TIMP-1 c.T372C Genetic Polymorphism as a Possible Predictor for Acute Aortic Dissection

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ABSTRACT

While single nucleotide polymorphisms (SNP) have been extensively researched in atherosclerotic aortic aneurysms, there are too few data about acute aortic dissection (AAD). The matrix metalloproteinase regulation system has shown a high biological relevance to the development of aortic aneurysms and AAD. The *TIMP-1 c.T372C* (rs4898, nt+434) SNP was previously associated with the onset of abdominal aortic and other aneurysms. Therefore, we chose this SNP to search for a connection with AAD and to find its place among the other risk factors. 115 patients with AAD were studied for their *TIMP-1 c.T372C* genotype by means of conventional restrictase analysis. To confirm the biological relevance of our findings, immunohistochemistry for TIMP-1 was performed on tissue samples from the same patients with AAD and compared with a control group of 23 autopsy cases. The *TIMP-1 c.T372C* showed a significant difference in allele frequency in the AAD patients compared to the general population ($p < 0.0001$ for both sexes). This genotype did not show any association with any other co-morbid condition, nor with age. The immunohistochemistry results also showed significantly lower TIMP-1 expression in the dissected aortas. The C allele of *TIMP-1 c.T372C* shows a strong association with the onset of AAD.

Key words: *Aortic dissection, SNP, TIMP-1*

Although the pathogenesis of aortic wall disease has been well discussed, there still remain unresolved problems in the genetic field, especially concerning acute aortic dissection (AAD). Whereas a strong family linkage is observed[8], no single gene has been appointed responsible for this etiology. There are several established connective tissue disorder syndromes (CTDS) which are known to be associated with a higher prevalence of AAD: Marfan, Loeys-Dietz, Ehlers-Danloss and Turner[15]. Besides these, there are numerous genetic variations which are blamed for causing aortic aneurysms and dissections[8,15]. They are suspected of contributing to AAD by mere assumption, being proven more frequent in aortic aneurysm patients. However, there are too few studies specifically related to AAD patients, beyond the data known for CTDS.

The role of the matrix metalloproteinases (MMPs) in the pathogenesis of aortic aneurysms and AAD has been researched in several studies[5,11,17,20]. MMPs have also been linked to aneurysm formation in other arteries[12]. Their function is to degrade and remodel the extracellular matrix. High blood pressure combined with overactive MMPs is likely to cause substantial damage to the aortic wall, leading to aneurysm formation or dissection. Arterial hypertension has also been proven to be highly prevalent in cases with aortic aneurysms and dissections[8,15]. Kalay et al reported a deletion of the angiotensin converting enzyme (*ACE*) gene, associated with a higher risk of AAD.

In this study we focused on TIMP-1 (tissue

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inhibitor of metalloproteinases-1). TIMP-1 is an extracellular protein from the TIMP family which inhibits all known MMPs except MMP-14[22].

In previous studies a single nucleotide polymorphism (SNP) of *TIMP-1* showed an association with the onset of abdominal aortic and other arterial aneurysms[2,12,18,23]. AAD has never been investigated in such a study.

We examined the *TIMP-1 c.T372C* (nt+434, rs4898) SNP genotype in patients with AAD and compared the data obtained to the normal allele frequency in the general population. We investigated the biological relevance of our findings and evaluated the significance of this SNP as a predictor of AAD.

The *TIMP-1* gene lies in the X-chromosome within an intron of the synapsin gene. The researched SNP is located in exon 5, at the third position in the codon for the 124th amino acid in the TIMP-1 molecule. It is a synonymous polymorphism with two alleles - C and T.

MATERIALS AND METHODS

Study populations:

115 patients (61 male, 54 female, all Japanese), operated for AAD in the Department of Cardiovascular Surgery, Hiroshima University Hospital, between 1995 and 2012, were retrospectively recruited for the study. Among them, 13 (6 male, 7 female) had clinically confirmed CTDS: Marfan, 11 cases, Ehlers-Danloss, 1 case and Loeys-Dietz, 1 case. All CTDS patients were diagnosed with type A aortic dissection. 84 patients (41 male, 43 female) from the non-CTDS group were diagnosed with AAD type A, and 18 patients (14 male, 4 female) with AAD type B. All diagnoses were verified during surgery.

