In conclusion, inverse correlations between occlusion time and VWF:Ag, VWF:RCo, RBCs, hemoglobin and hematocrit were detected in the GTT analysis; especially, relations between VWF and the occlusion time in the GTT was reported for the first time. Further diagnostic values in the GTT should be determined in patients with atherosclerotic disorders.

## References


1 Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL. Platelets and shear stress. *Blood* 1996; **88**:1525–1536.
2 O'Brien JR. Shear-induced platelet aggregation. *Lancet* 1990; **335**:711–721.
3 McCrary JK, Nolasco LH, Hellums JD, Kroll MH. Direct demonstration of radiolabeled von Willebrand factor binding to platelet glycoprotein Ib and IIb–IIIa in the presence of shear stress. *Ann Biomed Eng* 1995; **23**:787–793.
4 Goto S, Salomon DR, Ikeda Y, Ruggeri ZM. Characterization of unique mechanisms mediating the shear-dependent binding of soluble von Willebrand factor to platelets. *J Biol Chem* 1995; **270**: 23353–23364.
5 Brubaker DB. An in vitro bleeding time test. *Am J Clin Pathol* 1989; **91**:422–429.
6 Gorog DA, Kovacs IB. Thrombotic status analyzer. Measurement of platelet-rich thrombus formation and lysis in native blood. *Thromb Haemost* 1995; **73**:511–520.
7 Gorog P, Ahmed A. Haemostatmeter: a new in vitro technique for assessing haemostatic activity of blood. *Thromb Res* 1984; **34**:341–357.
8 Kundu SK, Heilmann EJ, Aio R, Garcia C, Davidson RM, Ostgaard RA. Description of an in vitro platelet function analyzer PFA-100. *Semin Thromb Hemost* 1995; **21 (Suppl 2)**:106–112.
9 Yamamoto J, Yamashita T, Ikarugi H, Taka T, Hashimoto M, Ishii H, *et al.* Gorog Thrombosis Test: a global in-vitro test of platelet function and thrombosis. *Blood Coagul Fibrinolysis* 2003; **14**:31–39.
10 Yamamoto J, Kovacs IB. Shear-induced in vitro haemostasis/thrombosis tests: the benefit of using native blood. *Blood Coagul Fibrinolysis* 2003; **14**:697–702.
11 Ikarugi H, Yamashita T, Aoki R, Ishii R, Kanki K, *et al.* Impaired spontaneous thrombolytic activity in eldery and in habitual smokers, as measured by a new global thrmbosis test. *Blood Coagul Fibrinolysis* 2003; **14**:781–784.
12 Wurzinger LJ, Optiz R, Wolf M, Schmid SH. Shear-induced platelet activation — a critical reappraisal. *Biorhenology* 1985; **22**:399–413.
13 Anderson GH, Hellums JD, Moake JL, Alfrey CP. Platelet lysis and aggregation in shear fields. *Blood Cells* 1978; **4**:499–507.
14 Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, *et al.* The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest* 1991; **87**:1234–1240.
15 Sakariassen KS, Ottenhof RM, Sixma JJ. Factors VIII–von Willebrand factor requires calcium for facilitation of platelet adherence. *Blood* 1984; **63**:99–1003.
16 Belch JJ, McArdle BM, Burns P, Lowe GD, Forbes CO. The effects of acute smoking on platelet behaviour, fibrinolysis and haemorheology in habitual smokers. *Thromb Haemost* 1984; **51**:6–8.
17 Reimers RC, Sutera SP, Joist JH. Potentiation by red cells of shear-induced platelet aggregation: relative importance of chemical and physical mechanisms. *Blood* 1984; **64**:1200–1206.
18 Joist JH, Bauman JE, Sutera SP. Platelet adhesion and aggregation in pulsatile shear flow: effects of red blood cells. *Thromb Res* 1998; **9219 (6 Suppl 2)**:47–52.
19 Peerschke EIB, Silver RTS, Grigg SE, Savion N, Varon D. Ex vivo evaluation of erythrocytosis-enhanced platelet thrombus formation using the cone and platelet analyzer: effect of platelet antagonists. *Br J Haematol* 2004; **127**:195–203.
20 Goldsmith HL, Bell DN, Braovac S, Steinberg A, McIntosh F. Physical and chemical effects of red cells in the shear-induced aggregation of human platelets. *Biophys J* 1995; **69**:1584–1595.
21 Cadrory Y, Hanson SR. Effects of red blood cell concentration on hemostasis and thrombus formation in a primate model. *Blood* 1990; **75**:2185–2193.
22 Bell DN, Spain S, Goldsmith HL. The effect of red blood cells on the ADP-induced aggregation of human platelets in flow through tubes. *Thromb Haemost* 1990; **63**:112–121.
23 Aarts PAMM, Heetaar RM, Sixma JJ. Red blood cell deformability influences platelets-vessel wall interaction in flowing blood. *Blood* 1984; **64**:1228–1233.
24 Bozzo J, Hernandez MR, Ordians A. Reduced red cell deformability associated with blood flow and platelet activation: improved by dipyrimidamole alone or combined with aspirin. *Cardiol Res* 1995; **30**:725–730.
25 Valles J, Santos MT, Aznar J, Marxus AJ, Sales VM, Portoles M, *et al.* Erythrocytes metabolically ehnace collagen-induced platelet responsiveness via increased thromboxan production.adenosine diphosphate release,and recruitment. *Blood* 1991; **78**:154–162.


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Short Communication

# A561C polymorphism of E-selectin is associated with ischemic cerebrovascular disease in the Japanese population without diabetes mellitus and hypercholesterolemia

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ABSTRACT

E-selectin, which is a member of the selectin superfamily of adhesion molecules, contributes to the leukocyte–endothelial cell attachments and is involved in the pathogenesis of thrombovascular diseases as a consequence. We investigated the A561C mutation in the E-selectin gene in 235 Japanese patients with ischemic cerebrovascular disease (CVD) and 301 age- and sex-matched healthy controls. Excluding the subjects with diabetes mellitus and hypercholesterolemia, the AC genotype frequencies of patients with ischemic CVD were higher than those of controls: 12.7% vs. 5.8% ($P=0.04$). Our results show that E-selectin gene polymorphisms represent an increased risk for ischemic CVD in the Japanese population without diabetes mellitus and hypercholesterolemia.

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Leukocyte–endothelial attachments contribute to acute and chronic inflammation and atherosclerosis (Yoshida et al., 2003). In the normal physiological state, endothelial cells have low adhesiveness for leukocytes. However, inflammation or atherosclerosis activates both leukocytes and endothelial cells (Fassbender et al., 1999). E-selectin, L-selectin, and P-selectin are members of the selectin superfamily of adhesion molecules. Selectins are expressed on activated endothelial cells (E-selectin and P-selectin), leukocytes (L-selectin), and activated platelets (P-selectin) (Haring et al., 1996). They have in common an epidermal growth factor (EGF)-like domain connected with variable repeats of amino acid units to a membrane and cytoplasmic domain, and they bind to specific carbohydrate molecules on leukocyte molecules (Bevilacqua, 1993). These molecules, especially E-selectin, were revealed to facilitate leukocyte–endothelial cell attachments and contribute to the pathogenesis of thrombovascular diseases as a consequence (Cherian et al., 2003).

Recent studies showed that E-selectin plasma levels in homozygous C561C subjects and heterozygous A561C subjects were statistically higher than in wild-type A561A subjects (Mlekusch et al., 2004) and the A561C allele enhanced thrombin generation and fibrin formation significantly (Jilma et al., 2005). Positive results for C561 were associated with myocardial infarction in Japanese patients (Yoshida et al., 2003).

Interestingly, the E-selectin and P-selectin serum levels in patients with acute ischemic stroke were significantly higher than in controls, suggesting that these selectins are directly associated with the development of stroke (Cherian et al.,

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**Table 1 – Clinical characteristics of patients with CVD and controls**

| | Controls ($n=301$) | Patients with CVD ($n=235$) | P[a] |
|---|---|---|---|
| Male, % | 77.1 | 77.9 | NS[b] |
| Age, mean±SD, years | 58.7±4.4 | 58.3±7.8 | NS[b] |
| Hypertension, % | 25.5 | 56.2 | <0.001 |
| Hypercholesterolemia, % | 32.6 | 38.6 | 0.146 |
| Diabetes mellitus, % | 6.31 | 25.8 | <0.001 |
| Smoking, % | 37.9 | 53.3 | <0.001 |
| Body mass index >27.3kg/m$^2$, % | 5.17 | 9.02 | 0.155 |
| Family history, % | 24.2 | 29.3 | 0.296 |

[a] $\chi^2$tests were used to compare values of patients with CVD and controls for all parameters except for age, which was compared by Student's t test.
[b] Not significant.

2003). Although several studies have showed the positive relationship between E-selectin A561C polymorphism and atherosclerotic disease (Mlekusch et al., 2004; Wenzel et al., 1994), the association between the polymorphism and ischemic stroke remains unclear. The primary aim of this study was to determine whether the E-selectin single nucleotide polymorphism is associated with cerebrovascular disease in the Japanese population.

We analyzed 235 unrelated Japanese patients with ischemic cerebrovascular disease (CVD) and 301 age- and sex-matched controls. All patients with CVD had attended the outpatient clinic of Keio University Hospital, Tokyo, for regular follow-up examinations. All controls who worked for Keio University had visited to Keio University Hospital for their annual health check-up. We selected patients with CVD aged ≦70years at the onset of CVD. On the basis of the Classification of Cerebrovascular Diseases III report from the committee established by the National Institute of Neurological Disorders and Stroke, patients with CVD and a diagnosis of atherothrombotic infarction (AT), lacunar infarction, or transient ischemic attack (TIA) were enrolled in this study. Those with cardioembolic cerebral infarction or cerebral hemorrhage were excluded. Controls were patients who had had regular check-ups. Those with a clinical history of CVD, myocardial infarction, or peripheral vascular disease were excluded. Written informed consent was obtained from all subjects after a full explanation of the study and a guarantee of total confidentiality. Brain computed tomography (CT) and/or magnetic resonance imaging (MRI) studies were performed on all patients with CVD. Hypertension, hypercholesterolemia, and smoking were defined as described previously (Ito et al., 2000).

To analyze the A561C polymorphism of E-selectin, polymerase chain reaction (PCR) was carried out as described previously (Ito et al., 2000). Briefly, amplification of a 358-bp fragment of the E-selectin gene was performed with the 5′ primer 5′-ATGGCACTCTGTAGGACTGCT-3′ and 3′ primer 5′-GTCTCAGCTCACGATCACCAT-3′. Amplification by PCR consisted of an initial 3min denaturation at 94 °C, 35 cycles of 30s at 94 °C, 1min at 60 °C, and 1min at 72 °C, followed by 7min at 72 °C in a Gene Amp PCR system 2400 (Perkin Elmer, Foster City, CA, USA). The PCR product (5μl) was cleaved with 15 U PstI restriction enzyme (Takara Shuzo, Ohtu, Shiga, Japan). The digested PCR products yielded bands of 221 and 137bp in AA homozygotes, and 358, 221, and 137bp in AC heterozygotes. There were no CC homozygotes among our subjects.

