6 months of age, and six (/25) were diagnosed within 6 years. As a whole, 25 of 43 (58%) USS patients were correctly diagnosed before they reached 15 years of age, including 12 females and 13 males, indicating that there is no gender disparity in diagnosing USS during childhood. These 25 patients would be unanimously considered to have the 'early-onset phenotype'. However, the remaining 18 USS patients were diagnosed after 15 years of age. This raises the question of whether these patients were the true 'late-onset phenotype' or not. One particularly interesting result was that 15 (/18) patients were diagnosed between 15 and 45 years of age, and interestingly they were all female. Furthermore, among these 15 female patients, nine were diagnosed in association with pregnancy. The remaining three patients (USS-H3, -BB3, and -GG2) were diagnosed after 45 years of age, and they were all male, which sharply contrasts the previous scenario. Thus, the natural history of these three male patients appeared to be an excellent means to analyse the pathogenesis of the 'late-onset phenotype'. Among these patients, USS-H3 with a p.A250V/c.330 + 1G > A genotype had an episode of thrombocytopenia, but there are few clinical details and the patient died of renal failure in 2002 [42], Thus, no further results on USS-H3 are available.

However, two other males, USS-BB3 and USS-GG2, had received annual health examinations during adulthood, and there were no apparent abnormalities until sudden and overt TTP developed at 55 and 63 years of age, respectively. This may indicate that the clinical signs of TTP were very mild during their childhood and adulthood, and any symptoms might have been attributed to isolated mild thrombocytopenia. Interestingly, these two elderly men carried two different homozygous *ADAMTS13* gene mutations, p.R193W/p.R193W and p.C1024R/p.C1024R, respectively. We previously reported that the p.R193W protein was present in the plasma of patient USS-Z3 [12,73]. In this study we also determined that the p.C1024R protein was present in the plasma of patient USS-GG2 (data not shown). Furthermore, *in vitro* expression studies using HeLa cells that were transfected with either of these two mutant gene plasmids showed that each protein was consistently secreted into the culture medium but had much reduced activity compared to the wild-type protein ([39] and unpublished data). Consistent with these observations, the ADAMTS13 activity of patient USS-GG2 was mildly reduced (2.4–3.4% of the normal) on three different occasions. As for the homozygous p.R193W/p.R193W mutation, we identified another female patient (USS-Z3) who was correctly diagnosed with USS at 27 years of age as a result of pregnancy-associated TTP at 25 years of age. Her past history was well recorded, and indicated that she had mild jaundice as a newborn and thus did not receive an exchange blood transfusion. However, she was diagnosed with ITP with isolated thrombocytopenia at 7 years of age. Taken together, these results indicate that the phenotype of the homozygous p.R193W/p.R193W mutation is mild. Therefore, patients carrying this mutation would presumably have mild thrombocytopenia during childhood, as shown in USS-Z3, unless they are exposed to strong stimuli such as a cytokine storm during influenza virus infection. However, after adolescence the gender disparity apparently determines the fate of these USS-patients. Pregnancy undoubtedly is a strong inducer of overt TTP in female USS-patients, although the pathogenesis is not fully elucidated. However, it is now well established that plasma VWF levels remarkably increase as gestation progresses, along with the appearance of UL-VWFMs, which are accompanied by reduced ADAMTS13 activity due to consumption, even in normal pregnant women [74,75]. Thus, in pregnant USS women, an enormous excess of the substrate (larger VWFM) relative to the ADAMTS13 enzyme is the most plausible pathogenic mechanism.

As a consequence, our studies here have re-confirmed that pregnancy, influenza infection, and DDAVP administration can be the strong triggers inducing overt TTP in USS-patients. Besides, now it is indicated that the aging, interferon therapy, and heavily drinking alcohol could be additional modifiers aggravating clinical signs of USS-patients.

Given that the p.R193W mutation is a frequent DCM for USS in Japan, male patients carrying this mutation might not exhibit clinical signs of thrombosis at a younger age. However, as they age, multi-factorial endogenous and exogenous causes mentioned above would facilitate thrombotic events, leading to brain infarctions and chronic renal failure as a result of microcirculation disturbances. We speculate that thrombotic events in the brain or kidney, which still have an unknown pathogenesis, might result from *ADAMTS13* gene abnormalities. Our examination of the natural history in this large cohort of USS-patients with *ADAMTS13* mutations may shed light on these important diseases. Thus, here we emphasise again an importance of the assay for ADAMTS13 activity as a routine test to make and/or exclude a diagnosis of USS, when physicians meet the patients with thrombocytopenia of unknown aetiology, not only in childhood but also in adulthood.

Acknowledgements

This work was supported in part by research grants from the Ministry of Health, Labor, and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science and Technology of Japan. Y. Fujimura dedicates this paper to the late Professors Theodore S. Zimmerman and Hiromu Fukui, who both encouraged me to be involved in the research on VWF and its clinical application.

Disclosure of Conflict of Interests

YF is a clinical advisory board for Baxter Bioscience.

References

1 Fujimura Y, Matsumoto M, Yagi H, Yoshioka A, Matsui T, Titani K. Von Willebrand factor-cleaving protease and Upshaw–Schulman syndrome. *Int J Hematol* 2002; **75**: 25–34.
2 Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *J Biol Chem* 2001; **276**: 41059–63.

3 Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, Yang AY, Siemieniak DR, Stark KR, Gruppo R, Sarode R, Shurin SB, Chandrasekaran V, Stabler SP, Sabio H, Bouhassira EE, Upshaw JD Jr, Ginsburg D, Tsai HM. Mutations in a member of the *ADAMTS* gene family cause thrombotic thrombocytopenic purpura. *Nature* 2001; **413**: 488–94.

4 Soejima K, Mimura N, Hirashima M, Maeda H, Hamamoto T, Nakagaki T, Nozaki C. A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? *J Biochem* 2001; **130**: 475–80.

5 Sadler JE. Von Willebrand factor, ADAMTS13, and thrombotic thrombocytopenic purpura. *Blood* 2008; **112**: 11–8.

6 Waters AM, Licht C. aHUS caused by complement dysregulation: new therapies on the horizon. *Pediatr Nephrol* 2011; **26**: 41–57.

7 George JN. Clinical practice. Thrombotic thrombocytopenic purpura. *N Engl J Med* 2006; **354**: 1927–35.

8 Veyradier A, Obert B, Haddad E, Cloarec S, Nivet H, Foulard M, Lesure F, Delattre P, Lakhdari M, Meyer D, Girma JP, Loirat C. Severe deficiency of the specific von Willebrand factor-cleaving protease (ADAMTS 13) activity in a subgroup of children with atypical hemolytic uremic syndrome. *J Pediatr* 2003; **142**: 310–7.

9 Remuzzi G, Galbusera M, Noris M, Canciani MT, Daina E, Bresin E, Contaretti S, Caprioli J, Gamba S, Ruggenenti P, Perico N, Mannucci PM. von Willebrand factor cleaving protease (ADAMTS13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Blood* 2002; **100**: 778–85.

10 Moake JL. Thrombotic microangiopathies. *N Engl J Med* 2002; **347**: 589–600.

11 Kinoshita S, Yoshioka A, Park YD, Ishizashi H, Konno M, Funato M, Matsui T, Titani K, Yagi H, Matsumoto M, Fujimura Y. Upshaw–Schulman syndrome revisited: a concept of congenital thrombotic thrombocytopenic purpura. *Int J Hematol* 2001; **74**: 101–8.

12 Fujimura Y, Matsumoto M, Kokame K, Isonishi A, Soejima K, Akiyama N, Tomiyama J, Natori K, Kuranishi Y, Imamura Y, Inoue N, Higasa S, Seike M, Kozuka T, Hara M, Wada H, Murata M, Ikeda Y, Miyata T, George JN. Pregnancy-induced thrombocytopenia and TTP, and the risk of fetal death, in Upshaw–Schulman syndrome: a series of 15 pregnancies in 9 genotyped patients. *Br J Haematol* 2009; **144**: 742–54.

13 Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood* 2001; **98**: 1662–6.