Samples for another 4 patients (2 male, 2 female, all type A) were obtained from necropsy material. Their diagnoses were verified at autopsy.

The age of all patients was adjusted to the time of the first acute event. Co-morbidity data was obtained from hospital documentation. Arterial hypertension was defined as arterial blood pressure repeatedly measured as above140/90 mmHg. Subsequent aortic surgery was defined as reoperation caused by advancing changes in the aortic wall, not by operative complications from the first surgery. Occlusive vascular events were defined as a history of myocardial or cerebral infarction, angina pectoris or occlusive atherosclerosis of the limbs. Death was defined as early death if it occurred within one month after surgery. The demographic data of the patients is summarized in Table 1.

The normal frequency of the researched SNP in the general population was revealed using the genotype data of a randomly recruited group of

Table 1. Main characteristics of the patient group

	Sex	Number	Age (SD)	AH (%)	OVE (%)	SAS (%)
CTDS	M	6	36 (12)	1 (17)	0	2 (33)
	F	7	40 (14)	1 (14)	0	2 (29)
Non-syndrome	M	57	64 (11)	27 (49)	5 (9)	3 (5)
	F	49	72 (9)	20 (42.5)	9 (19)	2 (4)
Type A*	M	43	65 (10)	21 (48)	5 (11)	3 (7)
	F	45	72 (9)	18 (41)	8 (18)	2 (5)
Type B*	M	14	62 (12)	6 (54)	0	0
	F	4	75 (17)	2 (67)	1 (33)	0

AH : arterial hypertension known with certainty

OVE : occlusive vascular events - myocardial or cerebral infarction, angina pectoris, occlusive atherosclerosis of the peripheral arteries

SAS : subsequent aortic surgery

*All the data is given only within the non-syndrome group.

110 volunteers (55 male, 55 female, all Japanese). The representativeness of this data was estimated and ensured by adjusting it to the Hardy-Weinberg equilibrium.

This study has been approved by the ethics committee (No 2012-68). The review of the records was also approved by the Institutional Review Board.

Genotyping:

The DNA from the AAD group was extracted from paraffin tissue blocks. It was performed using QIAamp® DNA FFPE Tissue Kit (Qiagen®), following the protocol of the manufacturer.

The DNA used for estimating the normal allele frequency was extracted from peripheral leukocytes using Wako DNA Extractor WB Kit, according to the recommendations of the manufacturer (Wako Pure Chemical Industries).

Nested PCR was performed using Quick Taq™ HS Dye Mix.

The primers for the first PCR were (660 bp product):

TIMP1 - 01: tggggacaccagaagtcaac

TIMP1 - 02: taagctcaggctgttccagg

For the second PCR (344 bp product):

TIMP1 - 03: aggctgttccagggagtcgc

TIMP1 - SP5: ccgccatggagagtgtctgc

The original sequence of the polymorphism area does not make a restriction enzyme site. For this reason the TIMP1-03 primer differs in two bases from the referent sequence and lies close to the polymorphic spot.

Thus, the original sequence of the locus is TT(C) GTGG while our PRC product was in fact TT(C) GCGA. In its C-variant it is a palindrome which forms a site for the NruI restriction enzyme. The genotype was revealed using NruI restriction enzyme (New England BioLabs®) at 37℃ for two hours, followed by Agilent 2100 bioanalyzer assessment with Agilent DNA 1000 kit, according to the instructions of the manufacturer (Agilent

Technologies). The restriction of the C-allele PCR product resulted in 325 bp and 19 bp digest products, while the T-allele products remained unrestricted.

This polymorphism exists in two alleles and their combination results in 5 possible genotypes. Because men lack a second X-chromosome, the possible genotypes are CY and TY hemizygotes (Y states for the Y-chromosome). As for women, there are CC and TT homozygotes and CT hetero-zygotes. After revealing the genotypes of the AAD patients, we estimated the allele frequencies in the general population. Next we looked at whether the allele frequency found in the AAD excerpt was different from the expected one.