The differences in genotype frequencies and other risk factors were analyzed by the $\chi^2$ test. Mean age and allele frequencies in the two groups were compared by Student's t test. Multiple logistic regression methods were used to control for possible confounding factors. All statistical analyses were performed using Statview software (version 5.0 for Windows, SAS Institute, Cary, NC, USA).

The 235 patients with CVD and 301 controls were well matched in terms of age and sex (Table 1). The risk factors hypertension, diabetes mellitus, and smoking were significantly more common in patients with CVD than in controls ($P<0.001$). The frequencies of the A561C polymorphism showed no deviation from Hardy–Weinberg equilibrium.

The frequencies of AC genotype carrier were not statistically different between patients with CVD and controls: 9.4% vs. 8.3%, odds ratio (OR)=1.14 [95% confidence interval (CI)=0.63–2.05, $P=0.669$]. The C allele frequencies were not significantly different between patients with CVD and controls: 4.7% vs. 4.2%, OR=1.13 (95% CI=0.64–2.03, $P=0.705$) (Table 2).

The frequency of AC genotype carrier without diabetes mellitus and hypercholesterolemia was statistically higher in patients with CVD compared with healthy controls: 12.7% vs. 5.8%, OR=2.37 (95% CI=1.03–5.44, $P=0.040$). The C allele excluding diabetes mellitus and hypercholesterolemia was significantly more frequent in CVD patients than healthy controls: 6.4% vs. 2.9%, OR=2.28 (95 CI=1.04–4.99, $P=0.045$) (Table 2).

**Table 2 – Genotype and allele frequencies of the Ser128Arg (A561C) polymorphism of the E-selectin gene in patients with CVD and controls**

| Genotype | All patients ($n=235$) | AT ($n=69$) | Lacunar ($n=142$) | TIA ($n=24$) | Controls ($n=301$) |
|---|---|---|---|---|---|
| AC, % | 9.4 (22) | 11.6 (8) | 7.7 (11) | 12.5 (3) | 8.3 (25) |
| AA, % | 90.6 (213) | 88.4 (61) | 92.3 (131) | 87.5 (21) | 91.7 (276) |
| P* | 0.669 | 0.402 | 0.840 | 0.504 | – |
| C, % | 4.7 | 5.8 | 3.9 | 6.3 | 4.2 |
| A, % | 95.3 | 94.2 | 96.1 | 93.7 | 95.8 |
| P* | 0.705 | 0.414 | 0.843 | 0.516 | – |
| *Without diabetes mellitus and hyperlipidemia* | | | | | |
| AC, % | 12.7 (14) | 13.8 (4) | 10.9 (7) | 17.6 (3) | 5.8 (11) |
| AA, % | 87.3 (96) | 86.2 (25) | 89.1 (57) | 82.4 (14) | 94.2 (179) |
| P* | 0.040 | 0.150 | 0.184 | 0.108 | – |
| C, % | 6.4 | 6.9 | 5.5 | 8.8 | 2.9 |
| A, % | 93.6 | 93.1 | 94.5 | 91.2 | 97.1 |
| P* | 0.045 | 0.159 | 0.193 | 0.118 | – |

The number of subjects (X). *$\chi^2$ tests were used to compare genotype and allele frequencies between controls and all patients with CVD and between controls and individual groups of patients with CVD.

In logistic regression analysis, sex, age, hypertension, hypercholesterolemia, diabetes mellitus, current smoking, body mass index, family history of stroke, and E-selectin genotypes (AA vs. AC or A allele vs. C allele) were included as independent variables. This analysis revealed that presence of the C allele was independent of these acquired risk factors.

The present study examined the relation between ischemic CVD and E-selectin single nucleotide polymorphisms, and the results indicated that A561C single nucleotide polymorphism was one of the genetic risk factors of ischemic CVD in Japanese persons without diabetes mellitus and hypercholesterolemia.

The A561C polymorphism within the epidermal growth factor-like domain of the human E-selectin gene results in the substitution of arginine for serine at position 128 (Ser128Arg) of the mature protein (Wenzel et al., 1994). The A561C polymorphism has a profound effect on ligand recognition and binding (Ellsworth et al., 2001). There is evidence that human umbilical vein endothelial cells carrying the A561C mutation shows more rolling and adhesion of neutrophils and mononuclear cells than the wild-type cells (Yoshida et al., 2003). Additionally, Mlekusch et al. (2004) demonstrated that E-selectin plasma levels in subjects with the A561C mutation were statistically higher than in subjects with wild-type A561A. In summary, A561C polymorphism could facilitate the attachment between neutrophils and endothelial cells in the first stage of atherosclerosis and increase the expression of E-selectin in the human body, suggesting that A561C is one of the genetic risk factors in atherothrombotic CVD.

Our study failed to show the relation between ischemic CVD and A561C in all participants, including those with diabetes mellitus and/or hypercholesterolemia. The two groups in Japan showed that serum levels of soluble E-selectin were higher in patients with type 2 diabetes mellitus and hyperlipidemia or other complications than in healthy controls (Nomura et al., 2003; Matsumoto et al., 2002). On the other hand, smoking did not increase serum E-selectin level in the acute or chronic phases (Patiar et al., 2002). These facts indicated that the expression of E-selectin levels could be essentially increased in diabetes mellitus and/or hypercholesterolemia even if the participants in our study had the wild-type A561A genotype; therefore, the genetic effect of E-selectin genotype may be attenuated in these patients.

In conclusion, this study revealed a significant association between A561C polymorphisms in the E-selectin gene and ischemic CVD without diabetes mellitus and hypercholesterolemia. Although further studies are needed to evaluate whether E-selectin may play a role in the pathogenesis of CVD, our results and those of recent studies indicate that E-selectin polymorphism and expression levels in the human body may have an effect on the occurrence of ischemic CVD.

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## REFERENCES

Bevilacqua, M.P., 1993. Endothelial–leukocyte adhesion molecules. Annu. Rev. Immunol. 11, 767–804.

Cherian, P., Hankey, G.J., Eikelboom, J.W., Thom, J., Baker, R.I., McQuillan, A., Staton, J., Yi, Q., 2003. Endothelial and platelet activation in acute ischemic stroke and its etiological subtypes. Stroke 34, 2132–2137.

Ellsworth, D.L., Bielak, L.F., Turner, S.T., Sheedy 2nd, P.F., Boerwinkle, E., Peyser, P.A., 2001. Gender- and age-dependent relationships between the E-selectin S128R polymorphism and coronary artery calcification. J. Mol. Med. 79, 390–398.

Fassbender, K., Bertsch, T., Mielke, O., Muhlhausser, F., Hennerici, M., 1999. Adhesion molecules in cerebrovascular diseases. Evidence for an inflammatory endothelial activation in cerebral large- and small-vessel disease. Stroke 30, 1647–1650.

Haring, H.P., Berg, E.L., Tsurushita, N., Tagaya, M., del Zoppo, G.J., 1996. E-selectin appears in nonischemic tissue during experimental focal cerebral ischemia. Stroke 27, 1386–1392.

Ito, D., Murata, M., Tanahashi, N., Sato, H., Sonoda, A., Saito, I., Watanabe, K., Fukuuchi, Y., 2000. Polymorphism in the promoter of lipopolysaccharide receptor CD14 and ischemic cerebrovascular disease. Stroke 31, 2661–2664.

Jilma, B., Marsik, C., Kovar, F., Wagner, O.F., Jilma-Stohlawetz, P., Endler, G., 2005. The single nucleotide polymorphism Ser128Arg in the E-selectin gene is associated with enhanced coagulation during human endotoxemia. Blood 105, 2380–2383.

Matsumoto, K., Sera, Y., Ueki, Y., Inukai, G., Niiro, E., Miyake, S., 2002. Comparison of serum concentrations of soluble adhesion molecules in diabetic microangiopathy and macroangiopathy. Diabet. Med. 19, 822–826.

Mlekusch, W., Exner, M., Schillinger, M., Sabeti, S., Mannhalter, C., Minar, E., Wagner, O., 2004. E-selectin and restenosis after femoropoliteal angioplasty: prognostic impact of the Ser128Arg genotype and plasma levels. Thromb. Haemostasis 91, 171–179.

Nomura, S., Kanazawa, S., Fukuhara, S., 2003. Effect of eicosapentaenoic acid on platelet activation markers and cell adhesion molecules in hyperlipidemic patients with type 2 diabetes mellitus. J. Diabetes Its Complicat. 17, 153–159.

Patiar, S., Slade, D., Kirkpatrick, U., McCollum, C.N., 2002. Smoking causes a dose-dependent increase in granulocyte-bound L-selectin. Thromb. Res. 106, 1–6.

Wenzel, K., Felix, S., Kleber, F.X., Brachold, R., Menke, T., Schattke, S., Schulte, K.L., Glasser, C., Rohde, K., Baumann, G., Speer, A., 1994. E-selectin polymorphism and atherosclerosis: an association study. Hum. Mol. Genet. 3, 1935–1937.

Yoshida, M., Takano, Y., Sasaoka, T., Izumi, T., Kimura, A., 2003. E-selectin polymorphism associated with myocardial infarction causes enhanced leukocyte–endothelial interactions under flow conditions. Arterioscler., Thromb., Vasc. Biol. 23, 783–788.

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特集 循環器疾患における遺伝子診療の現状と将来

# 血小板，凝固異常における遺伝子診療*

横山健次**
池田康夫**

**Key Words**：protein S, plasminogen, plasminogen activator inhibitor 1, ADAMTS 13, GPIbα

## はじめに

心筋梗塞などの動脈血栓症，肺血栓症などの静脈血栓症は，遺伝的要因，後天的要因が複雑に絡み合って発症すると考えられている．血栓症発症に関与する可能性がある遺伝的要因としてはさまざまなものが考えられるが，凝固線溶系因子あるいは血小板膜蛋白の先天的異常により血液が過凝固状態になることも，その一つと考えられる．これらの異常をきたす遺伝子変異には，頻度は低いが病的意義がほぼ明らかにされている病的変異〔アンチトロンビンⅢ欠損症，プロテインC(protein C：PC)欠損症など〕と，比較的頻度が高いが必ずしも病的意義の有無は明らかではない遺伝子多型がある．血栓症発症の遺伝的要因を明らかにすることを目的として，遺伝子多型と血栓症発症との関連の有無を検討する多数の疫学的研究，*in vitro*の実験が行われてきた．しかし，それらの結果は報告により相反することも稀ではなく，現時点ではそれぞれの遺伝子多型と血栓症発症の関連の有無に関しては一定の見解が得られていないものが多い．本稿では，これらの凝固線溶系因子，あるいは血小板膜蛋白の遺伝子多型の中で，日本人に特異的，あるいは頻度が高く，心筋梗塞，肺血栓症などの血栓症発症と関連する可能性がある遺伝子多型を中心に紹介し解説を加える．