14 Gerritsen HE, Robles R, Lämmle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. *Blood* 2001; **98**: 1654–61.

15 Tsai HM. Pathophysiology of thrombotic thrombocytopenic purpura. *Int J Hematol* 2010; **91**: 1–19.

16 Fujimura Y, Matsumoto M. Registry of 919 patients with thrombotic microangiopathies across Japan: database of Nara Medical University during 1998–2008. *Intern Med* 2010; **49**: 7–15.

17 Dacie JV, Mollison PL, Richardson N, Selwyn JG, Shapiro L. Atypical congenital haemolytic anaemia. *Q J Med* 1953; **22**: 79–98.

18 Schulman I, Pierce M, Lukens A, Currimbhoy Z. Studies on thrombopoiesis. I. A factor in normal human plasma required for platelet production; chronic thrombocytopenia due to its deficiency. *Blood* 1960; **16**: 943–57.

19 Upshaw JD Jr. Congenital deficiency of a factor in normal plasma that reverses microangiopathic hemolysis and thrombocytopenia. *N Engl J Med* 1978; **298**: 1350–2.

20 Rennard S, Abe S. Decreased cold-insoluble globulin in congenital thrombocytopenia (Upshaw–Schulman syndrome). *N Engl J Med* 1979; **300**: 368.

21 Koizumi S, Miura M, Yamagami M, Horita N, Taniguchi N, Migita S. Upshaw–Schulman syndrome and fibronectin (cold insoluble globulin). *N Engl J Med* 1981; **305**: 1284–5.

22 Goodnough LT, Saito H, Ratnoff OD. Fibronectin levels in congenital thrombocytopenia: Schulman's syndrome. *N Engl J Med* 1982; **306**: 938–9.

23 Miura M, Koizumi S, Miyazaki H. Thrombopoietin in Upshaw–Schulman syndrome. *Blood* 1997; **89**: 4663–4.

24 Moake JL, Rudy CK, Troll JH, Weinstein MJ, Colannino NM, Azocar J, Seder RH, Hong SL, Deykin D. Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med* 1982; **307**: 1432–5.

25 Furlan M, Robles R, Solenthaler M, Wassmer M, Sandoz P, Lämmle B. Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. *Blood* 1997; **89**: 3097–103.

26 Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, Tamai H, Konno M, Kamide K, Kawano Y, Miyata T, Fujimura Y. Mutations and common polymorphisms in *ADAMTS13* gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci U S A* 2002; **99**: 11902–7.

27 Furlan M, Robles R, Lämmle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood* 1996; **87**: 4223–34.

28 Kato S, Matsumoto M, Matsuyama T, Isonishi A, Hiura H, Fujimura Y. Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity. *Transfusion* 2006; **46**: 1444–52.

29 Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol* 2005; **129**: 93–100.

30 Ferrari S, Mudde GC, Rieger M, Veyradier A, Kremer Hovinga JA, Scheiflinger F. IgG subclass distribution of anti-ADAMTS13 antibodies in patients with acquired thrombotic thrombocytopenic purpura. *J Thromb Haemost* 2009; **7**: 1703–10.

31 Kokame K, Kokubo Y, Miyata T. Polymorphisms and mutations of ADAMTS13 in Japanese population and estimation of the number of patients with Upshaw-Schulman syndrome. *J Thromb Haemost* (in press).

32 Plaimauer B, Fuhrmann J, Mohr G, Wernhart W, Bruno K, Ferrari S, Konetschny C, Antoine G, Rieger M, Scheiflinger F. Modulation of ADAMTS13 secretion and specific activity by a combination of common amino acid plymorphisms and a missense mutation. *Blood* 2006; **107**: 118–25.

33 Schettert IT, Pereira AC, Lopes NH, Hueb WA, Krieger JE. Association between ADAMTS13 polymorphisms and risk of cardiovascular events in chronic coronary disease. *Thromb Res* 2010; **125**: 61–6.

34 Ruan C, Dai L, Su J, Wang Z. The frequency of P475S polymorphism in von Willebrand factor-cleaving protease in the Chinese population and its relevance to arterial thrombotic disorders. *Thromb Haemost* 2004; **91**: 1257–8.

35 Bongers TN, De Maat MP, Dippel DW, Uitterlinden AG, Leebeek FW. Absence of Pro475Ser polymorphism in ADAMTS-13 in Caucasians. *J Thromb Haemost* 2005; **3**: 805.

36 Akiyama M, Kokame K, Miyata T. ADAMTS13 P475S polymorphism causes a lowered enzymatic activity and urea lability in vitro. *J Thromb Haemost* 2008; **6**: 1830–2.

37 Yagi H, Konno M, Kinoshita S, Matsumoto M, Ishizashi H, Matsui T, Titani K, Fujimura Y. Plasma of patients with Upshaw–Schulman syndrome, a congenital deficiency of von Willebrand factor-cleaving protease activity, enhances the aggregation of normal platelets under high shear stress. *Br J Haematol* 2001; **115**: 991–7.

38 Konno M, Yoshioka A, Takase T, Imai T. Partial clinical improvement in Upshaw–Schulman syndrome following prostacyclin infusion. *Acta Paediatr Jpn* 1995; **37**: 97–100.

39 Miura M, Koizumi S, Nakamura K, Ohno T, Tachinami T, Yamagami M, Taniguchi N, Kinoshita S, Abildgaard CF. Efficacy of several plasma components in a young boy with chronic thrombocytopenia and hemolytic anemia who responds repeatedly to normal plasma infusions. *Am J Hematol* 1984; **17**: 307–19.

40 Matsumoto M, Kokame K, Soejima K, Miura M, Hayashi S, Fujii Y, Iwai A, Ito E, Tsuji Y, Takeda-Shitaka M, Iwadate M, Umeyama H, Yagi H, Ishizashi H, Banno F, Nakagaki T, Miyata T, Fujimura Y. Molecular characterization of *ADAMTS13* gene mutations in Japanese patients with Upshaw–Schulman syndrome. *Blood* 2004; **103**: 1305–10.

41 Shinohara T, Miyamura S, Suzuki E, Kobayashi K. Congenital microangiopathic hemolytic anemia: report of a Japanese girl. *Eur J Pediatr* 1982; **138**: 191–3.

42 Uchida T, Wada H, Mizutani M, Iwashita M, Ishihara H, Shibano T, Suzuki M, Matsubara Y, Soejima K, Matsumoto M, Fujimura Y, Ikeda Y, Murata M. Identification of novel mutations in ADAMTS13 in an adult patient with congenital thrombotic thrombocytopenic purpura. *Blood* 2004; **104**: 2081–3.

43 Shibagaki Y, Matsumoto M, Kokame K, Ohba S, Miyata T, Fujimura Y, Fujita T. Novel compound heterozygote mutations (H234Q/R1206X) of the *ADAMTS13* gene in an adult patient with Upshaw–Schulman syndrome showing predominant episodes of repeated acute renal failure. *Nephrol Dial Transplant* 2006; **21**: 1289–92.

44 Kato R, Shinohara A, Sato J. ADAMTS13 deficiency, an important cause of thrombocytopenia during pregnancy. *Int J Obstet Anesth* 2009; **18**: 73–7.

45 Kokame K, Aoyama Y, Matsumoto M, Fujimura Y, Miyata T. Inherited and de novo mutations of ADAMTS13 in a patient with Upshaw–Schulman syndrome. *J Thromb Haemost* 2008; **6**: 213–5.

46 Saitoh H, Murakami H, Mori C. Upshaw–Schulman syndrome in two siblings. *Acta Paediatr Jpn* 1990; **32**: 373–6.

47 Hara T, Kitano A, Kajiwara T, Kondo T, Sakai K, Hamasaki Y. Factor VIII concentrate-responsive thrombocytopenia, hemolytic anemia, and nephropathy. Evidence that factor VIII: von Willebrand factor is involved in its pathogenesis. *Am J Pediatr Hematol Oncol* 1986; **8**: 324–8.

48 Sasahara Y, Kumaki S, Ohashi Y, Minegishi M, Kano H, Bessho F, Tsuchiya S. Deficient activity of von Willebrand factor-cleaving protease in patients with Upshaw–Schulman syndrome. *Int J Hematol* 2001; **74**: 109–14.

49 Kentouche K, Budde U, Furlan M, Scharfe V, Schneppenheim R, Zintl F. Remission of thrombotic thrombocytopenic purpura in a patient with compound heterozygous deficiency of von Willebrand factor-cleaving protease by infusion of solvent/detergent plasma. *Acta Paediatr* 2002; **91**: 1056–9.