Immunohistochemistry

Immunohistochemistry for TIMP-1 was per-formed using anti TIMP-1 monoclonal mouse antibodies (Novacastra lab.), following a standard protocol in accordance with the recommendations of the manufacturer.

The results were evaluated using a 0-4 scale, as follows:

    0 - no staining in aortic media,
    1 - slight and occasional staining in aortic media,
    2 - moderate staining,
    3 - strong and rather abundant staining,
    4 - very strong and very abundant staining in aortic media.

Zones with atherosclerotic changes and hemor-rhage were avoided because of the abundance of TIMP-1 in blood cells.

The normal expression of TIMP-1 was evaluated using aortic tissue from autopsy cases (n = 23: 16 male, 7 female), where death was from causes other than AAD and aneurysms. The average age of these cases was significantly different from the average age of the AAD group (44 vs. 67, respectively), therefore we performed correla-tion analysis for age and sex difference in the immunohistochemically estimated tissue expres-sion of TIMP-1. No such correlation was found, which allowed us to employ this group as a control group. The average interval between death and taking the aortic tissue sample was 25 hr.

The causes of death in the control group were as follows: cancer (6), hemorrhage (7), asphyxia (5), sepsis (2), autoimmune hepatitis (1), trauma (1), encephalitis (1).

Statistics and modeling

As the *TIMP-1* gene lies in the X-chromosome, the results for male and female patients were processed separately. The CTDS group was also processed separately.

Analysis for possible deviations of the genotype distribution from that expected for a population in Hardy-Weinberg equilibrium was done with the χ^2 test. For categorical values the χ^2 and Mann-Whitney U-test for variables of a non-Gaussian distribution were used. As a measure of rank correlation, Kendall's τ was used. Computations were made with Maple-13 and Mathematica 7. The results were considered significant if $p < 0.05$.

For confirming the feasibility of the risk formulas, we used a Monte Carlo simulation, over a population with the same size and structure as Hiroshima (based on the demographic data for Hiroshima Prefecture from the last census in Japan, January 2010), then compared the results with the study group.

RESULTS

Significant deviation in the C and T allele frequencies was observed both for male and female patients with AAD compared with the normal allele distribution ($p < 0.0001$ for both, Pearson's χ^2-test). The C allele was much more common in the dissection group (0.75 vs. 0.4 CY genotype in the general population for men and 0.55 vs 0.22 CC genotype for women, respectively) than the T allele (0.25 vs. 0.6 TY genotype in the general population for men and 0.08 vs 0.29 TT for women, respectively). The detected heterozygocy was 0.37 vs 0.49 CT genotype in the general population. These results are illustrated in Fig. 1.

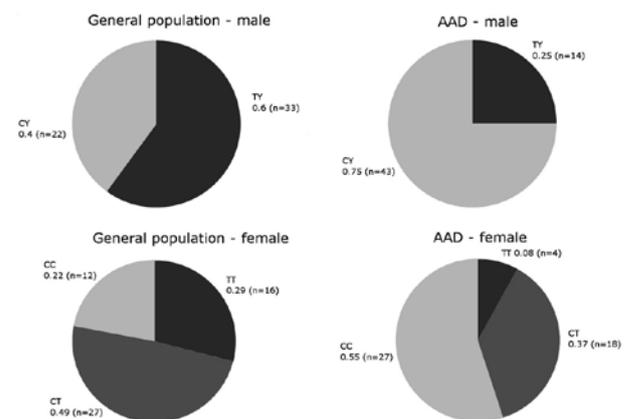The A and B types of dissection were examined separately, but no differences were found between the groups.

Fig. 1. Genotype results for the *TIMP-1 c.T372C* (nt+434, rs4898) SNP.

The chart shows the share of different genotypes in the general population and in the dissection group, separately for men and for women.

CY: C-allele male hemizygote;

TY: T-allele male hemizygote;

CC: C-allele female homozygote;

TT: T-allele female homozygote;

CT - female heterozygote;

n - patient number.