## プロテインS(PS)

PSは主に肝臓で合成されるビタミンK依存性の糖蛋白質であり，PS分子はアミノ末端から順にGlaドメイン，トロンビン感受性ドメイン，4個の上皮増殖因子(epidermal growth factor：EGF)様ドメイン，カルボキシ末端の性ホルモン結合グロブリン(sex hormone binding globulin：SHBG)様ドメインの各ドメインで構成されている．PSは，ヒト血漿中には遊離型，およびC4b結合蛋白質(C4BP)と結合した結合型として存在しており，活性型プロテインC(activated protein C：APC)と結合し，補酵素として抗凝固作用を示す．また，PSは直接活性化第X因子(Xa)の活性を抑制し，さらにプロトロンビン(prothrombin：PT)の活性化第V因子(Va)への結合を抑制することによっても抗凝固作用を示す．PS欠損症にはPS抗原量，活性ともに低下するI型，PSの分子異常により抗原量は正常ながら活性のみ低下するII型，遊離型PS抗原量が低下するIII型があり，PS欠損症患者では深部静脈血栓症(deep venous thrombosis：DVT)を発症しやすいと考えられている．日本人ではPS欠損症の頻度が1.12～2.04%と報告されており[1)2)]，白人の0.03～0.13%に比し高い[3)]．なかでも日本人ではII型PS欠損症が多く，日本人でみられるII型PS欠損症の大部分はEGF

* Genetic analysis of platelet and coagulation disorders.
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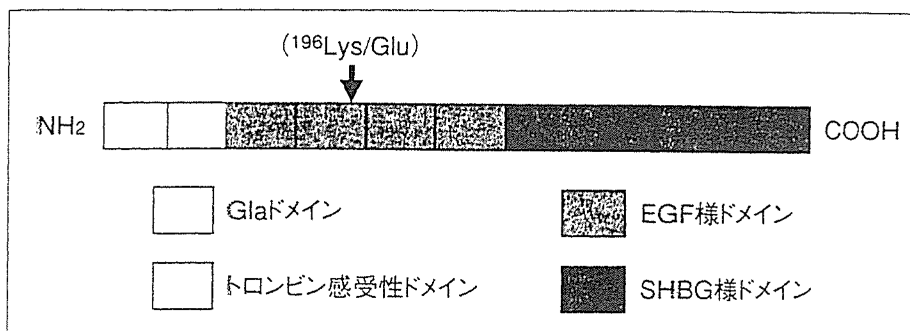


図1　PS分子の構造と遺伝子多型
PS分子はアミノ末端から順にGlaドメイン，トロンビン感受性ドメイン，4個のEGF様ドメイン，カルボキシ末端のSHBG様ドメインの各ドメインで構成されている．PS K196E(PS Tokushima)では2番目のEGF様ドメイン内の196番目のLysがGluに置換されている．

表1　PS K196Eの機能

| 低　下 | 正　常 |
|---|---|
| APC補酵素活性 | リン脂質への結合 |
| APCへの結合 | C4BPへの結合 |
| Xaへの結合 | Vaへの結合 |
| XaとVaの結合促進 | |

リコンビナントPS K196Eを用いた*in vitro*の実験の結果では，PS K196EではAPC補酵素活性，APCへの結合，Xaへの結合，XaとVaの結合を促進する作用が低下している．一方，リン脂質への結合，C4BPへの結合，Vaへの結合は野生型と差がない．

様ドメイン内の196番目のLysがGluに置換されたPS K196Eである(図1)[4]．このPS K196Eは，以前はPS K155EあるいはPS Tokushimaと呼ばれていたが[5]，アミノ酸番号の付け方が替わりPS K196Eと呼ばれるようになったものであり，日本人の1.65～1.8%がPS K196Eのヘテロのキャリアーであることが報告されている[6][7]．リコンビナントPS K196Eを用いた実験によりPS K196EはAPC補酵素活性を失い，APCあるいはXaに結合しないことなどが示されている(表1)[8][9]．日本人DVT患者161人中13人がヘテロ，2人がホモのPS K196Eキャリアーであったこと[7]，などが報告されており，日本人DVT患者の10%程度がPS K196E変異を有するII型PS欠損症と考えられる．これらの結果からは，PS K196Eは日本人におけるDVT発症の危険因子であるといってよいと思われる．今後は前向きの臨床研究を行い，実際にII型PS欠損症，あるいはPS K196EキャリアーでDVTを発症する危険性はどの程度か，危険性が高いのであれば手術，妊娠などDVT発症の危険性が増す際にはあらかじめ予防策をとる必要はあるか，またDVTのみならず心筋梗塞，脳梗塞などの動脈血栓症の危険因子となる可能性はあるか，などを明らかにしていくことが必要であろう．

## プラスミノーゲン(PLNG)

プラスミノーゲン(plasminogen：PLNG)は，組織プラスミノーゲンアクティベーター(tissue plasminogen activator：t-PA)により酵素活性を発現するプラスミンに変化する．血栓形成に重要な役割を果たしているフィブリンが，プラスミンにより分解されてフィブリン分解産物となり，その結果血栓が溶解するのが線溶系の主要な反応である．PLNG分子はアミノ末端から順にプレアクティベーションペプチド，5個のクリングルドメイン，カルボキシ末端のセリンプロテアーゼドメインの各ドメインで構成されている．日本人の再発性のDVT患者で発見された620番目のAlaがThrに置換されたPLNG A620Tのヘテロのキャリアーでは，PLNG活性が50～60%に低下している(図2)[10]．このPLNG A620Tは，以前はPLNG A601TあるいはPLNG Tochigiと呼ばれていたものであり，日本人ではアリル頻度が1.1～2.1%，中国人では1.5%，韓国人では1.6%と東洋人には高頻度でみられ，一方白人にはみられない[11][12]．PLNGは線溶系開始の重要な因子であり，PLNG A620TはDVT患者で最初に発見されたが，その後施行された4,517人を対象とした大規模な臨床研究の結果，日本人では抗原量，活性とも

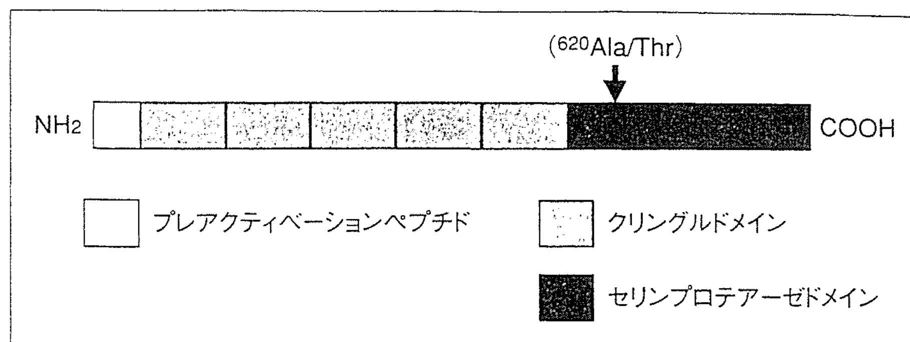


図2 PLNG分子の構造と遺伝子多型
PLNG分子はアミノ末端から順にプレアクティベーションペプチド，5個のクリングルドメイン，カルボキシ末端のセリンプロテアーゼドメインの各ドメインで構成されている．PLNG A620T(PLNG Tochigi)ではセリンプロテアーゼドメイン内の620番目のAlaがThrに置換されている．

に低下するⅠ型PLNG欠損症の頻度が0.42%，抗原量は正常であるが活性が低下しているⅡ型PLNG欠損症(この中にはPLNG A620Tが含まれる)の頻度がヘテロ3.83%，ホモ0.04%とⅡ型PLNG欠損症の頻度が高いが，PLNG欠損症群と対象群でDVTないし動脈血栓症発症の危険性に差はないことが示された[12]．

## プラスミノーゲンアクティベーターインヒビター1(PAI-1)

プラスミノーゲンアクティベーターインヒビター1(plasminogen activator inhibitor 1：PAI-1)はt-PAを阻害して線溶系を抑制する．したがって，血漿PAI-1が高値となればフィブリン分解が阻害され血栓形成が進行することが予測される．実際，動脈硬化病変部位ではPAI-1 mRNA発現量が亢進していること，心血管死にはPAI-1高値が関連すること，などが報告されており，PAI-1高値は血栓症発症に関与すると考えられている[13]．PAI-1発現量は種々のサイトカイン，ホルモン，増殖因子，などにより調節されているが，さらにPAI-1遺伝子のプロモーター領域-675bpにある4G(GTGGGGAGTC)/5G(GTGGGGGAGTC)の遺伝子多型がPAI-1発現量に影響することが知られている．*In vitro*の実験では，4Gアリルには転写抑制因子が結合できないためPAI-1発現量が亢進することが示されており，4G/4Gのキャリアーでは5G/5Gのキャリアーよりも約25%PAI-1値が高値となることが報告されている[14]．PAI-1[-675]4G/5G遺伝子多型と血栓症の関連はいくつかの報告で解析されている．Erikssonらは，45歳以前に心筋梗塞を発症した男性患者では4Gのキャリアーが有意に多いことを報告した[15]．しかし，その後報告された高齢男性を対象としたUS Physician's, Health Studyでは，心筋梗塞と4G/5G遺伝子多型の間に関連はみられなかった[16]．その他のいくつかの報告でも，4G/5G遺伝子多型と心筋梗塞発症あるいはDVT発症との関連の有無に関する解析結果はさまざまである[14]．さらに，日本人を対象としてYamadaらが施行した候補遺伝子アプローチ法を用いた解析では，5Gキャリアーが女性の心筋梗塞発症の危険因子であった[17]．これらの結果から，現時点ではPAI-1[-675]4G/5G遺伝子多型と血栓症発症の関連に関しては結論できないと考えるのが妥当であろう．

## ADAMTS 13

ヴォンヴィレブランド因子(von Willebrand factor：vWF)を分解する酵素であるADAMTS 13は，アミノ末端から順に，プレプロペプチドドメイン，メタロプロテアーゼドメイン，ディスインテグリン様ドメイン，トロンボスポンジン様ドメイン，システインリッチドメイン，スペーサードメイン，さらに7個のトロンボスポンジン様ドメイン，カルボキシ末端のCUBドメインで構成されている．アミノ酸変異により活性を失ったADAMTS 13分子異常の患者では，先天性の血栓性血小板減少性紫斑病(thrombotic thrombocytopenic purpura：TTP)を発症することがあることが知られている[18]．ADAMTS 13の遺伝子

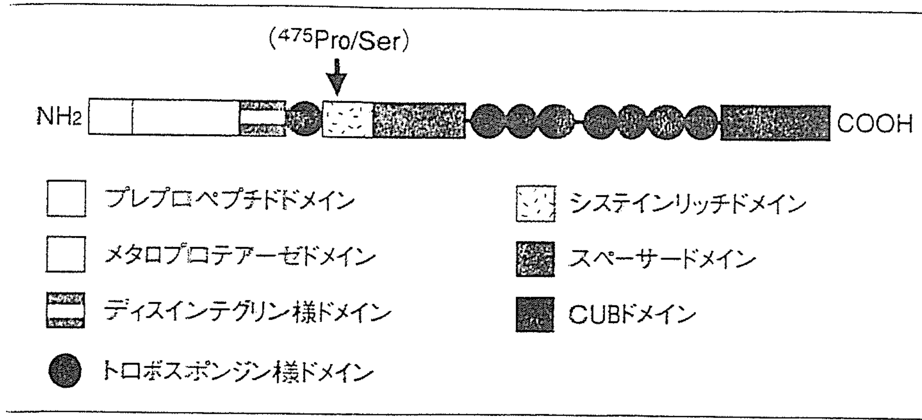


図3 ADAMTS 13の構造と遺伝子多型
ADAMTS 13はアミノ末端から順に，プレプロペプチドドメイン，メタロプロテアーゼドメイン，ディスインテグリン様ドメイン，トロンボスポンジン様ドメイン，システインリッチドメイン，スペーサードメイン，さらに7個のトロンボスポンジン様ドメイン，カルボキシ末端のCUBドメインで構成されている．ADAMTS 13 P475Sではシステインリッチドメイン内の475番目のProがSerに置換されている．