50 Schneppenheim R, Budde U, Oyen F, Angerhaus D, Aumann V, Drewke E, Hassenpflug W, Haberle J, Kentouche K, Kohne E, Kurnik K, Mueller-Wiefel D, Obser T, Santer R, Sykora KW. von Willebrand factor cleaving protease and ADAMTS13 mutations in childhood TTP. *Blood* 2003; **101**: 1845–50.

51 Antoine G, Zimmermann K, Plaimauer B, Grillowitzer M, Studt JD, Lämmle B, Scheiflinger F. ADAMTS13 gene defects in two brothers with constitutional thrombotic thrombocytopenic purpura and normalization of von Willebrand factor-cleaving protease activity by recombinant human ADAMTS13. *Br J Haematol* 2003; **120**: 821–4.

52 Savasan S, Lee SK, Ginsburg D, Tsai HM. ADAMTS13 gene mutation in congenital thrombotic thrombocytopenic purpura with previously reported normal VWF cleaving protease activity. *Blood* 2003; **101**: 4449–51.

53 Assink K, Schiphorst R, Allford S, Karpman D, Etzioni A, Brichard B, van de Kar N, Monnens L, van den Heuvel L. Mutation analysis and clinical implications of von Willebrand factor-cleaving protease deficiency. *Kidney Int* 2003; **63**: 1995–9.

54 Pimanda JE, Maekawa A, Wind T, Paxton J, Chesterman CN, Hogg PJ. Congenital thrombotic thrombocytopenic purpura in association with a mutation in the second CUB domain of ADAMTS13. *Blood* 2004; **103**: 627–9.

55 Veyradier A, Lavergne JM, Ribba AS, Obert B, Loirat C, Meyer D, Girma JP. Ten candidate ADAMTS13 mutations in six French families with congenital thrombotic thrombocytopenic purpura (Upshaw–Schulman syndrome). *J Thromb Haemost* 2004; **2**: 424–9.

56 Licht C, Stapenhorst L, Simon T, Budde U, Schneppenheim R, Hoppe B. Two novel ADAMTS13 gene mutations in thrombotic thrombocytopenic purpura/hemolytic-uremic syndrome (TTP/HUS). *Kidney Int* 2004; **66**: 955–8.

57 Noris M, Bucchioni S, Galbusera M, Donadelli R, Bresin E, Castelletti F, Caprioli J, Brioschi S, Scheiflinger F, Remuzzi G. Complement factor H mutation in familial thrombotic thrombocytopenic purpura with ADAMTS13 deficiency and renal involvement. *J Am Soc Nephrol* 2005; **16**: 1177–83.

58 Tao Z, Anthony K, Peng Y, Choi H, Nolasco L, Rice L, Moake JL, Dong JF. Novel ADAMTS-13 mutations in an adult with delayed onset thrombotic thrombocytopenic purpura. *J Thromb Haemost* 2006; **4**: 1931–5.

59 Schneppenheim R, Kremer Hovinga JA, Becker T, Budde U, Karpman D, Brockhaus W, Hrachovinova I, Korczowski B, Oyen F, Rittich S, von Rosen J, Tjonnfjord GE, Pimanda JE, Wienker TF, Lämmle B. A common origin of the 4143insA ADAMTS13 mutation. *Thromb Haemost* 2006; **96**: 3–6.

60 Donadelli R, Banterla F, Galbusera M, Capoferri C, Bucchioni S, Gastoldi S, Nosari S, Monteferrante G, Ruggeri ZM, Bresin E, Scheiflinger F, Rossi E, Martinez C, Coppo R, Remuzzi G, Noris M. In-vitro and in-vivo consequences of mutations in the von Willebrand factor cleaving protease ADAMTS13 in thrombotic thrombocytopenic purpura. *Thromb Haemost* 2006; **96**: 454–64.

61 Peyvandi F, Lavoretano S, Palla R, Valsecchi C, Merati G, De Cristofaro R, Rossi E, Mannucci PM. Mechanisms of the interaction between two ADAMTS13 gene mutations leading to severe deficiency of enzymatic activity. *Hum Mutat* 2006; **27**: 330–6.

62 Manea M, Kristoffersson A, Tsai HM, Zhou W, Winqvist I, Oldaeus G, Billstrom R, Bjork P, Holmberg L, Karpman D. ADAMTS13 phenotype in plasma from normal individuals and patients with thrombotic thrombocytopenic purpura. *Eur J Pediatr* 2007; **166**: 249–57.

63 Hommais A, Rayes J, Houllier A, Obert B, Legendre P, Veyradier A, Girma JP, Ribba AS. Molecular characterization of four ADAMTS13 mutations responsible for congenital thrombotic thrombocytopenic purpura (Upshaw–Schulman syndrome). *Thromb Haemost* 2007; **98**: 593–9.

64 Meyer SC, Jeddi R, Meddeb B, Gouider E, Lammle B, Kremer Hovinga JA. A first case of congenital TTP on the African continent due to a new homozygous mutation in the catalytic domain of ADAMTS13. *Ann Hematol* 2008; **87**: 663–6.

65 Garagiola I, Valsecchi C, Lavoretano S, Oren H, Bohm M, Peyvandi F. Nonsense-mediated mRNA decay in the ADAMTS13 gene caused by a 29-nucleotide deletion. *Haematologica* 2008; **93**: 1678–85.

66 Cermakova Z, Hrdlikova R, Sulakova T, Koristka M, Kovarova P, Hrachovinova I. Thrombotic thrombocytopenic purpura: incidence of congenital form of disease in north Moravia (region Moravia-Silesia). *Prague Med Rep* 2009; **110**: 239–44.

67 Ma E, Li Y, Kwok J, Ling S, Pau P, Chau G. ADAMTS13 mutationmal analysis in Chinese patients wtih chronic relapsing thrombotic thrombocytopenic purpura. *HK J Paediatr* 2006; **11**: 22–7.

68 Park HW, Oh D, Kim N, Cho HY, Moon KC, Chae JH, Ahn HS, Choi Y, Cheong HI. Congenital thrombotic thrombocytopenic purpura associated with unilateral moyamoya disease. *Pediatr Nephrol* 2008; **23**: 1555–8.

69 Furlan M, Lämmle B. Aetiology and pathogenesis of thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome: the role of von Willebrand factor-cleaving protease. *Best Pract Res Clin Haematol* 2001; **14**: 437–54.

70 Bernardo A, Ball C, Nolasco L, Moake JF, Dong JF. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. *Blood* 2004; **104**: 100–6.

71 Motto DG, Chauhan AK, Zhu G, Homeister J, Lamb CB, Desch KC, Zhang W, Tsai HM, Wagner DD, Ginsburg D. Shigatoxin triggers thrombotic thrombocytopenic purpura in genetically susceptible ADAMTS13-deficient mice. *J Clin Invest* 2005; **115**: 2752–61.

72 Banno F, Kokame K, Okuda T, Honda S, Miyata S, Kato H, Tomiyama Y, Miyata T. Complete deficiency in ADAMTS13 is prothrombotic, but it alone is not sufficient to cause thrombotic thrombocytopenic purpura. *Blood* 2006; **107**: 3161–6.

73 Ishizashi H, Yagi H, Matsumoto M, Soejima K, Nakagaki T, Fujimura Y. Quantitative Western blot analysis of plasma ADAMTS13 antigen in patients with Upshaw–Schulman syndrome. *Thromb Res* 2007; **120**: 381–6.

74 Stirling Y, Woolf L, North WR, Seghatchian MJ, Meade TW. Haemostasis in normal pregnancy. *Thromb Haemost* 1984; **52**: 176–82.