A significant difference in age was observed between the male and female patients (64 and 72 years respectively, p = 0.03), but no age differences were observed in the accordance of *TIMP-1* genotype within the same sex groups. The CTDS and the non-syndrome group also differed significantly in age (38 and 68 years respectively, p < 0.001).

Multivariate analysis with risk factors for cardiovascular diseases (age, sex, diabetes, hypertension) did not show any connection with the researched SNP, nor was any explicit connection found with other conditions present in the patients (Table 2).

Table 2. Co-morbidity (non-syndrome group)

	Male % (n)		Female % (n)		
Genotype	TY	CY	TT	CT	CC
AH	64 (9)	40 (17)	100 (4)	39 (7)	33 (9)
OVE	14 (2)	7 (3)	0	6 (1)	30 (8)
Arrhythmia	7 (1)	7 (3)	25 (1)	11 (2)	4 (1)
Diabetes	7 (1)	2 (1)	0	0	4 (1)
Obesity	7 (1)	0	0	6 (1)	0
Effusion at admission	7 (1)	16 (7)	0	28 (5)	11 (3)
Early death	0	2 (1)	0	11 (2)	11 (3)

AH : arterial hypertension known with certainty

OVE : occlusive vascular events - myocardial or cerebral infarction, angina pectoris, occlusive atherosclerosis of the peripheral arteries
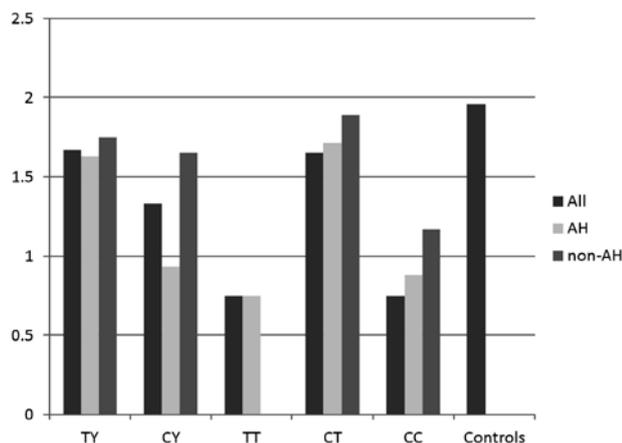

Fig. 2. Immunohistochemistry results.

The chart shows the mean values of immunohistochemically evaluated expression of TIMP-1: dissection vs controls. Results for AH patients and non-AH patients are given separately.

CY: C-allele male hemizygote;

TY: T-allele male hemizygote;

CC: C-allele female homozygote;

TT: T-allele female homozygote;

CT - female heterozygote;

AH - arterial hypertension.

All - the whole dissection group (AH+nonAH)

Ordinate - TIMP-1 expression level (mean values).

Although the number of subsequent aortic operations was significantly higher in the CTDS group (36% vs 5%, p < 0.001), we did not find any correlation with the *TIMP-1* genotype.

While processing the data, we found an anomalous allele distribution at different time periods. Before the year 2000 there were only two cases bearing T-allele (both CT).

Immunohistochemistry showed a significant difference (p = 0.0132) between the dissection group and the control group (1.17 vs. 1.96). Hypertensive patients showed significantly lower TIMP-1 expression compared to patients with no known hypertension (0.98 vs. 1.6, p = 0.0084). While the hypertensive patients showed a significantly lower expression compared to the control group (0.98 vs. 1.96 p = 0.0047), there was no significant difference between the control group and the non-hypertensive patients (1.96 vs. 1.6, p = 0.1) (Fig. 2). TIMP-1 expression was similar in male and female patients in both the hypertension (0.8 vs. 1.1, p = 0.0918) and non-hypertension (1.53 vs. 1.7, p = 0.1562) groups.

With a set of logical assumptions we obtained a formula for personal risk assessment based directly on our study group, and then checked for feasibility by Monte Carlo simulations over the adult population of Hiroshima. We consistently obtained results with similar frequencies of the different factors studied.