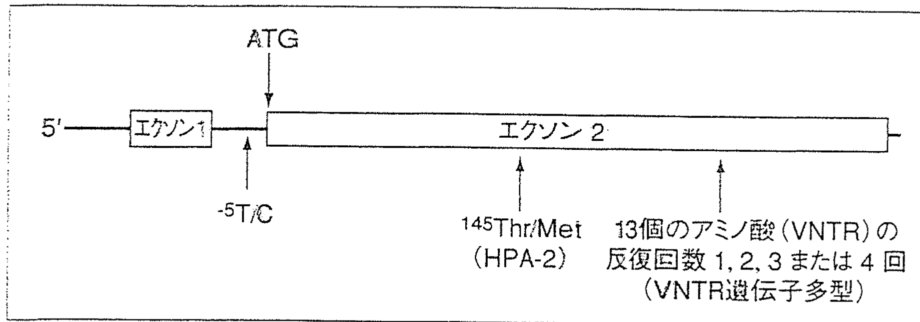


図4 GPIbαの遺伝子多型
GPIbαにはいくつかの遺伝子多型が報告されているが，本図に示すKozak配列の-5T/C，145番目のThr/Met, 13個のアミノ酸の繰り返し配列(VNTR)の反復回数の遺伝子多型と血栓症との関連について研究されている．

表2 GPIbαの145Thr/MetとVNTRの反復回数の人種差

| | 145Thr | 145Met | VNTRの反復回数 | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| 日本人 | 92.0 | 8.0 | 33.9 | 59.8 | 0.6 | 5.7 |
| 白人 | 92.4 | 7.6 | 8.4 | 84.0 | 7.6 | 0.0 |

日本人ではGPIbαの145番目のアミノ酸がThrの場合，VNTRは1回または2回の反復であり，Metの場合には3回または4回の反復である．一方白人では，145番目がMetの場合にはほとんどが3回の反復である．
(文献23 24)を参照して作成)

多型はいくつか報告されているが，日本人に頻度が多いものはシステイン-リッチドメイン内の475番目のProがSerに置換されたADAMTS 13 P475Sである(図3)[19]．この遺伝子多型の日本人でのアリル頻度は5.1%であるが，中国人では1.5%[20]，さらに白人ではみられないと報告されている[21]．また，リコンビナントADAMTS 13 P475Sを用いた*in vitro*の実験では酵素活性が低下していることが示されているが[19]，われわれのグループで行った解析では，心血管疾患患者，脳梗塞患者でこの変異を有する割合は健常人と有意差はなかった[22]．vWFが血栓形成に重要な役割を果たしているのはよく知られているが，その分解酵素と血栓症の関連に関する研究は始まったばかりである．今後さらに大規模な前向き研究を行い，ADAMTS 13 P475Sを含めたADAMTS 13の遺伝子多型の臨床的意義を明らかにしていくことが必要であろう．

## GPIbα

GPIbαはGPIbβ，GPV, GPIXと複合体を形成している血小板膜蛋白である．この複合体は，

表3　最近報告された血栓症と遺伝子多型のメタ解析の結果

| | DVT再発の危険比 | 冠動脈疾患発症の危険比 | 白人/日本人でのアリル頻度(%) |
|---|---|---|---|
| FV Leiden | 1.41 | 1.17 | 3/0 |
| PT G20210A | 1.72 | 1.31 | 1/0 |
| PAI-1[-675]4G/5G | — | 1.06 | 49/39* |
| GPIbα[-5]T/C | — | 1.05 | 13/20** |

*5Gアリルの頻度，**-5Gアリルの頻度．最近報告されたメタ解析の結果，FV LeidenおよびPT G20210AはDVT再発との間に関連があり，CAD発症にも弱い関連があった．一方，本文中に紹介したPAI-1[-675]4G/5GとCAD発症にも弱い関連がある可能性があるが，この結果は選択した論文のバイアスの影響があると考えられている．図4に示したGPIbα[-5]T/Cの遺伝子多型とCAD発症の間には関連はなかった．　（文献[28,29]より改変して作成）

高ずり応力下ではvWFと結合して血小板粘着，血栓形成に重要な役割を果たしている．GPIbαにはいくつかの遺伝子多型が報告されているが，145番目のThr/Metの遺伝子多型と13個のアミノ酸の繰り返し配列(VNTR)の反復回数の関連が日本人と白人で違いがみられる(図4)．日本人では，145番目のアミノ酸がThrの場合VNTRは1回または2回の反復であり，Metの場合には3回または4回の反復である(表2)[23]．一方白人では，145番目がMetの場合にはほとんどが3回の反復である[24]．GPIbαの145番目がMetである，あるいはVNTRの3回または4回反復を有することが心筋梗塞，脳梗塞などの血栓症発症が関連する[25]，との報告がある一方で，これらに相関関係は認められない[26]，とする報告もあり，このGPIbαの遺伝子多型が血栓症の危険因子であるか否かは，まだ一定の見解は得られていない．

## 第5因子(FV)とプロトロンビン(PT)

血液凝固因子の一つであるFVはトロンビンまたはXaで切断されると凝固促進活性を有する活性化FV(FVa)を形成し，このFVaはさらに抗凝固因子であるAPCにより切断され不活化される．一方，FVが最初にAPCにより切断されると，抗凝固活性をもちAPCの補酵素として働く．APCレジスタンスの原因として知られているFV Leidenは，506番目のArgがGlnに置換されてAPCにより切断されなくなったFVであり，FV Leidenのキャリアーの血液は血栓傾向を呈している．また，PTの遺伝子多型としては非翻訳領域に変異を有するPT G20210Aが重要であり，この多型を有するキャリアーは血漿PT値が高値となり血栓傾向を呈することが知られている．白人ではFV Leidenの頻度が2～7％，PT G20210Aの頻度が2～3％と比較的高く，いくつかの臨床研究の結果，両者はともにDVT発症の危険因子であると考えられている[27]．最近報告されたいくつかの臨床研究をメタ解析した結果では，計3,104人の初発のDVT患者中21.4％がFV Leidenキャリアーであり，FV LeidenキャリアーではDVT再発の危険性は1.41倍であった．計2,903人の初発のDVT患者中9.7％がPT G20210Aキャリアーであり，PT G20210AキャリアーではDVT再発の危険性は1.72倍であった．これらの結果から，この両者はDVT再発の危険因子でもあるが，その程度は再発予防のために必ずしも長期の抗凝固療法が必須とされるほどではない，と結論されている[28]．

一方，この両者の変異は従来必ずしも心筋梗塞などの動脈血栓症の危険因子とは考えられていなかったが，最近報告された66,155人の冠動脈疾患(coronary artery disease：CAD)患者と91,307人の対照群という大規模なメタ解析の結果では，FV G1691AのキャリアーでCAD発症の相対危険比が1.17, PT G20210Aでは1.31と上昇しており，この両者の変異はCAD発症に強くはないが関連していることが示された．なお，この研究ではその他5種類の遺伝子多型に関しても解析されているが，それらとCAD発症の関連は明らかではなかった[29]．この結果は，FV Leiden, PT G20210AはDVTのみならず弱いながらもCAD発症の危険因子であることを示唆するものといえる(表3)．したがって，血栓症，ことに肺血栓

症などのDVT患者ではこの両者の変異を有するかは重要な情報ではあるが，実際は日本人にはいずれの変異も存在しない．したがって，日本国内で日本人を対象として診療を行っているかぎりこれらの変異を検査する必要はまったくない．しかし外国人，とくに白人患者を診療する場合，あるいは外国で診療を行う場合にはこの両遺伝子多型の検査が必要となることがある．

## おわりに

以上述べてきたように，日本人ではおそらくPS K196Eが深部静脈血栓症（DVT）発症と関連すると考えられる．したがって，現時点では保険適応の問題はあるが，日本人でDVTと診断した場合には可能であればPS K196Eのキャリアーであるか否かを検査することが望ましいと思われる．ただし，その結果をいかに診療に役立てて行くべきかは今後の検討課題である．一方現時点では，日本人において心筋梗塞，脳梗塞などの動脈血栓症発症との関連が明らかにされている遺伝子多型はない．最初に述べたように血栓症発症にはさまざまな要因が複雑に絡み合っている．その中で遺伝子多型と血栓症の関連を明らかにするためには，PS K196Eも含め今後多くの遺伝子多型に関して前向きの大規模な試験を行う必要がある．

## 文　献


1) Nomura T, Suehisa E, Kawasaki T, et al. Frequency of protein S deficiency in general Japanese population. Thromb Res 2000 ; 100 : 367.
2) Sakata T, Okamoto A, Mannami T, et al. Prevalence of protein S deficiency in the Japanese general population : The Suita Study. J Thromb Hamemost 2004 ; 2 : 1012.
3) Dykes AC, Walker ID, McMahon AD, et al. A study of protein S antigen levels in 3788 healthy volunteers : influence of age, sex and hormone use, and estimate for prevalence of deficiency state. Br J Haematol 2001 ; 113 : 636.
4) Miyata T, Kimura R, Kokubo Y, et al. Geneticrisk factors for deep vein thrombosis among Japanese : Importance of protein S K196E mutation. Int J Haematol 2006 ; 83 : 217.
5) Shigekiyo T, Uno Y, Kawauchi S, et al. Protein S Tokushima : An abnormal protein S found in Japanese family with thrombosis. Thromb Haemost 1993 ; 70 : 244.
6) Yamazaki T, Sugiura I, Matsushita T, et al. A phenotypically neutral dimorphism of protein S : the substitution of Lys 155 by Glu in the second EGF domain predicted by an A to G base exchange in the gene. Thromb Res 1993 ; 70 : 395.
7) Kimura R, Honda S, Kawasaki T, et al. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. Blood 2006 ; 107 : 1737.
8) Hayashi T, Nishioka J, Shigekiyo T, et al. Protein S Tokushima : Abnormal molecule with a substitution of Glu for Lys-155 in the second epidermal growth factor-like domain of protein S. Blood 1994 ; 83 : 683.
9) Hayashi T, Nishioka J, Suzuki K. Molecular mechanism of the dysfunction of protein $S_{Tokushima}$ ($Lys^{155}$ →Glu) for the regulation of the blood coagulation system. Biochim Biophys Acta 1995 ; 1272 : 159.
10) Miyata T, Iwanaga S, Sakata Y, et al. Plasminogen Tochigi : Inactive plasmin resulting from replacement of alanine-600 by threonine in the active site. Proc Natl Acad Sci USA 1982 ; 79 : 6132.
11) Ooe A, Kida M, Yamazaki T, et al. Common mutation of plasminogen defected in three Asian populations by an amplification refractory mutation system and rapid automated capillary electrophoresis. Thromb Haemost 1999 ; 82 : 1342.
12) Okamoto A, Sakata T, Mannami T, et al. Population-based distribution of plasminogen activity and estimated prevalence and relevance to thrombotic disease of plasminogen deficiency in the Japanese: the Suita Study. J Thromb Haemost 2003 ; 1 : 2397.
13) Kohler HP, Grant PJ. Plasminogen-activator inhibitor type 1 and coronary artery disease. N Engl J Med 2000 ; 342 : 1792.
14) Francis CW. Plasminogen activator inhibitor-1 levels and polymorphisms. Association with venous thromboembolism. Arch Pathol Lab Med 2002 ;

126 : 1401.