75 Sánchez-Luceros A, Farías CE, Amaral MM, Kempfer AC, Votta R, Marchese C, Salviú MJ, Woods AI, Meschengieser SS, Lazzari MA. von Willebrand factor-cleaving protease (ADAMTS13) activity in normal non-pregnant women, pregnant and post-delivery women. *Thromb Haemost* 2004; **92**: 1320–6.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 29, pp. 26158–26165, July 22, 2011

NDRG4 Protein-deficient Mice Exhibit Spatial Learning Deficits and Vulnerabilities to Cerebral Ischemia*[S]

Received for publication, May 2, 2011, and in revised form, May 29, 2011 Published, JBC Papers in Press, June 2, 2011, DOI 10.1074/jbc.M111.256446

Hitomi Yamamoto[‡], Koichi Kokame[‡1], Tomohiko Okuda[‡], Yukako Nakajo[§], Hiroji Yanamoto[§2], and Toshiyuki Miyata[‡]

From the [‡]Department of Molecular Pathogenesis and [§]Laboratory of Neurology and Neurosurgery, National Cerebral and Cardiovascular Center, Suita, Osaka 565-8565, Japan

The N-myc downstream-regulated gene (NDRG) family consists of four related proteins, NDRG1-NDRG4, in mammals. We previously generated NDRG1-deficient mice that were unable to maintain myelin sheaths in peripheral nerves. This condition was consistent with human hereditary motor and sensory neuropathy, Charcot-Marie-Tooth disease type 4D, caused by a nonsense mutation of NDRG1. In contrast, the effects of genetic defects of the other NDRG members remain unknown. In this study, we focused on NDRG4, which is specifically expressed in the brain and heart. *In situ* mRNA hybridization on the brain revealed that NDRG4 was expressed in neurons of various areas. We generated NDRG4-deficient mice that were born normally with the expected Mendelian frequency. Immunochemical analysis demonstrated that the cortex of the NDRG4-deficient mice contained decreased levels of brain-derived neurotrophic factor (BDNF) and normal levels of glial cell line-derived neurotrophic factor, NGF, neurotrophin-3, and TGF-β1. Consistent with BDNF reduction, NDRG4-deficient mice had impaired spatial learning and memory but normal motor function in the Morris water maze test. When temporary focal ischemia of the brain was induced, the sizes of the infarct lesions were larger, and the neurological deficits were more severe in NDRG4-deficient mice compared with the control mice. These findings indicate that NDRG4 contributes to the maintenance of intracerebral BDNF levels within the normal range, which is necessary for the preservation of spatial learning and the resistance to neuronal cell death caused by ischemic stress.

N-myc downstream-regulated gene (NDRG)[3] family members NDRG1-NDRG4 are intracellular proteins, consist of 340–394 amino acid residues, and share 53–65% sequence identity with each other. Furthermore, accumulating evidence implicates their roles in development, cancer metastasis, and the immune system (1–5).

We originally identified RTP (NDRG1) as a homocysteine-responsive gene in human umbilical vein endothelial cells (6), which is also called DRG1, Cap43, Rit42, Ndr1, and PROXY-1. NDRG1 expression is induced by a number of conditions, such as DNA damage, hypoxia, and intracellular calcium ion elevation (4). Overexpression of NDRG1 suppresses the metastatic potency of some types of cancer cells (4) and enhances the degranulation of mast cells in response to various stimuli (7). A nonsense mutation of *NDRG1* causes hereditary motor and sensory neuropathy, Charcot-Marie-Tooth disease type 4D, which presents as distal muscle wasting and atrophy, foot and hand deformities, tendon areflexia, sensory loss, and deafness in afflicted individuals (8). We previously generated NDRG1-deficient mice and revealed the essential role of NDRG1 in the cytoplasm of Schwann cells for the maintenance of myelin sheaths in peripheral nerves (9). A frame shift deletion of *Ndrg1* in Greyhounds also causes polyneuropathy (10).

Similar to NDRG1, the expression of NDRG2 is induced by stress conditions such as hypoxia (1). NDRG2 expression is up-regulated in cortical pyramidal neurons, senile plaques, and the cellular process of dystrophic neurons in the Alzheimer's brain, whereas expression is decreased in the rat frontal cortex after antidepressant treatment and electroconvulsive therapy (11). NDRG2 also plays a role in aldosterone-mediated epithelial sodium channel function (12), dendritic cell differentiation (13), and insulin action (14). NDRG3, on the other hand, may play a role in spermatogenesis because it is found in the outer layers of the seminiferous epithelium (3). Overexpression of NDRG3 contributes to the angiogenesis of tumors via up-regulation of chemokines (3).

In contrast to other NDRG members, NDRG4 expression is detected specifically in the brain and heart (15). In the embryonic mouse heart, NDRG4 expression is down-regulated under severe ventricular hypoplasia caused by Tbx2 misexpression, implying that NDRG4 is involved in cell growth and proliferation (16). However, information on the physiological function of NDRG4 is lacking. In the mouse brain, NDRG4 is identified in the neuronal cytoplasm of the cerebrum and cerebellum (17). Down-regulation of NDRG4 in PC12 cells results in extending shorter neurites in response to NGF (18). Considering that NDRG4 expression is induced by treatment with homocysteine in rat aortic smooth muscle cells (19), we speculated that

* This work was supported by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

[1] To whom correspondence may be addressed: 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Fax: 81-6-6835-1176; E-mail: kame@ri.ncvc.go.jp.

[2] To whom correspondence may be addressed: 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Fax: 81-6-6835-6894; E-mail: hyanamot@res.ncvc.go.jp.

[3] The abbreviations used are: NDRG, N-myc downstream-regulated gene; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; NT-3, neurotrophin-3; GFAP, glial fibrillary acidic protein; MCAO, middle cerebral artery occlusion; rCBF, regional cerebral blood flow.

NDRG4 is stress-related and has a cell-protective role in neurological disorders and cerebrovascular disease. This was supported by our finding that NDRG4 mRNA expression is decreased in the brain of patients with Alzheimer's disease (15).

Neurotrophins such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), NGF, neurotrophin-3 (NT-3), and TGF-β1 are essential for the survival and homeostatic maintenance of central neurons (20). BDNF, especially, is a potent modulator of synaptic connectivity in the central nervous system, influencing synaptic structure and function. The reduced levels of BDNF in the entorhinal cortex or forebrain are associated with poor memory (21, 22). BDNF also has neuroprotective action in models of ischemia. Increased BDNF levels in the brain for an appropriate period prior to the ischemic insult increases the resistance of the brain against lethal stresses caused by severe ischemia (23, 24). In contrast, a deficiency in endogenous BDNF renders the brain more susceptible to ischemic injury (25) and more suppressive to infarct tolerance by the preconditioning of spreading depression (26).

In this study, we generated NDRG4-deficient mice to reveal the roles of NDRG4 in the brain. As a result, we found that under the condition of NDRG4 deficiency, mice showed impaired phenotypes in spatial learning and neuroprotection with decreased levels of BDNF.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-NDRG1 rabbit antiserum was raised against recombinant glutathione S-transferase-fusion protein of human NDRG1 (27). Anti-NDRG2, anti-NDRG3, and anti-NDRG4 rabbit antisera were raised against the synthetic peptides Q^{351}SSESGTLPSGPPGH^{365} for mouse NDRG2 (17), F^{343}SRSVTSNQSDGTQE^{357} for mouse NDRG3 (17), and C-N^{214}RPGTVPNAKTLR^{226}-$CONH_2$ for mouse NDRG4, respectively, which were conjugated with keyhole limpet hemocyanin. Polyclonal antibodies in the antisera were purified by antigen-immobilized affinity column chromatography. Anti-NeuN and anti-glial fibrillary acidic protein (GFAP) were purchased from Millipore and Dako, respectively.

Construction of the Targeting Vector—We previously isolated and characterized genomic clones carrying *NDRG4* (15). The NDRG4-B and NDRG4-B^{var} isoforms are the alternative splicing products, whereas the NDRG4-H isoform is produced by the alternative promoter usage. The initiating Met codons for NDRG4-B/B^{var} and NDRG4-H exist in exons 5 and 3, respectively. Exon 6 is common to all isoforms. The loxP-flanked pST-neoB cassette (28) was inserted within exon 6. The ~11-kb sequence was inserted into the diphtheria toxin A fragment cassette vector (29), and the DNA was linearized by SalI digestion for electroporation.