DISCUSSION

The different distribution of the two alleles of the *TIMP-1 c.T372C* polymorphism in patients operated for AAD compared to the general population is described for the first time in this study. Previous studies have shown somewhat contradictory results concerning this polymorphism and aortic aneurysms. While Ogata et al[18] show a higher prevalence of the T-allele in non-familial cases of abdominal aortic aneurysms, Ailawadi et al[2] cite results (again in abdominal aortic aneurysms) very similar to those we found in our AAD patients. Although the number of patients is rather small, the high statistical significance and the fact that the theoretical model based on them (the results of Monte Carlo simulation) is consistent with previous research on the topic, suggest that the association we found is not likely to be a chance observation.

Interestingly, the CTDS dissections did not show much difference in *TIMP-1* genotype compared to the non-syndrome group, but the number of patients with known CTDS was insufficient for statistical evaluation.

The differences in expression of TIMP-1 molecule in patients with hypertension together with the higher prevalence of hypertension in T-allele bearers once more suggest the simulta-

neous involvement of many factors in the etiology and pathogenesis of AAD.

The tissue ratio between TIMP-1 and MMP-9 expression was reported to be lower in patients with AAD[11], but a significant difference in the TIMP-1 expression was not found. We report such differences for the first time, confirming the biological relevance of our genetic observations.

Many previous studies[3,4,6,16,19,21,24] have shown the importance of the MMP/TIMP system for maintaining the mechanical properties of the aortic wall. Inhibiting the MMP activity in an animal model using a nonselective inhibitor (doxycycline) showed delayed aneurysm rupture in a mouse model of Marfan syndrome[24]. In *TIMP-1* knockout mice, the absence of the *TIMP-1* gene leads to aneurysm formation[16] and medial degradation[16,19]. TIMP-1 acts also as a cytokine enhancing smooth muscle cell proliferation in the aortic wall[3]. In animal experiments expression of TIMP-1 molecule in the aortic tissue in vivo was proven to be enhanced by angiotensine II[6] and in human and mouse fibroblasts by TGF-beta[21]. Allaire et al[4] have pointed out the great significance of the paracrine effect of the smooth muscle cells in maintaining the integrity of the aortic wall.

Considering all this data and comparing it to our own findings, we hypothesized that the TIMP-1 molecule has a very important impact on maintaining the integrity of the aortic wall, especially in cases of high blood pressure. In such cases, angiotensine levels rise. This would normally lead to higher expression of TIMP-1[6] in the aortic media. It may be considered a natural mechanism for maintaining the stress from this higher pressure. If there is impairment in the system, the aortic media would fail to respond in an adequate way (MMP inhibition and smooth muscle cell proliferation) to pressure stress conditions. Moreover, it is known that mutual

Fig. 3. Proposed mechanism of the normal function of the MMP/TIMP system in normal and high blood pressure.

feedback regulation of MMPs and TIMP-1 is mediated by TGF-beta[7,25]. The association between TGF-beta and AAD has been much discussed[7]. All of these speculations are illustrated in Fig. 3. Our results are in line with the suggested involvement of the TIMP-1 molecule in the natural history of AAD. Another somewhat oblique proof of the proposed mechanism is the reported association between *ACE* gene deletion and the ADD[10]. Investigating the coincidence of *TIMP-1* and *ACE* genotypes will be the objective of further research.

At this point we cannot provide a conclusive explanation of how this SNP affects TIMP-1 activity. Further research is necessary. One possible explanation is that the C-allele forms a CpG site, which is a well-known target of DNA-methylation. Another is the codon usage bias[14] - in the human genome there is no gene responsible for tRNA complementary to TTT codon[13] and there are 14 genes whose tRNA bear anticodon complementary to TTC codon, which affects the transcription speed.

The age of onset data is consistent with previous studies[15]. The same is known about CTDS compared to non-syndrome patients[9].

The results of multivariate analysis suggest that the *TIMP-1* genotype may be an independent risk factor.

Formulas for the risk

The frequency of AAD in the general population is reported about 4 per 100,000 per year[15] and the cases studied form 25-30% of all the dissections in Hiroshima for the given period. This allows us to draw conclusions about the risk of AD in different people. What we give here is a rough estimation of the risk, according to genotype, arterial hypertension and the existence of connective tissue syndromes.

