorphism may not be a crucial factor in the local regulation of MGP gene expression. Furthermore, because numerous factors including hypertension, hyperglycemia, hyperlipidemia and especially aging are known to independently and strongly affect formation of atherosclerosis and vascular calcification, the contribution of simple promoter polymorphism at -138 by the MGP gene may not have been an independent factor in the calcification of abdominal aorta in our limited study.

An assumed molecular mechanism that T-138C polymorphism affects calcification of the coronary artery and thereby confers an increased risk of plaque calcification and myocardial infarction has shown a different binding of the AP-1 transcription factor to the polymorphic site at -138 as a result of the presence (T-138) or absence (C-138) of a functional AP-1 binding site in the MGP promoter sequence (10, 14). Our results also confirmed that the T-138 genotype showed a higher steady-state expression level than the C-138 genotype, the difference being greatly enhanced by TPA treatment (Fig. 2). Immunohistochemically, MGP was localized mainly in the media of the aorta and was slightly increased around the calcifying foci and greatly decreased at the foci (Fig. 4B). These histological findings are consistent with a previous report (28), where increased MGP expression around the calcification foci functions as a feedback system to prevent or minimize further calcification. Since vascular smooth muscle cells are rich in c-Fos, an important component of the steady-state AP-1 complex (Fig. 4C), MGP expression should also be different according to the difference in the genotypes at -138, especially at the site of atherosclerotic plaques or at that of strong shear stress where AP-1 expression is usually enhanced. Comparison of the immunohistochemical expression of MGP revealed no significant difference among the TT,

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TC and CC genotypes (one representative case with the TT genotype is shown in Fig. 4). Although quantitation by immunohistochemical staining may not be so sensitive as to detect differences between the T-138 and C-138 genotypes by transient transfection studies, we speculate that polymorphism at -138 does not contribute much to the local regulation of MGP expression by inflammatory cytokines or hormones produced at the site of atherosclerotic calcification or of shear stress. Therefore transcription factor binding sites other than the AP-1 site at T-138 may be crucial in controlling the local regulation of MGP expression.

Screening the T-138C polymorphism among populations in Northern Ireland, The Netherlands and France has shown that the TT genotype is dominant around 60%, with the CT and CC genotypes around 35 and 5%, respectively (10, 14). Furthermore, populations bearing the C-138 genotype have been linked to increased risk of coronary artery plaque calcification and myocardial infarction. In the present study, the CC genotype, although still numerically small, was as high as 16.8%, with the CC and CT genotypes together exceeding half of the population studied. This difference in the ratios among the genotypes is probably due to the difference in racial background, materials for polymorphism analysis and population biases. We speculate that the traditional Japanese lifestyle, especially with regard to food has been a factor in greatly reducing the risk of coronary artery disease despite the genomic susceptibility of the Japanese to coronary artery calcification and myocardial infarction related to MGP polymorphism. Herrman *et al.* analyzed MGP polymorphism in the ECTIM study which included myocardial infarction patients and control subjects and the AXA study which consisted of health volunteers (14). They could result in different conclusion if they analyzed control subjects in the ECTIM study or AXA study.

In conclusion, we investigated the relation between the risk factors for vascular calcification including a MGP promoter gene polymorphism and calcification of aorta, trachea and costal cartilage by multivariant analysis of 108 autopsy cases. Although MGP promoter activity and the binding of the AP-1 transcription factor were clearly different between T-138 and C-138 MGP promoter polymorphism *in vitro*, and although the CC and TC genotypes tended to show a higher calcification index of abdominal aorta than the TT-type, T-138C polymorphism was not statistically an independent factor of atherosclerosis and atherosclerotic vascular calcification in the abdominal aorta.

ACKNOWLEDGEMENTS

We thank Dr. Takeshi Kondo, Division of Molecular Pathology, Department of Biomedical Informatics and Dr. Mitsuhiro Yokoyama and Dr. Ken-ichi Hirata, Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan for helpful suggestions and encouragement. We thank Dr. Cees Vermeer, Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, the Netherlands for helpful suggestions and for kindly providing the antibody against MGP. We also thank Shuichi Matsuda and Noriko Sakamoto for excellent technical assistance.

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