Generation of NDRG4-deficient Mice—R1 mouse embryonic stem cells (30) were electroporated with the targeting vector and selected in medium containing G418. Targeted clones were identified by Southern blotting using the Gene Images Random-prime system (GE Healthcare) with 5′- and 3′-external probes. These cells were injected into blastocysts to obtain chimeras, which were crossed with wild-type C57BL/6 mice (Japan SLC) for germ line transmission of the disrupted *Ndrg4* allele. The genotypes of the offspring were examined by PCR analysis of DNA isolated from ear biopsy using three primers; P1 (CATCTCTCCAAGAGCCAGAGTGT), P2 (AAGATGCAGCCACACTTACGATT), and P3 (AACAGTAACAGCTTCCCACATC). Heterozygous mice with the disrupted *Ndrg4* allele were backcrossed with wild-type C57BL/6 mice. The mouse experiments were approved by the Animal Care and Use Committee of the National Cerebral and Cardiovascular Center in Japan, and were performed in accordance with the institutional and national guidelines and regulations.

Western Blotting Analysis—Protein expression was analyzed by Western blotting as described previously (31). Briefly, organs perfused with PBS (10 mM sodium phosphate, 150 mM NaCl (pH 7.4)) were homogenized in SDS sample buffer (10 mM Tris-HCl, 2% SDS, 50 mM DTT, 2 mM EDTA, 0.02% bromphenol blue, 6% glycerol (pH 6.8)), boiled for 7 min, and subjected to SDS-PAGE. Proteins in the gels were transferred to an immunoblot PVDF membrane (Bio-Rad). Following a blocking step with 5% skim milk, the membrane was incubated with anti-NDRG4, anti-NDRG1, anti-NDRG2, or anti-NDRG3 and probed with HRP-labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories). The membrane was developed using Immobilon Western chemiluminescent HRP substrate (Millipore), and chemiluminescence was detected by a LAS-3000 image analyzer (GE Healthcare).

In Situ mRNA Hybridization—Digoxigenin-labeled riboprobes were prepared for nucleotide positions 1269–1777 (NDRG4-a) and 1811–2343 (NDRG4-b) of mouse NDRG4 (NM_145602). The paraffin-embedded brain sections (6 μm thick) were dewaxed, rehydrated, and treated with proteinase K (8 μg/ml) for 30 min at 37 °C. The sections were acetylated by 0.25% acetic anhydride and hybridized with the riboprobes (300 ng/ml) for 16 h at 60 °C. Following treatment with RNase A (50 μg/ml) for 30 min at 37 °C and 0.5% blocking reagent (Roche), the sections were incubated with anti-DIG alkaline phosphate conjugate (Roche). Colorimetric reactions were performed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate solution (Sigma), and then the sections were counterstained with Kernechtrot stain solution (Mutoh). Because NDRG4-a and NDRG4-b riboprobes exhibited quite similar performance, only the data from NDRG4-a riboprobes are shown.

Immunohistochemistry—Serial sections for *in situ* mRNA hybridization were deparaffinized, rehydrated, and boiled by microwave irradiation in 10 mM citrate buffer (pH 6.0). After incubation with 0.3% hydrogen peroxide, the sections were blocked, incubated with anti-NeuN, and stained using Histofine MOUSESTAIN Kit (Nichirei) and diaminobenzidine according to the manufacturer's instructions. Alternate sections were blocked with Protein Block Serum-Free (Dako) and the Avidin/Biotin blocking kit (Vector Laboratories), and then incubated with anti-GFAP. Following incubation with biotin-conjugated goat anti-rabbit Ig (Dako), the sections were treated with HRP-conjugated streptavidin (Nichirei) and stained with diaminobenzidine. The sections were counterstained with Mayer's hematoxylin (Mutoh).

Measurement of Neurotrophin Levels—Protein levels of BDNF, GDNF, NGF, NT-3, and TGF-β1 were measured as

described (32). The PBS-perfused cerebral cortex described above was excised from each mouse (7–18 weeks old) and homogenized. The protein levels were measured using a two-site sandwich ELISA, Emax Immunoassay System (Promega). The protein concentration in each sample was measured using a BCA protein assay kit (Thermo Scientific).

Morris Water Maze (MWM) Test—We conducted the MWM test (33) using modifications as described previously (32). In a 64 × 91 cm-sized pool of opaque water (from a non-toxic agent), a 10 × 10 cm-square-shaped platform was hidden at a fixed position 2 cm under the surface of the water. The temperature of the water was kept at 24–25 °C during the procedure. Each mouse (6–8 weeks old) performed four trials per day, over five consecutive days, without any prior or subsequent training. We defined a successful escape, *i.e.* standing on the platform, as a stop for more than 1 s with all limbs on the platform. The cut-off time in a trial was set at 300 s. Mice that failed to reach the platform in 300 s were removed from the water, and the time needed to escape to the platform (escape latency) became 300 s. In each trial, the escape latency, the total path length needed to navigate to the platform, and the maximum swimming speed were analyzed using a video-tracking system, Smart (Panlab).

Middle Cerebral Artery Occlusion (MCAO) Model—Temporary focal ischemia was induced using the three-vessel occlusion technique as described previously (34). Briefly, under halothane-inhalation anesthesia, the left middle cerebral artery of each mouse (8–19 weeks old) was cauterized at the lateral border of the olfactory tract, and bilateral common carotid arteries were clip-occluded for 15 min followed by reperfusion. After opening the skull and subsequent cauterization of the MCA, the wound for the surgical MCA obstruction was closed within 3 min to avoid hypothermic neuroprotection against reperfusion injury (35). The rectal temperature was regulated so that it stayed within the physiological range (36.5–37.5 °C) using a temperature controller (NS-TC10, Neuroscience) during the operation. The heart rate and mean blood pressure were monitored via the tail artery using indirect blood pressure meter BP-98AW (Softron).

Regional Cerebral Blood Flow (rCBF) in the Penumbra-like Peripheral Area—The rCBF was monitored using the laser-Doppler blood flowmetry meter TBF-LN1 (Unique Medical) (34). The measurement area was set in the penumbra-like peripheral area of the ischemic region at 2 mm caudal and 1 mm dorsal to the crossover point of the left middle cerebral artery and the lateral surface of the olfactory tract. The rCBF was measured just before (control), during, and after MCAO.

Cerebral Function—Twenty-four hours or 7 days after MCAO, neurological deficits were examined according to a published scoring scale, with some modifications (35). Balance in the body trunk while being lifted by the tail was graded according to the following criteria: 0, no deficit (no twisting of the body); 1, mild deficit (asymmetric twisting tendency of the body); and 2, severe deficit (repeated asymmetric twisting of the body). Motor function of the extremities while being lifted by the tail was graded as follows: 0, no deficit (symmetrical movement of the forelimbs); 1, mild deficit (intermittent asymmetrical flexion of the forelimbs); and 2, severe deficit (continuous asymmetrical flexion of the forelimbs). The neurological deficit score (from 0 to 4) comprises the sum of the grades of the balance in body trunk and motor function of extremities.

Measurement of Infarcted Volume—Mice were perfused transcardially with heparinized PBS at 24 h or 7 days after MCAO to wash out any blood components from the brain tissue, which visualizes intraluminal blood coagulation or thrombosis formation, if any. The brain was removed and cut from the frontal tip into 1-mm-thick slices. Viable tissue was stained red with 2% 2,3,5-triphenyltetrazolium chloride followed by fixation with 4% paraformaldehyde in PBS. The infarct and total hemispheric areas of each slice were measured by tracing the borders in a computer-assisted image-analysis system, WinROOF (Mitani). To assess the total infarct volume after MCAO, an edema index was calculated by dividing the total volume of the hemisphere ipsilateral to the MCAO by the volume of the contralateral hemisphere. The infarcted volume was adjusted by dividing the volume by the edema index. The value of edema index at 7 days after MCAO could be considered 1.00, as found in our previous study (35).

Statistical Analysis—Data are presented as the means ± S.D. We used unpaired Student's *t* tests for comparisons within each parameter. Probability values of < 0.05 were considered statistically significant.

RESULTS

Localization of NDRG4 in Mouse Brain—We performed *in situ* mRNA hybridization to investigate the cellular localization of NDRG4 in the adult mouse brain. NDRG4 mRNA was widely distributed in various parts of brain (Fig. 1, *A* and *B*), including the olfactory bulb, olfactory tuberculum, cerebral cortex, striatum, hippocampus, dentate gyrus, thalamus, hypothalamus, mesencephalon, cerebellum, pons, and medulla oblongata (supplemental Fig. S1). To identify the cell types that were positive for the NDRG4 riboprobe, we analyzed serial sections by *in situ* mRNA hybridization for NDRG4 (Fig. 1, *C* and *D*) in combination with immunostaining for NeuN, a marker of neurons (*E* and *F*) or GFAP, a marker of astrocytes (*G* and *H*). NDRG4 expression was mainly observed in NeuN-positive cells but not in GFAP-positive cells, indicating that NDRG4 was specifically expressed in neurons. This was consistent with our previous finding that NDRG4 protein was expressed in neurons of the cerebral cortex and Purkinje cells of the cerebellum (17).