We make the preliminary assumption that genotype, arterial hypertension and the connective tissue disorder syndromes are unrelated, thus their corresponding variables are independent. All variables are Boolean. In a future and more detailed study, one could use discrete variables, based on 5 or 7-point scales, instead of Boolean variables for hypertension, CTDS and other conditions. We chose to search a formula where risks from genotype and hypertension interact multiplicatively, since the percentage of hypertensive patients in different sub-groups based on sex and genotype is relatively constant. The two exceptions are the TT patients, who are too few, and the syndrome group, where the number of hypertensive patients is considerably higher. This can also be attributed to better diagnosis since the patients with known genetic syndrome are more frequently examined and generally better controlled. A high proportion of
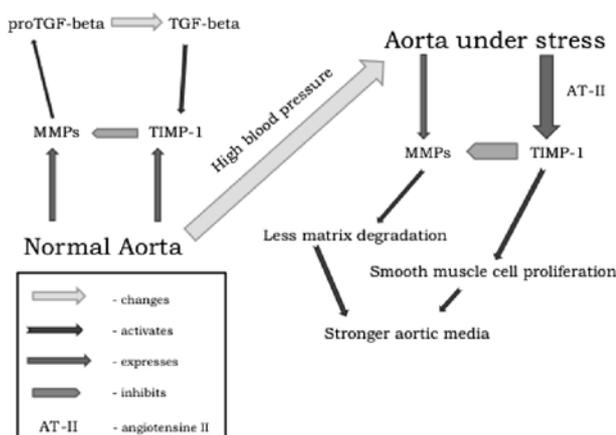
average non-syndrome patients do not know before dissection that they had arterial hypertension.

The formulas, which are obtained directly from the studied data, give results in complete accord with the research of previous authors[1,8,15] and Monte Carlo simulations over population of the same profile as the one in Hiroshima show results, statistically identical to the data obtained. We give the following formulas for the approximate risk per year:

$$Risk = \kappa \times f\,(AH) \times g\,(TIMP) \times 10^{(-5)}$$

Here $\square$ denotes the number of aortic dissections per 100,000 per year. In publications different authors have reported $\square$ between 2.6 and 5.2[1], $g(TT) = 0.33$, $g(CT) = 0.66$, $g(CC) = 2$, $g(T) = 0.58$, $g(C) = 2.09$ and $f(1) = 6.85$, $f(0) = 1$ (i.e. the risk for patients with hypertension is almost 7 times greater). If we take into consideration the above stated fact that many patients were not diagnosed with hypertension at the onset of the dissection, this risk rate may increase additionally. We also give an approximation for the risk in patients with connective tissue disorders. As a comparison, the risk of a non-hypertensive Marfan patient is $250 \times \kappa \times 10^{(-5)}$ if we accept Elefteriades'[8] conclusion that 5% of all aortic dissections are caused by connective tissue disorders, which is consistent with other data. We consider Elefteriades` figures for the risk for aortic dissection overestimated, i.e. falling in the extremes of the model. However, risks in patients with connective tissue syndromes and arterial hypertension are extremely high. We conjecture that a longitudinal study would be very useful in refining the prediction and risk assessment of such patients.

As seen in this study, age is not an explicit variable and the results gathered in the study group indicate against models in which a simple function based solely on age is included. Instead, age is an important factor for other predispositions, such as arterial hypertension. The existing literature suggests that several other age-related conditions, including atherosclerosis, diabetes and other endocrine disorders, may modify the risk for aortic dissection.

LIMITATIONS

The patient group includes mainly cases that have undergone surgery. Furthermore, most of the patients were admitted in circumstances of emergency, so preoperative data is often scarce and inconclusive, especially when it was gathered in retrospect.

The different allele distribution in the different time periods could not predict long term survival.

CONCLUSION

The C allele of *TIMP-1 c.T372C* shows strong association with the onset of AAD.

It can be speculated that TIMP-1 function may interfere with aortic wall integrity by weakening the inhibition of TIMP-1 over MMPs and failing to stimulate smooth muscle cell proliferation.

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