15) Eriksson P, Kallin B, van't Hooft FM, et al. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. Proc Natl Acad Sci USA 1995 ; 92 : 1851.

16) Ridker PM, Hennekens CH, Lindpaintner K, et al. Arterial and venous thrombosis is not associated with the 4G/5G polymorphism in the promotor of the plasminogen activator inhibitor gene in a large cohort of US men. Circulation 1997 ; 95 : 59.

17) Yamada Y, Izawa H, Ichihara S, et al. Prediction of the risk of myocardial infarction from the polymorphisms in candidate gene. N Engl J Med 2002 ; 347 : 1916.

18) Levy GG, Nichols WC, Lian EC, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature 2001 ; 413 : 488.

19) Kokame K, Matsumoto M, Soejima K, et al. Mutations and common polymorphisms in ADAMTS 13 gene responsible for von Willebrand factor-cleaving protease activity. Proc Natl Acad Sci USA 2002 ; 99 : 11902.

20) Ruan C, Dai L, Su J, et al. The frequency of P475S polymorphisms in von Willebrand factor-cleaving protease in the Chinese population and its relevance to arterial thrombotic disorders. Thromb Haemost 2004 ; 91 : 1257.

21) Bongers TN, De Maat MPM, Dippel DWJ, et al. Absence of Pro475Ser polymorphism in ADAMTS-13 in Caucasians. J Thromb Haemost 2005 ; 3 : 805.

22) Murata M, Uchida T, Suzuki M, et al. Screening of single nucleotide polymorphisms in the ADAMTS 13 (von Willebrand factor-cleaving protease) gene and studies on their association with stroke and coronary artery disease. Blood 2003 ; 102 : 801a.

23) Murata M, Matsubara Y, Kawano K, et al. Coronary artery disease and polymorphisms in a receptor mediating shear stress-dependent platelet activation. Circulation 1997 ; 96 : 3281.

24) Aramaki KM, Reiner AP. A novel isoform of platelet glycoprotein Ibα is prevalent in African Americans. Am J Haematol 1999 ; 60 : 77.

25) Sonoda A, Murata M, Ito D, et al. Association between platelet glycoprotein Ibα genotype and ischemic cerebrovascular disease. Stroke 2000 ; 31 : 493.

26) Carter AM, Catto AJ, Bamford JM, et al. Platelet GP IIIa PIA and GPIb variable number tandem repeat polymorphisms and markers of platelet activation in acute stroke. Arterioscler Thromb Vasc Biol 1998 ; 18 : 1124.

27) Reitsma PH. Genetic heterogeneity in hereditary thrombophilia. Haemostasis 2000 ; 30 Suppl 2 : 1.

28) Ho WK, Hankey G, Quinlan DJ, et al. Risk of recurrent venous thromboembolism in patients with common thrombophilia. Arch Intern Med 2006 ; 166 : 729.

29) Ye Z, Liu EHC, Higgins JPT, et al. Seven haemostatic gene polymorphisms in coronary disease : meta-analysis of 66155 cases and 91307 controls. Lancet 2006 ; 367 : 651.

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# Telomere length of normal leukocytes is affected by a functional polymorphism of hTERT

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**Abstract**

Transcriptional regulation of human telomerase reverse transcriptase (hTERT), a catalytic subunit of telomerase, is essential for telomerase activity associated with telomere length. In this study, we investigated the effects of a $^{-1327}$T/C polymorphism within the hTERT promoter region on the hTERT promoter activity and leukocyte telomere length in normal individuals. The promoter activity in the $^{-1327}$T-sequence was significantly higher than that in the $^{-1327}$C-sequence ($p = 0.0004$). For leukocyte telomere length, the $^{-1327}$T-allele carriers had significantly longer than the $^{-1327}$T-allele non-carriers ($p = 0.0007$). Also, there was no age-related shortening in leukocyte telomere length in the $^{-1327}$T/T ($p = 0.6633$) and $^{-1327}$T/C subjects ($p = 0.1691$), whereas there was clear age-related telomere shortening in the $^{-1327}$C/C subjects ($p = 0.0117$). These findings suggest that the functional $^{-1327}$T/C polymorphism of hTERT is associated with leukocyte telomere length in normal individuals.
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*Keywords:* Human telomerase reverse transcriptase; Polymorphism; Telomere length

Telomerase synthesizes telomeric repeats for addition to the end of linear chromosomes, although replication of the telomeric end is sometimes incomplete [1,2]. Thus, telomere shortening occurs after repeated cell divisions and has a key role in cellular senescence, differentiation, immortalization, and transformation [3]. A recent study showed that telomere shortening is assumed to contribute to mortality in older subjects or age-related diseases [4].

Telomere length is mainly regulated by telomerase activity associated with transcriptional activity of human telomerase reverse transcriptase (hTERT), a subunit of telomerase [5–7]. The hTERT promoter region located with the 1375 bp upstream of the transcription-starting site is rich in transcription factor binding sites [8,9]. Although the regulation of hTERT transcription has been widely studied, little is known about the genetic variations in relation to hTERT transcriptional activity.

In this study, the hTERT promoter region was sequenced for screening of genetic polymorphisms in a healthy population. A T to C transition 1327 bp upstream of the transcription-starting site of hTERT ($^{-1327}$T/C) was frequently observed (nucleotide numbering according to Horikawa et al.) [9]. Further, we investigated the association between the $^{-1327}$T/C polymorphism and (a) hTERT transcriptional activity in normal human umbilical vein endothelial cells (HUVECs), and (b) telomere length and telomerase activity in peripheral leukocytes in normal individuals.

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## Materials and methods

*Screening of the sequence variations within the hTERT promoter region.* Written informed consent was obtained from all subjects enrolled into the study. Study subjects were genetically unrelated Japanese subjects.

We recruited 46 healthy subjects for screening of polymorphisms within the hTERT promoter region. Among these subjects, the variation(s) in the hTERT sequence (nucleotide number [9] $^{-}1665$ to $^{+}20$) for 17 subjects and the sequence variation at position $^{-}1327$ for 29 subjects were analyzed by a direct DNA sequence analysis.

*Luciferase assay.* A dual-luciferase reporter assay system (Promega, Madison, WI) was used according to the manufacturer's protocol. A 1.6-kb DNA fragment (nucleotide number [9] $^{-}1623$ to $^{+}20$) with the $^{-1327}$T- or $^{-1327}$C-sequence was subcloned using the TA Cloning Kit (Invitrogen, Carlsbad, CA). Each hTERT insert was subsequently cloned into a firefly luciferase reporter plasmid pGL-3-Basic, a promoter- and enhancer-less vector (Promega), designated pGL3-$^{-1327}$T and pGL3-$^{-1327}$C. Thus, we prepared four types of firefly luciferase reporter plasmids, pGL3-$^{-1327}$T, pGL3-$^{-1327}$C, pGL3-Basic, and pGL3-Control, with the SV40 enhancer/promoter for the normalization of hTERT promoter activity and one *Renilla* luciferase reporter plasmid for standardization of transfection efficiency.

HUVECs (passage number, 3) were purchased from TAKARA (Tokyo, Japan). Early passage HUVECs (passage number, 5–7) were used to avoid the influence of any transformation by subculture on this assay. Transfection with luciferase plasmid into HUVECs was performed using FuGene 6 Transfection Reagent (Roche, Nutley, NJ). Luminescence was measured in each transfectant 24 h and 48 h after transfection. The value corresponding to the transcriptional activity of hTERT promoter for pGL3-$^{-1327}$T or -$^{-1327}$C was calculated using the formula: relative luciferase activity (%) = [(pGL3-$^{-1327}$T or -$^{-1327}$C) − (pGL3-Basic)]/[(pGL3-Control) − (pGL3-Basic)] × 100.

*Assay for telomere length.* To measure telomere length of leukocyte DNA, as assessed by mean length of terminal restriction fragments (TRF), we used Southern hybridization of telomeric DNA [10] and real-time kinetics quantitative polymerase chain reaction (PCR) [11], and correlation of results by these two methods was previously confirmed [11]. After the confirmation of correlation between these two different methods for measuring telomeres of our samples, we calculated telomere length. Study subjects were 133 males over 40 years of age because the rate of telomere shortening decreases after 40 years of age and is higher in males [12,13]. Genotyping of the $^{-1327}$T/C polymorphism was performed using Megabase 1000 (General Electric, Fairfield, CT), according to the manufacturer's protocol for the single nucleotide primer extension-based method.

*Telomerase activity.* Telomerase activity in leukocyte from healthy subjects was measured using the method for real-time quantitative PCR telomeric repeat amplification protocol (TRAP) assay, as described previously [14], and telomerase activity in each genotype of the $^{-1327}$T/C polymorphism was analyzed by the values of threshold cycle of telomeric repeat amplification in the real-time quantitative PCR TRAP assay. Nine study subjects were selected to match in age among three genotypes of the $^{-1327}$T/C polymorphism.

*Statistics.* Mean values of the two groups in this study were compared by Student's $t$ test. Mean values of the three groups in this study were compared by ANOVA. Single regression analysis was used to detect a correlation coefficient ($r$) in TRF length assay. Statistical analyses were performed using StatView (ver 5.0, for Macintosh, SAS, Cary, NC). A $p$ value less than 0.05 was considered to be statistically significant.

## Results

We analyzed the sequence of the hTERT promoter region to screen for genetic variations in 17 subjects, and 2 subjects were showed to be heterozygous for a T to C transition at 1327 bp upstream of the transcription-starting site [9]. This $^{-1327}$T/C transition has been reported (rs 2735940) in the database of single nucleotide polymorphism (http://www.ncbi.nlm.nih.gov/SNP/index.html), although there is no report of epidemiologic or experimental data for this substitution. To examine whether this T/C substitution is polymorphism or not, i.e., this substitution is present more than 1% among population, the genotype distribution of the $^{-1327}$T/C substitution was analyzed in an expanded population of 46 subjects. As a result, the genotype distribution was 15.2% for the $^{-1327}$T/T genotype, 39.0% for the $^{-1327}$T/C genotype, and 45.8% for the $^{-1327}$C/C genotype, suggesting that this T/C substitution is a polymorphism.

To investigate the effects of the $^{-1327}$T/C polymorphism on hTERT transcriptional activity, we performed an experimental study using a dual-luciferase reporter assay system. The mean value of relative luciferase activity representative of hTERT promoter activity in HUVECs transfected with pGL3-$^{-1327}$T was significantly higher than that in HUVECs transfected with pGL3-$^{-1327}$C at 24 h or 48 h after the transfection: $4.592 \pm 0.285$ (%, mean ± SD) for the pGL3-$^{-1327}$T and $3.711 \pm 0.686$ for the pGL3-$^{-1327}$C after 24 h of the transfection ($p = 0.0026$), and $6.368 \pm 1.017$ for the pGL3-$^{-1327}$T and $4.842 \pm 0.203$ for the pGL3-$^{-1327}$C after 48 h of the transfection ($p = 0.0004$) (data were obtained from three independent experiments performed in triplicate). The results are indicative of the relationship between the $^{-1327}$T-sequence and higher hTERT transcriptional activity.