Generation of NDRG4-deficient Mice—To elucidate the effects of NDRG4 deficiency in neuronal cells, we generated NDRG4 knockout mice using gene targeting strategies (Fig. 2*A*). The *Ndrg4* gene covers all NDRG4 protein isoforms, NDRG4-B, NDRG4-B^{var}, and NDRG4-H (15). The genomic DNA fragment encompassing exon 6, which is the most upstream common coding region of NDRG4 isoforms, was used to construct the targeting vector. The genotype was confirmed by genomic PCR analysis (Fig. 2*B*). The F1 mice with one *Ndrg4*-disrupted allele (*Ndrg4*$^{+/-}$) were backcrossed with wild-type C57BL/6 mice (*Ndrg4*$^{+/+}$). *Ndrg4*$^{+/-}$ mice were then crossed to generate the NDRG4-deficient mice (*Ndrg4*$^{-/-}$). *Ndrg4*$^{-/-}$ mice were born normally with the expected Mendelian distribution. The numbers of *Ndrg4*$^{+/+}$, *Ndrg4*$^{+/-}$, and *Ndrg4*$^{-/-}$ live births were 56, 115, and 51, respectively (p =

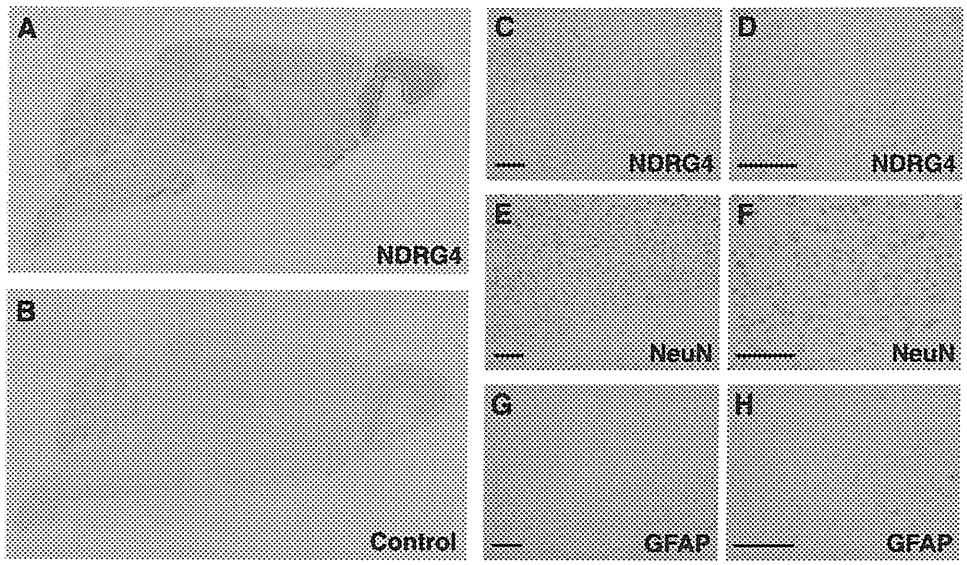

FIGURE 1. **Localization of NDRG4 in the mouse brain.** Sagittal sections were prepared from 8-week-old wild-type mice and subjected to *in situ* mRNA hybridization of NDRG4 (*A* and *B*). Digoxigenin-labeled antisense riboprobes for NDRG4 (*A*) but not sense riboprobes (*B*, negative control) produced positive signals (*blue*). The nuclei were counterstained in *red*. *C–H*, serial coronal sections of wild-type mouse brain were subjected to *in situ* mRNA hybridization of NDRG4 (*C* and *D*, *blue* signals) and immunostaining of NeuN (*E* and *F*, *brown* signals) or GFAP (*G* and *H*, *brown* signals). The nuclei were counterstained in *red* (*C* and *D*) and *blue* (*E–H*). *D*, *F*, and *H* are higher-magnification images of *C*, *E*, and *G*, respectively. *Scale bars* = 100 μm.

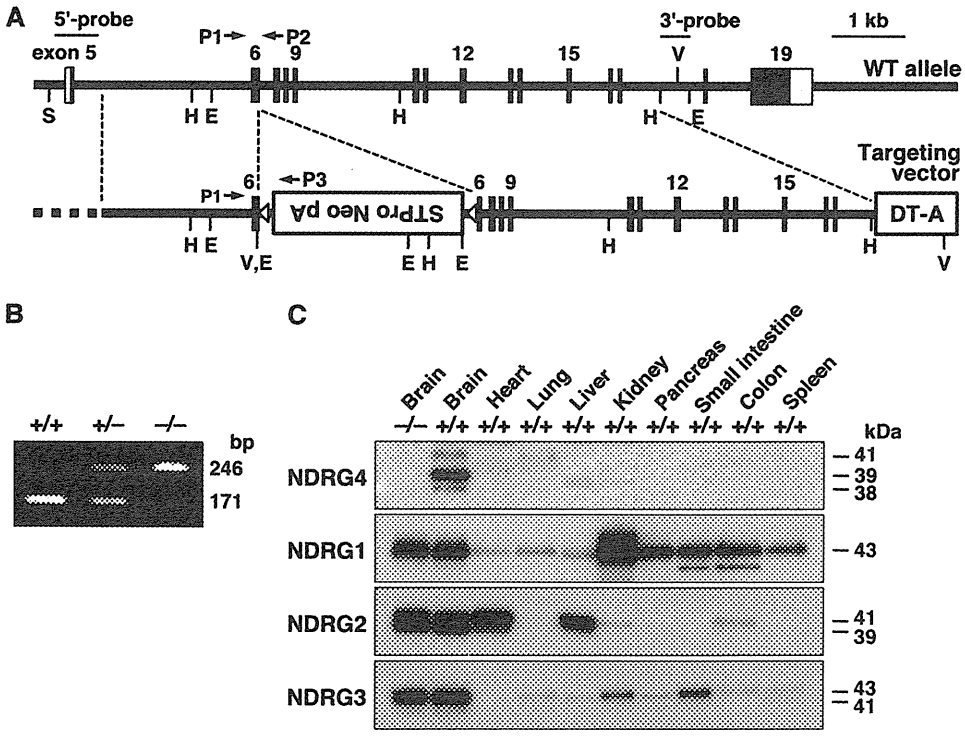

FIGURE 2. **Generation of NDRG4-deficient mice and expression pattern of NDRG family proteins in mouse organs.** *A*, targeting strategy for the *Ndrg4* gene knockout. *Solid boxes* represent open reading frames of *Ndrg4*. The loxP-flanked (*open triangles*) pSTneoB cassette with a polyadenylation signal (*STPro Neo pA*) was inserted into exon 6, and the diphtheria toxin A fragment cassette (*DT-A*) was included at the 3′ end of the vector. The 5′- and 3′-external probes used for Southern blotting selection of ES clones are shown by *bars*. The PCR primers (*P1*, *P2*, and *P3*) for genotyping are shown by *arrows*. *S*, SalI; *H*, HindIII; *E*, EcoRI; *V*, EcoRV. *B*, genotyping of wild-type (*Ndrg4*$^{+/+}$), heterozygous NDRG4-deficient (*Ndrg4*$^{+/-}$), and homozygous NDRG4-deficient (*Ndrg4*$^{-/-}$) mice. PCR amplification of the *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ alleles resulted in products of 171 and 246 bp, respectively. *C*, expression patterns of NDRG family proteins in mouse organs. Equal protein amount of organ homogenates from 17-week-old *Ndrg4*$^{-/-}$ and *Ndrg4*$^{+/+}$ mice were subjected to Western blotting analysis using each antibody. Anti-NDRG4 detected NDRG4-B (38 kDa), NDRG4-B^{var} (39 kDa), and NDRG4-H (41 kDa) in the *Ndrg4*$^{+/+}$ brain but not in the *Ndrg4*$^{-/-}$ brain. The expression of NDRG1, NDRG2, and NDRG3 was not affected by the lack of NDRG4 in the brain.

0.77, chi-square test). Both male and female *Ndrg4*$^{-/-}$ mice were fertile.

NDRGs Expression in Mouse Organs—To examine the expression patterns of NDRG family proteins in *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice, we performed a Western blotting analysis of their organs as adults. NDRG4 was specifically expressed in the brain, and little or no signal was detected in other tissues (Fig. 2*C*). The three isoforms, NDRG4-B (38 kDa), NDRG4-B^{var} (39

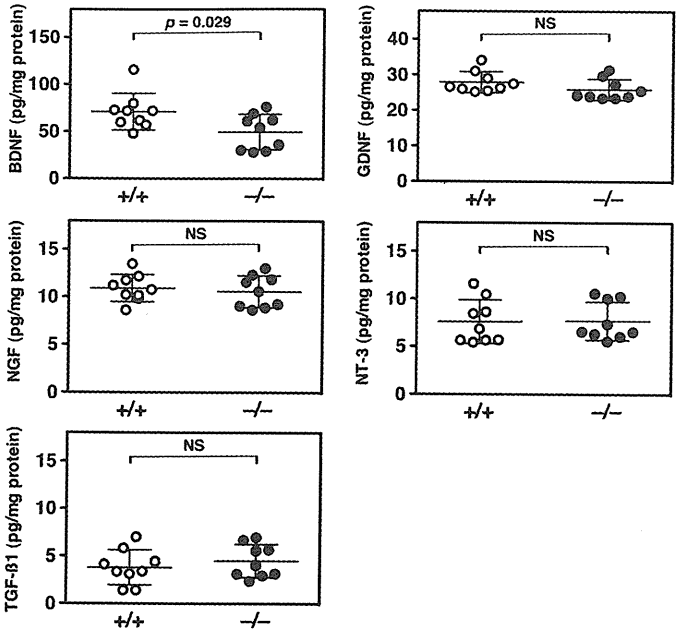

FIGURE 3. **Neurotrophins in the mouse cortex.** The protein levels of BDNF, GDNF, NGF, NT-3, and TGF-β1 in the cortex isolated from the *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice were measured by ELISA. Data are expressed as the mean ± S.D. ($n = 9$). *NS*, $p > 0.05$.

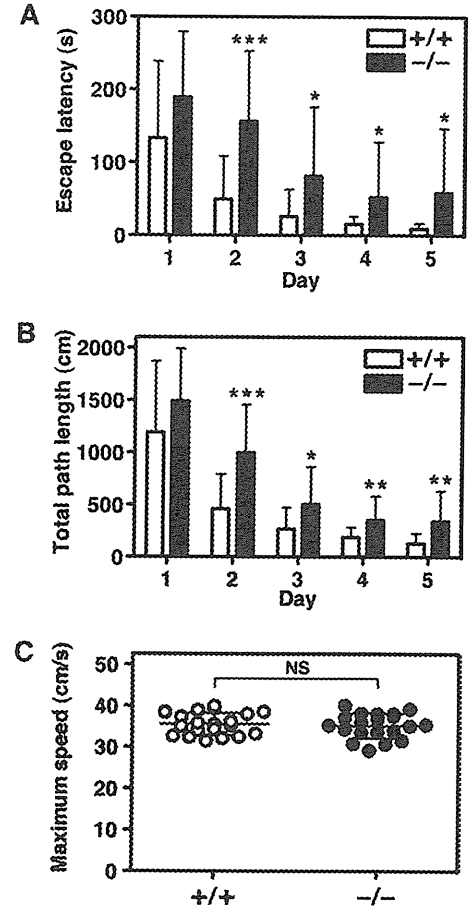

FIGURE 4. **MWM test.** Spatial learning and memory function of the *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice were tested in the MWM task. The escape latency (*A*), total path length (*B*), and maximum swimming speed (*C*) to the hidden platform on trials over five consecutive days are shown. The *Ndrg4*$^{-/-}$ mice exhibited inferior performance in escape latency and total path length in the MWM task as compared with *Ndrg4*$^{+/+}$ mice. However, the maximum swimming speed was equivalent between groups. Data are mean with error bars of S.D. ($n = 20$ in each experimental group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *NS*, $p > 0.05$.

kDa), and NDRG4-H (41 kDa), were detectable in the brain of *Ndrg4*$^{+/+}$ mice, whereas they were absent in the *Ndrg4*$^{-/-}$ brain. As described previously (15), NDRG1 was ubiquitously expressed in all tested organs. NDRG2 was mainly expressed in the brain, heart, and liver, with weaker expression in the kidney and colon. NDRG3 was observed in the brain, kidney, and small intestine. The expression levels of NDRG1, NDRG2, and NDRG3 in the brain were not affected by a lack of NDRG4, suggesting that there were no compensatory up-regulation mechanisms of gene expression.

Neurotrophin Levels—To investigate whether NDRG4 deficiency impacts brain function, we measured the protein levels of major neurotrophins in the brain (Fig. 3). The quantification of BDNF in the cortex homogenates revealed a significant decrease of BDNF in *Ndrg4*$^{-/-}$ (49.4 ± 18.8 pg/mg protein, $n = 9$) compared with *Ndrg4*$^{+/+}$ (71.1 ± 19.5 pg/mg protein, $n = 9$) mice. In contrast, the levels of GDNF, NGF, NT-3, and TGF-β1 in the cortex were not significantly different between the *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice (GDNF, 28.0 ± 3.0 *versus* 26.0 ± 3.0 pg/mg protein; NGF, 10.9 ± 1.4 *versus* 10.6 ± 1.7 pg/mg protein; NT-3, 7.6 ± 2.3 *versus* 7.7 ± 2.0 pg/mg protein; TGF-β1, 3.8 ± 1.8 *versus* 4.5 ± 1.7 pg/mg protein; $n = 9$). Therefore, we expected that abnormal regulation of BDNF protein levels may be involved in the development of the phenotypes of *Ndrg4*$^{-/-}$ mice.

Spatial Learning Ability—To confirm whether the lack of NDRG4 affects the ability of spatial learning and memory, we analyzed the performance of the mice in the MWM task. We found that escape latency to the hidden platform was significantly longer after the first trial for *Ndrg4*$^{-/-}$ mice compared with *Ndrg4*$^{+/+}$ mice (Fig. 4*A*). The total path length needed to navigate to the platform was also significantly longer in *Ndrg4*$^{-/-}$ than in *Ndrg4*$^{+/+}$ mice after the first trial (Fig. 4*B*). In contrast, there were no significant differences in the maximum swimming speed between *Ndrg4*$^{+/+}$ (35.5 ± 2.7 cm/s) and *Ndrg4*$^{-/-}$ (35.2 ± 3.0 cm/s) mice, indicating that *Ndrg4*$^{-/-}$ mice have normal sensorimotor function (Fig. 4*C*). These results indicated that poor performance of *Ndrg4*$^{-/-}$ mice in the MWM test was caused by the attenuation of spatial learning ability accompanied with BDNF reduction.