Next, we measured leukocyte TRF length to test the hypothesis that the $^{-1327}$T/C polymorphism affects telomere length, closely related to the final stages of the telomere system. This speculation was also raised by previous reports that an inter-individual variation in leukocyte telomere length was genetically determined [15,16]. The TRF length in normal leukocytes was significantly different among the three genotypes: $7.80 \pm 1.23$ (kb, mean ± SD) for the $^{-1327}$C/C genotype ($n = 67$), $8.47 \pm 1.04$ for the $^{-1327}$T/C genotype ($n = 52$), and $8.53 \pm 0.96$ for the $^{-1327}$T/T genotype ($n = 14$) ($p = 0.0031$; Fig. 1). When analyzing the telomere length between the subjects without or with the $^{-1327}$T-allele, we obtained the results which showed that the genotypes with $^{-1327}$T/T and $^{-1327}$T/C were significantly longer than that in the $^{-1327}$C/C genotype: $7.80 \pm 1.23$ (kb, mean ± SD) for the $^{-1327}$C/C genotype ($n = 67$), $8.47 \pm 1.04$ for the $^{-1327}$T/C and $^{-1327}$T/T genotypes ($n = 66$) ($p = 0.0007$). Mean age was not significantly different between groups: $53.4 \pm 5.0$ (years, mean ± SD) for the $^{-1327}$C/C, $52.7 \pm 4.4$ for the $^{-1327}$T/C, and $51.9 \pm 4.4$ for the $^{-1327}$T/T ($p = 0.5200$). Also, there was no age-related shortening in TRF length in the $^{-1327}$T/T ($r = 0.128$, $p = 0.6633$) and $^{-1327}$T/C subjects ($r = -0.194$, $p = 0.1691$), whereas there was clear age-related telomere shortening in the $^{-1327}$C/C subjects ($r = -0.306$, $p = 0.0117$; Fig. 1). These observations suggest that the $^{-1327}$T/C polymorphism is strongly associated with telomere length in peripheral leukocytes in normal individuals.

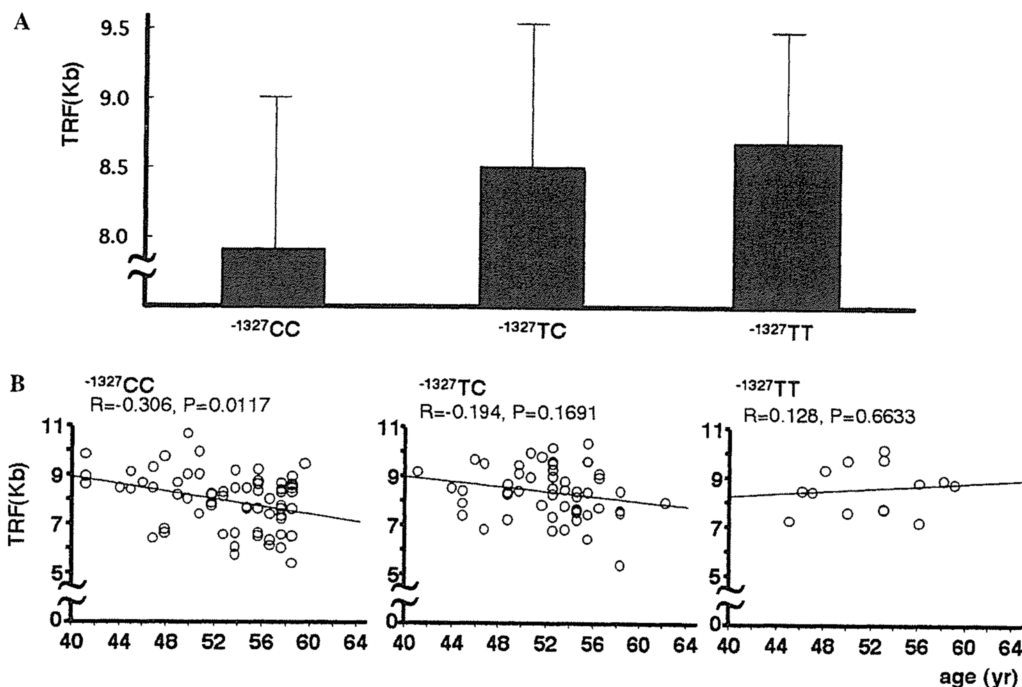


Fig. 1. Relationship between leukocyte telomere length and the $^{-1327}$T/C polymorphism. (A) Bars show mean TRF length (i.e., telomere length) (mean ± SD) in each genotype. (B) Plot of leukocyte TRF length against age and regression line are shown separately for the $^{-1327}$CC, $^{-1327}$TC, and $^{-1327}$TT genotypes.

Telomere length is mainly regulated by telomerase activity that is generally associated with hTERT transcriptional activity. However, several reports showed that transcriptional activity of hTERT did not always correlate directly with telomerase activity and the presence of post-translational modification [17]. Thus, we analyzed the relationship between the $^{-1327}$T/C polymorphism and telomerase activity in leukocyte from healthy subjects, and mean age in each genotype group was 36.0 ± 11.0 (y, mean ± SD) for the $^{-1327}$C/C genotype ($n = 4$), 36.0 ± 7.1 for the $^{-1327}$T/C genotype ($n = 2$), and 36.0 ± 12.2 for the $^{-1327}$T/T genotype ($n = 3$). Telomerase activity was examined using the threshold cycle values ($C_t$) of telomeric repeat amplification in the real-time quantitative PCR TRAP assay, thus higher $C_t$ indicating lower telomerase activity. Telomerase activity in the subjects with the $^{-1327}$T-allele was higher than that in the subjects without the $^{-1327}$T-allele: 29.9 ± 5.6 ($C_t$, mean ± SD) for the $^{-1327}$C/C genotype, 28.0 ± 4.2 for the $^{-1327}$T/C genotype, and 21.8 ± 4.0 for the $^{-1327}$T/T genotype, and this difference was marginally significant ($p = 0.0713$). Observation suggests that the $^{-1327}$T-allele is associated with higher telomerase activity in leukocyte.

## Discussion

The present study demonstrates for the first time that the $^{-1327}$T/C polymorphism within the hTERT promoter region has functional roles: the $^{-1327}$T sequence is associated with higher transcriptional activity, lack of age-dependent telomere shortening, longer telomere length, and telomerase activity. The relationship of the $^{-1327}$T/C polymorphism to telomere shortening, telomere length, and telomerase activity was found in normal peripheral leukocytes. Leukocyte telomere shortening has been highlighted as a critical marker in the research of cell senescence and cancer, thus, our observations show an impact in the fields.

Transcriptional regulation of hTERT has a key role in telomerase activity and telomere shortening; therefore, we focused on the hTERT promoter region in this study. In our promoter assay, we found approximately a 25% higher promoter activity in the $^{-1327}$T-sequence compared the $^{-1327}$C-sequence. Although the finding with such a modest effect, the data were so strong statistically significant. This significance was caused by the small range of the standard deviations, and possible reasons of the very little inter-assay are as follows; we used a dual-luciferase assay system for standardization of transfection efficiency and early passage HUVECs (passage number, 5–7) to avoid the influence of any transformation by long-term culture on this assay. Particularly, long-term culture of HUVECs showed cell senescence [18]. Although HUVECs have slight activity of telomerase [19], telomerase activity in senescent HUVECs is not fully understood. These suggest that long-term culture of HUVECs is not adapted to evaluate hTERT promoter assay. Thus, we used the present assay system that telomerase promoter with promoter gene works under transient condition using early passage HUVECs although it is important to examine the promoter assay under permanent condition in HUVEC. As a result of careful assay design, we found the relationship of the $^{-1327}$T/C polymorphism on hTERT transcription activity in HUVECs.

We measured leukocyte DNA TRF length, but not that of endothelial cells, because telomere length in both leukocytes and endothelial calls is inversely correlated with age (average decline 30–40 bp/year in normal leukocytes) [12,13,20–22], and leukocyte DNA was available for this study. Also, endothelial cells and leukocytes are exposed to the same hemodynamic stress, thus the rate of turnover is considered to correlate between these cells [21]. The $^{-1327}$T-sequence was strongly associated with longer telomere length. We postulated that $^{-1327}$T-sequence with higher hTERT transcriptional activity is associated with more effective extension of the telomeric end during cell division, and our results reveal a possible causative role of the $^{-1327}$T/C polymorphism in inter-individual variations in leukocyte telomere length.

In conclusion, we report a potential role of the $^{-1327}$T/C polymorphism within the hTERT promoter region in the hTERT promoter activity and leukocyte telomere shortening among normal individuals.

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## References

[1] G.B. Morin, The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats, Cell 59 (1989) 521–529.

[2] A.O. Wilkie, J. Lamb, P.C. Harris, R.D. Finney, D.R. Higgs, A truncated human chromosome 16 associated with alpha thalassaemia is stabilized by addition of telomeric repeat (TTAGGG)n, Nature 346 (1990) 868–871.

[3] J.M. Wong, K. Collins, Telomere maintenance and disease, Lancet 362 (2003) 983–988.

[4] R.M. Cawthon, K.R. Smith, E. O'Brien, A. Sivatchenko, R.A. Kerber, Association between telomere length in blood and mortality in people aged 60 years or older, Lancet 361 (2003) 393–395.

[5] T.M. Nakamura, G.B. Morin, K.B. Chapman, S.L. Weinrich, W.H. Andrews, J. Lingner, C.B. Harley, T.R. Cech, Telomerase catalytic subunit homologs from fission yeast and human, Science 276 (1997) 955–959.

[6] A. Kilian, D.D. Bowtell, H.E. Abud, G.R. Hime, D.J. Venter, P.K. Keese, E.L. Duncan, R.R. Reddel, R.A. Jefferson, Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types, Hum. Mol. Genet. 6 (1997) 2011–2019.

[7] A.L. Ducrest, H. Szutorisz, J. Lingner, M. Nabholz, Regulation of the human telomerase reverse transcriptase gene, Oncogene 21 (2002) 541–552.

[8] M. Takakura, S. Kyo, T. Kanaya, H. Hirano, J. Takeda, M. Yutsudo, M. Inoue, Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells, Cancer Res. 59 (1999) 551–557.

[9] I. Horikawa, P.L. Cable, C. Afshari, J.C. Barrett, Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene, Cancer Res. 59 (1999) 826–830.

[10] E. Hiyama, K. Hiyama, T. Yokoyama, T. Ichikawa, Y. Matsuura, Length of telomeric repeats in neuroblastoma: correlation with prognosis and other biological characteristics, Jpn. J. Cancer Res. 83 (1992) 159–164.

[11] R.M. Cawton, Telomere measurement by quantitative PCR, Nucleic Acid Res. 30 (2002) e47.

[12] H. Iwama, K. Ohyashiki, J.H. Ohyashiki, S. Hayashi, N. Yahata, K. Ando, K. Toyama, A. Hoshika, M. Takasaki, M. Mori, J.W. Shay, Telomeric length and telomerase activity vary with age in peripheral blood cells obtained from normal individuals, Hum. Genet. 102 (1998) 397–402.

[13] A. Benetos, K. Okuda, M. Lajemi, M. Kimura, F. Thomas, J. Skurnick, C. Labat, K. Bean, A. Aviv, Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity, Hypertension 37 (2001) 381–385.

[14] H. Wege, M.S. Chui, H.T. Le, J.M. Tran, M.A. Zern, SYBR green real-time telomeric repeat amplification protocol for the rapid quantification of telomerase activity, Nucleic Acid Res. 31 (2003) e3.

[15] P.E. Slagboom, S. Droog, D.I. Boomsma, Genetic determination of telomere size in humans: a twin study of three age groups, Am. J. Hum. Genet. 55 (1994) 876–882.

[16] B.A. Kosciolek, P.T. Rowley, Human lymphocyte telomerase is genetically regulated, Genes Chromosomes Cancer 21 (1998) 124–130.

[17] Y.H. Hsu, J.J. Lin, Telomere and telomerase as targets for anti-cancer and regeneration therapies, Acta Pharmacol. Sin. 26 (2005) 513–518.

[18] R. Hastings, M. Qureshi, R. Verma, P.S. Lacy, B. Williams, Telomere attrition and accumulation of senescent cells in cultured human endothelial cells, Cell Prolif. 37 (2004) 317–324.

[19] S.M. Bode-Boger, J. Martens-Lobenhoffer, M. Tager, H. Schroder, F. Scalera, Aspirin reduces endothelial cell senescence, Biochem. Biophys. Res. Commun. 334 (2005) 1226–1232.

[20] M. Ogami, Y. Ikura, M. Ohsawa, T. Matsuo, S. Kayo, N. Yoshimi, E. Hai, N. Shirai, S. Ehara, R. Komatsu, T. Naruko, M. Ueda, Telomere shortening in human coronary artery diseases, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 546–550.

[21] E. Chang, C.B. Harley, Telomere length and replicative aging in human vascular tissues, Proc. Natl. Acad. Sci. USA 92 (1995) 11190–11194.

[22] H. Vaziri, F. Schachter, I. Uchida, L. Wei, X. Zhu, R. Effros, D. Cohen, C.B. Harley, Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes, Am. J. Hum. Genet. 52 (1993) 661–667.

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Short Communication

# G501C polymorphism of oxidized LDL receptor gene (OLR1) and ischemic stroke

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ABSTRACT

The human lectin-like oxidized low-density lipoprotein receptor 1 (OLR1/LOX-1) is the major endothelial scavenger receptor against oxidized low-density lipoprotein (Ox-LDL), which has been implicated in the pathogenesis of atherosclerosis. We investigated the G501C mutation in the OLR1 gene in 235 Japanese patients with ischemic cerebrovascular disease (CVD) and 274 age- and sex-matched healthy controls using single nucleotide primer extension analysis (SNuPe). There was no significant difference in the polymorphism between patients with ischemic CVD and controls (GC+CC versus GG, $p=0.48$). The C allele was not significantly different between the patients and controls (C versus G, $p=0.91$). Our results show that the OLR1 gene polymorphism has little effect on an increased risk for ischemic CVD in the Japanese population.

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Human lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1), encoded by the OLR1 gene, was identified as a cell-surface endocytosis receptor for oxidized low-density lipoprotein (Ox-LDL) on vascular endothelial cells. In the physiological state, LOX-1 expression is seen *in vivo* in vascular rich organs, such as placenta, lungs, brain, and liver, and *in vitro* in normal aortic endothelial cells (Sawamura et al., 1997). On the other hand, Ox-LDL and inflammatory cytokines can upregulate the expression of LOX-1 and induce the endothelial expression of leukocyte adhesion molecules and smooth muscle growth factors, which are involved in atherosclerosis (Kume and Gimbrone, 1994). LOX-1 on the endothelium mediates the auto-activation of platelets, the platelet–endothelium interaction, and the release of endothelin-1 from endothelial cells that introduces endothelial dysfunction (Kakutani et al., 2000).

A single nucleotide polymorphism G501C of the OLR1 gene that results in an amino acid dimorphism (Lys/Asn) at residue 167 in LOX-1 protein was found in patients with ischemic heart disease from a single family, and G501C+C501C genotype increased the risk of myocardial infarction or the severity of coronary artery disease significantly (Tatsuguchi et al., 2003; Ohmori et al., 2004).

Interestingly, a recent study demonstrated that acetylsalicylic acid (aspirin), which could prevent ischemic stroke, inhibited Ox-LDL-mediated LOX-1 expression and metalloproteinase-1 in human coronary endothelial cells (Mehta et al., 2004). The expression of LOX-1 was greater than tenfold

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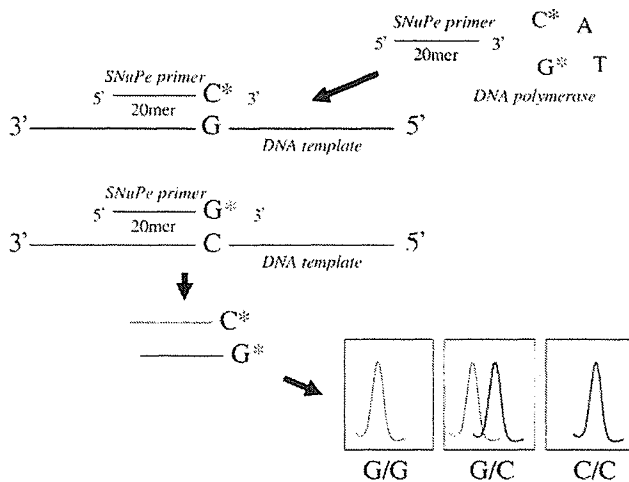


**Fig. 1 – The principal of SNuPe genotyping. The constructed SNuPe primers anneal the amplified DNA templates in front of 501 sites. Subsequently, the different fluorescent dyes labeled ddGTP or ddCTP react with the target SNP site and stop the annealing on and after 501 sites. Using a scanner, we can genotype the G501C single nucleotide polymorphism.**

at a transient ischemic core site compared with the non-ischemic side in the rat middle cerebral artery occlusion model (Schwarz et al., 2002). These data suggest that LOX-1 expression induces atherosclerosis in the brain and is the precipitating cause of ischemic stroke. Although several studies have shown the positive relationship between A501C polymorphism of OLR1 gene and atherosclerotic heart disease, the association between the polymorphism and ischemic stroke remains unclear. Using the SNuPe assay (Greenwood and Burke, 1996), we examined whether the OLR1 single nucleotide polymorphism is associated with ischemic cerebrovascular disease in the Japanese population.

We analyzed 235 unrelated Japanese patients with ischemic cerebrovascular disease (CVD) and 274 age- and sex-matched controls. All patients with CVD had attended the outpatient clinic of Keio University Hospital, Tokyo, for regular follow-up examinations. All controls worked for Keio University and had visited Keio University Hospital for their annual health examinations. We selected patients with CVD who were ≤70 years of age at the onset of CVD. On the basis of the Classification of Cerebrovascular Diseases III report from the committee established by the National Institute of Neurological Disorders and Stroke, patients with CVD who were given a diagnosis of atherothrombotic infarction (AT), lacunar infarction, or transient ischemic attack (TIA) were enrolled in this study. Those with cardioembolic cerebral infarction or cerebral hemorrhage were excluded. Those with a clinical history of cerebrovascular disease, myocardial infarction, or peripheral vascular disease were excluded. Written informed consent was obtained from all subjects after a full explanation of the study and a guarantee of total confidentiality. Brain computed tomography (CT) and/or magnetic resonance imaging (MRI) studies were performed on all patients with CVD. Hypertension, hypercholesterolemia, and smokers were defined as described previously (Ito et al., 2000).

Whole blood was collected into tubes containing sodium citrate. After genomic DNA was extracted, polymerase chain reaction (PCR) was carried out in a 25-μl volume containing 1.5 μl of 25 mM $MgCl_2$, 2 μl of 2 mM dNTP, 2.5 μl of 10× PCR buffer, 0.2 μl of each primer, 0.125 μl of Taq Gold, 17.475 μl of distilled water, and 1 μl of the extracted DNA. Amplification of a 441-bp fragment of the OLR1 gene was performed with the 5′ primer 5′-CTGGAGGGACAGATCTCAGC-3′ and 3′ primer 5′-TAAGTGGGGCATCAAAGGAG-3′. Amplification by PCR consisted of an initial 5 min of denaturation at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 61 °C, and 30 s at 72 °C, followed by 7 min at 72 °C in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) or a PLT-225 DNA Engine Tetrad (MJ Research, Waltham, MA, USA).

To analyze the G501C polymorphism of the OLR1 gene, a commercial MegaBACE SNuPe Genotyping Kit (Amersham Biosciences, Piscataway, NJ, USA) was used (Fig. 1). The 10-μl SNuPe reaction volume contained 1 μl purified PCR product, 4 μl SNuPe Premix, and 2 pmol SNuPe primer. The HPLC-purified SNuPe primer 5′-GGCTCATTTAACTGGGAAAA-3′ was constructed. The SNuPe reaction consisted of 25 cycles of 10 s at 94 °C, 5 s at 58 °C, and 10 s at 60 °C, and the sequence was completed by the MegaBACE 1000 DNA sequencing system (Amersham Biosciences, Piscataway, NJ, USA).

The differences in genotype frequencies and other risk factors were analyzed by the $\chi^2$ test. The mean age and allele frequencies in the two groups were compared by Student's t-test. Multiple logistic regression methods were used to control for possible confounding factors. All statistical analyses were performed using Statview software (Ver. 5.0 for Windows, SAS Institute, CA, USA).

The 235 patients with CVD and 274 controls were well matched in terms of age and sex (Table 1). The risk factors hypertension, hypercholesterolemia, diabetes mellitus, and smoking were significantly more common in patients with CVD than in controls ($P<0.001$). The frequencies of the G501C polymorphism showed no deviation from Hardy–Weinberg equilibrium.

The frequencies of GC+CC genotype carrier were not statistically different between all patients with CVD and

**Table 1 – Clinical characteristics of patients with CVD and controls**

| | Controls ($n$=274) | Patients with CVD ($n$=235) | P [a] |
|---|---|---|---|
| Male, % | 71.9 | 77.9 | NS [b] |
| Age, mean±SD, y | 59.1±3.4 | 58.3±7.8 | NS [b] |
| Hypertension, % | 25.3 | 56.2 | <0.001 |
| Hypercholesterolemia, % | 15.1 | 38.6 | <0.001 |
| Diabetes mellitus, % | 5.53 | 25.8 | <0.001 |
| Smoking, % | 25.6 | 53.3 | <0.001 |
| Body % mass index >27.3 kg/m$^2$, % | 5.68 | 9.02 | 0.062 |
| Family history, % | 18.5 | 29.3 | 0.012 |

[a] $\chi^2$ tests were used to compare values of patients with CVD and controls for all parameters except for age, which was compared by Student's t-test.
[b] Not significant.

**Table 2 – Genotype and allele frequencies of the G501C polymorphism of the OLR1 gene in patients with CVD and controls**

| Genotype | Patients with CVD ($n$=235) | AT ($n$=69) | Lacunar ($n$=142) | TIA ($n$=24) | Controls ($n$=274) |
|---|---|---|---|---|---|
| GC+CC, % | 39.1 (92) | 44.9 (31) | 37.3 (53) | 33.3 (8) | 36.1 (99) |
| GG, % | 60.9 (143) | 55.1 (38) | 62.7 (89) | 66.7 (16) | 63.9 (175) |
| P[a] | 0.484 | 0.182 | 0.811 | 0.783 | – |
| C, % | 21.1 | 24.6 | 20.1 | 16.7 | 21.4 |
| G, % | 78.9 | 75.4 | 79.9 | 83.3 | 78.6 |
| P[a] | 0.911 | 0.410 | 0.666 | 0.433 | – |

The number of subjects (X).
[a] $\chi^2$ tests were used to compare genotype and allele frequencies between controls and all patients with CVD and between controls and individual groups of patients with CVD.

controls: 39.1% versus 36.1%, odds ratio (OR)=1.14 [95% confidence interval (CI)=0.98 to 2.00, $p$=0.484]. The C allele frequencies were not significantly different between all patients with CVD and controls: 21.1% versus 21.4%, OR=0.98 (95% CI=0.73 to 1.33, $p$=0.911) (Table 2). As seen in Table 2, a stepwise increase in the percentage of patients with CVD and GC+CC genotype was found depending on the severity of CVD: 33.3% in TIA, 37.3% in lacunar infarction, and 44.9% in AT. However, we failed to show a statistical difference between these subtypes of CVD.

In logistic regression analysis, sex, age, hypertension, hypercholesterolemia, diabetes mellitus, current smoking, body mass index, family history of stroke, and OLR1 genotypes (GC+CC vs. GG or C allele vs. G allele) were included as independent variables. This analysis revealed that the presence of the C allele was independent of these acquired risk factors.

The present study examined the relation between ischemic CVD and the G501C single nucleotide polymorphism (SNP) on exon 4 of the OLR1 gene and did not show the statistical influence of the SNP. As shown in Table 3, there are three other studies regarding the relation between acute myocardial infarction (AMI) and the polymorphism, but two of the three failed to show any relation. In detail, Ohmori et al. identified the significant stepwise decrease in the percentage of patients with GC+CC genotype depending on the severity of coronary artery disease (Ohmori et al., 2004), but the other group showed a significant association between GG genotype and the severity of coronary artery disease when the patients having three obstructed vessels were compared to those with one or two obstructed vessels (Trabetti et al., 2006). In addition, we found a stepwise increase in the percentage of patients with CVD and GC+CC genotype depending on the severity of CVD, but it was not significant. Based on these findings, we suppose that the association between atherosclerotic diseases and the genotype seems weak because we found the paradoxical results associated with disease severity in both studies.

Several reasons may explain why the present study failed to show an association between stroke and G501C genotype. First, the G501C genotype and other polymorphisms on intron 4, intron 5, and 3′UTR of the OLR1 gene were studied by two other groups of researchers. They showed that there was a significant association between the 3′UTR/T allele carrier (TC+TT genotypes) and acute myocardial infarction or coronary artery disease severity (Mango et al., 2003; Chen et al., 2003). Although the G501C genotype of the OLR1 gene, which resulted in the missense mutation of K167N in the extracellular ligand-binding domain of the LOX-1 protein, may affect the binding affinity between Ox-LDL and LOX-1, the effect of these SNPs in the untranslated portion remains unclear. However, Mango et al. suggested that SNPs in the untranslated portion may affect the alternative splicing of the OLR1 gene, which could express not only LOX-1 but also LOXIN, which lacked exon 5. They also reported that LOXIN blocked the apoptosis of endothelial cells, smooth muscle cells, and macrophages associated with LOX-1 activation (Mango et al., 2005). In a comparative expression analysis between the wild-type and mutant OLR-1 in intron 4, intron 5, and 3′UTR, LOXIN expression in the mutant was higher than that in the wild-type plasmid. Taken together, they suggested that alteration of LOXIN expression in untranslated SNPs is directly associated with susceptibility to AMI. If these SNPs are also important to the susceptibility to ischemic stroke, LOX-1 activation without Ox-LDL binding is more important than the Ox-LDL scavenger function of LOX-1. Thus, G501C may not have such a strong effect on the susceptibility to stroke.

Second, the mechanism of the relation between LOX-1 and ischemic stroke was proposed to be based on oxidative stress (Gorelick, 2001). Primarily, angiotensin II stimulates oxidation of LDL and expression of LOX-1 in pathogenesis of ischemic stroke, and the binding of Ox-LDL to LOX-1 increased production of superoxide, which inactivated nitric oxide in a chemical reaction (Cominacini et al., 2001). Subsequently, this reduced nitric oxide has been linked to impaired endothelium-dependent relaxation and the progression of atherosclerosis. If that is the main reason of atherosclerotic progression in patients with ischemic CVD, the GC+CC genotype has adverse effects against atherosclerosis, which means that

**Table 3 – Association results between OLR1 gene polymorphism and atherosclerotic diseases in the previous studies and the present study**

| The first authors | Sample size[a] | Risk genotype | Odds ratio (95% CI) | P-value | Associated phenotype (results) |
|---|---|---|---|---|---|
| Tatsuguchi et al., 2003 | 102 vs. 102 | G501C+C501C | 2.89 (1.51–5.53) | <0.002 | Acute myocardial infarction (associated) |
| Ohmori et al., 2004 | 171 vs. 248 | G501C+C501C | 34 vs. 37%[a] | Not significant | Acute myocardial infarction (failed) |
| Trabetti et al., 2006 | 190 vs. 160 | G501C+C501C | 0.97 (0.55–1.68) | 0.88 | Acute myocardial infarction (failed) |
| Present study | 235 vs. 275 | G501C+C501C | 1.14 (0.98–2.00) | 0.484 | Ischemic cerebrovascular disease (failed) |

[a] Patients vs. controls.

the genotype may decrease atherosclerotic vessels. That is another reason of our failure.

In conclusion, this study showed no association between the G501C polymorphism in the OLR-1 gene and ischemic cerebrovascular disease in the Japanese population. G501C may not influence the susceptibility to ischemic stroke. One reason is that LOX-1 activation without Ox-LDL binding may be more important than the scavenger function of LOX-1 for intracranial atherosclerosis. Another reason is that the interaction between Ox-LDL and LOX-1 may have an accelerative effect on endothelium dysfunction. Although recent studies indicate that LOX-1 is implicated in the mechanism of atherosclerosis and ischemic stroke, further studies are needed to evaluate whether the G501C genotype may change the binding affinity of LOX-1.

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## REFERENCES

Chen, Q., Reis, S.E., Kammerer, C., Craig, W.Y., LaPierre, S.E., Zimmer, E.L., McNamara, D.M., Pauly, D.F., Sharaf, B., Holubkov, R., Bairey Merz, C.N., Sopko, G., Bontempo, F., Kamboh, I., 2003. Genetic variation in lectin-like oxidized low-density lipoprotein receptor 1 (LOX1) gene and the risk of coronary artery disease. Circulation 107, 3146–3151.

Cominacini, L., Rigoni, A., Pasini, A.F., Garbin, U., Davoli, A., Campagnola, M., Pastorino, A.M., Cascio, V.L., Sawamura, T., 2001. The binding of oxidized low-density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide. J. Biol. Chem. 276, 13750–13755.

Gorelick, P.B., 2001. Stroke prevention therapy beyond antithrombotics: unifying mechanisms in ischemic stroke pathogenesis and implication for therapy: an invited review. Stroke 33, 862–875.

Greenwood, A.D., Burke, D.T., 1996. Single nucleotide primer extension: quantitative range, variability, and multiplex analysis. Genome Res. 6, 336–348.

Ito, D., Murata, M., Tanahashi, N., Sato, H., Sonoda, A., Saito, I., Watanabe, K., Fukuuchi, Y., 2000. Polymorphism in the promoter of lipopolysaccharide receptor CD14 and ischemic cerebrovascular disease. Stroke 31, 2661–2664.

Kakutani, M., Masaki, T., Sawamura, T., 2000. A platelet–endothelium interaction mediated by lectin-like oxidized low-density lipoprotein receptor-1. Proc. Natl. Acad. Sci. U. S. A. 97, 360–364.

Kume, N., Gimbrone Jr., M.A., 1994. Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. J. Clin. Invest. 93, 907–911.

Mango, R., Clementi, F., Borgiani, P., Forleo, G.B., Federici, M., Contino, G., Giardina, E., Garza, L., Fahdi, I.E., Lauro, R., Mehta, J.L., Novelli, G., Romeo, F., 2003. Association of single nucleotide polymorphisms in the oxidized LDL receptor 1 (OLR1) gene in patients with acute myocardial infarction. J. Med. Genet. 40, 933–936.

Mango, R., Biocca, S., del Vecchio, F., Clementi, F., Sangiuolo, F., Amati, F., Filareto, A., Grelli, S., Spitalieri, P., Filesi, I., Favalli, C., Lauro, R., Mehta, J.L., Romeo, F., Novelli, G., 2005. In vivo and in vitro studies support that a new splicing isoform of OLR1 gene is protective against acute myocardial infarction. Circ. Res. 97, 152–158.

Mehta, J.L., Chen, J., Yu, F., Li, D.Y., 2004. Aspirin inhibits ox-LDL-mediated LOX-1 expression and metalloproteinase-1 in human coronary endothelial cells. Cardiovasc. Res. 64, 243–249.

Ohmori, R., Momiyama, Y., Nagano, M., Taniguchi, H., Egashira, T., Yonemura, A., Nakamura, H., Kondo, K., Ohsuzu, F., 2004. An oxidized low-density lipoprotein receptor gene variant is inversely associated with the severity of coronary artery disease. Clin. Cardiol. 27, 641–644.

Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H., Hoshikawa, H., Aiba, Y., Tanaka, T., Miwa, S., Katsura, Y., Kita, T., Masaki, T., 1997. An endothelial receptor for oxidized low-density lipoprotein. Nature 386, 73–77.

Schwarz, D.A., Barry, G., Mackay, K.B., Manu, F., Naeve, G.S., Vana, A.M., Verge, G., Conlon, P.J., Foster, A.C., Maki, R.A., 2002. Identification of differentially expressed genes induced by transient ischemic stroke. Brain Res. Mol. Brain Res. 101, 12–22.

Tatsuguchi, M., Furutani, M., Hinagata, J., Tanaka, T., Furutani, Y., Imamura, S., Kawana, M., Masaki, T., Kasanuki, H., Sawamura, T., Matsuoka, R., 2003. Oxidized LDL receptor gene (OLR1) is associated with the risk of myocardial infarction. Biochem. Biophys. Res. Commun. 303, 247–250.

Trabetti, E., Biscuola, M., Cavallari, U., Malerba, G., Girelli, D., Olivieri, O., Martinelli, N., Corrocher, R., Pignatti, P.F., 2006. On the association of the oxidized LDL receptor 1 (OLR1) gene in patients with acute myocardial infarction or coronary artery disease. Eur. J. Hum. Genet. 14, 127–130.