Neuronal Damage after Focal Ischemia—To elucidate whether NDRG4 is involved in the neuroprotective actions of BDNF, we explored the effect of NDRG4 deficiency on the development of neuronal damage after MCAO. We first performed transcardiac perfusion of PBS 24 h or 7 days after ischemia and confirmed that there was no thrombus formation except for the coagulated point in the proximal part of the middle cerebral artery in both *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice by visual inspection. A 2,3,5-triphenyltetrazolium chloride staining assay for viable cells at 24 h after a 15 min of MCAO demonstrated larger infarct lesion sizes in *Ndrg4*$^{-/-}$ mice compared with in *Ndrg4*$^{+/+}$ mice (Fig. 5, *A* and *B*). There were no differences in the edema index between the groups (1.07 ± 0.04 in *Ndrg4*$^{+/+}$ and 1.06 ± 0.03 in *Ndrg4*$^{-/-}$, $n = 10$). Corrobo-

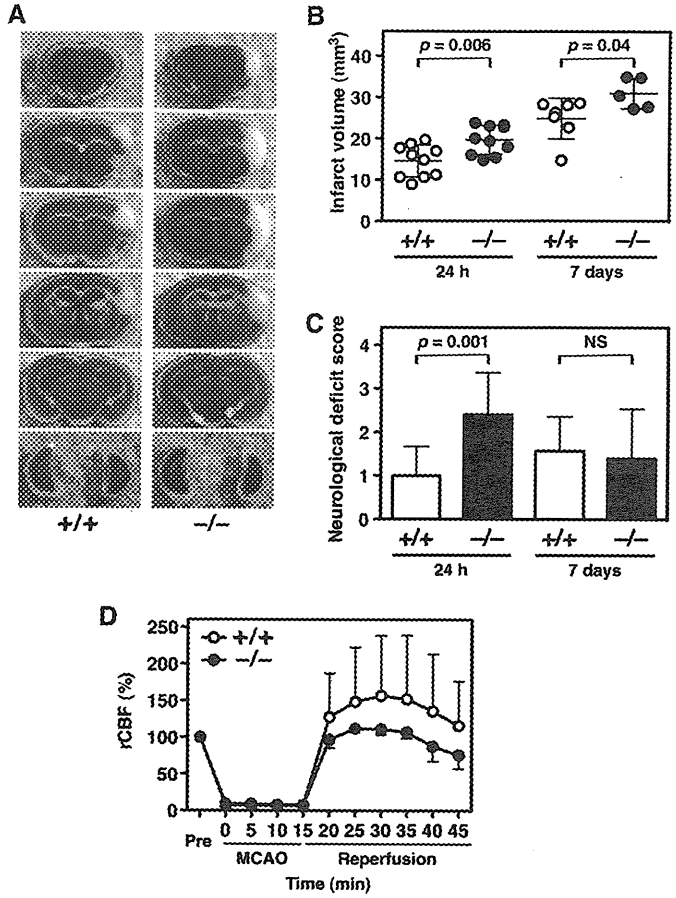

FIGURE 5. **Induction of temporary focal ischemia.** *A*, representative images of six corresponding coronal sections from *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mouse brains at 24 h after MCAO. The 1-mm-thick slices were stained with 2,3,5-triphenyltetrazolium chloride. The sizes of infarcted region (*white area*) were larger in the brain slices of *Ndrg4*$^{-/-}$ mice compared with *Ndrg4*$^{+/+}$ mice. *B*, quantification of infarct volumes (mm^3) at 24 h and 7 days after MCAO. *Ndrg4*$^{-/-}$ mice (●) had larger infarct volumes than *Ndrg4*$^{+/+}$ mice (○). Data are mean ± S.D. (n = 10). *C*, neurological deficit scored at 24 h and 7 days after MCAO. *Ndrg4*$^{-/-}$ mice had a severe neurological deficit score compared with *Ndrg4*$^{+/+}$ mice. Data are mean ± S.D. (n = 10). *D*, rCBF in the penumbra-like peripheral area of the ischemic legion. The rCBF was measured by laser-Doppler blood flowmetry system. The rCBFs were expressed as percentages of their preischemic normal values. During MCAO, rCBF was reduced to an equivalent level, and reperfusion was achieved in both groups, although the rCBF values of *Ndrg4*$^{-/-}$ mice were lower compared with *Ndrg4*$^{+/+}$ mice. Data are mean with error bars of S.D. in *Ndrg4*$^{+/+}$ (○, n = 7) and *Ndrg4*$^{-/-}$ mice (●, n = 5). The differences in rCBF between *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice at each point were not significant.

rating the histological results, MCAO-treated *Ndrg4*$^{-/-}$ mice showed more severe neurological deficits compared with *Ndrg4*$^{+/+}$ mice in the cerebral function scoring test (Fig. 5*C*). At 7 days after the 15-min MCAO, the infarction volumes were significantly larger in *Ndrg4*$^{-/-}$ mice compared with *Ndrg4*$^{+/+}$ mice, as seen in the acute phase study (Fig. 5*B*). However, differences in neurological deficit scores between *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice decreased at the end of the observation period (Fig. 5*C*).

Physiological measures of *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice, heart rate (24 h, 477 ± 100 *versus* 464 ± 95 beat/min, n = 10; 7 days, 497 ± 57 *versus* 483 ± 131 beat/min, n = 7 and 5, respectively) and mean blood pressure (24 h, 46 ± 8 *versus* 55 ± 13 mm Hg, n = 10; 7 days, 68 ± 10 *versus* 63 ± 25 mm Hg, n = 7 and 5, respectively) were not significantly different during ischemic treatment. These results indicate that NDRG4 is essential for the acquisition of normal resistance to the acute and chronic phase of cerebral ischemia through the retention of BDNF levels.

rCBF—We monitored the rCBF in the penumbra-like peripheral area of the ischemic legion to exclude the possibility that the larger infarct in the *Ndrg4*$^{-/-}$ brain was due to a decrease of rCBF during MCAO. Using laser-Doppler blood flowmetry, we found that rCBF values during the 15-min MCAO were equivalently reduced in *Ndrg4*$^{+/+}$ (8.4 ± 3.2%) and *Ndrg4*$^{-/-}$ (7.1 ± 2.0%) mice (Fig. 5*D*). After MCAO, perfusion was observed in both groups as expected, but the rCBF values were relatively lower in *Ndrg4*$^{-/-}$ than in *Ndrg4*$^{+/+}$ mice, which indicates that the sensitivity to ischemic stress is increased under a deficiency of NDRG4.

DISCUSSION

In this study, we revealed that NDRG4 was involved in the retaining of BDNF levels in the cortex. We also revealed that NDRG4-deficient mice showed cognitive deficits and impaired cerebral infarction tolerance. Although these phenomena in the brain seem to be physiologically distinct from each other, the abilities in learning/memory and neuroprotection are both appropriate indicators of biological activities involving BDNF (20).

BDNF participates in synaptic plasticity and memory processing in the adult brain (22). Indeed, mice that lack BDNF in their forebrain fail to learn the MWM task (21), whereas an increase in BDNF levels in the brain improves spatial learning and memory (22, 26). These observations are consistent with our findings that *Ndrg4*$^{-/-}$ mice have lower amounts of cortex BDNF than *Ndrg4*$^{+/+}$ and impaired spatial learning and memory function. Because BDNF also increases the survivability of neurons against ischemia, decreased levels of BDNF in the *Ndrg4*$^{-/-}$ cortex can explain the enlarged lesion sizes that appeared after the stress induced by temporary focal ischemia (23–26). BDNF-mediated production of prostacyclin (36) may be associated with the neuronal vulnerabilities of the *Ndrg4*$^{-/-}$ mice because prostacyclin has a potent neuroprotective effect against focal cerebral ischemia (37). Despite the decreased levels of BDNF in the cortex of *Ndrg4*$^{-/-}$ mice, the neurological deficits were recovered at 7 days after ischemia. It needs further investigations to clarify the mechanisms of neurologic recovery in *Ndrg4*$^{-/-}$ mice. Some signaling pathways mediated by BDNF receptors such as tropomyosin-related kinase B might be up-regulated by a sustained decrease of BDNF in *Ndrg4*$^{-/-}$ mice.

The expression of NDRG4 is decreased in the brains of patients with Alzheimer's disease (15), and BDNF expression is also decreased in the cortex of Alzheimer's patients (22), observations consistent with our current finding of decreased levels of BDNF in the *Ndrg4*$^{-/-}$ mouse brain. Therefore, it is likely that NDRG4 exists upstream of the BDNF production. A decrease of NDRG4 may cause neuronal vulnerability via an associated reduction of BDNF levels and thus may be a potential contributor or a risk factor in the pathogenesis of Alzheimer's disease.

Although the molecular mechanisms by which NDRG4 influences cerebral BDNF levels are unknown, NDRG4-mediated signaling pathways may play an essential role in BDNF synthesis and secretion. BDNF secretion is dependent on the activation of voltage-gated Na^+ channels and the subsequent of Ca^{2+} influx through voltage-gated N-type Ca^{2+} channels (38). In addition, BDNF release is involved in caffeine/ryanodine-sensitive Ca^{2+} release from intracellular stores. These findings support the idea that NDRG4 might regulate BDNF secretion via Ca^{2+} mobilization.

In contrast to the dysfunctional effects of NDRG4 deficiency on the central nervous system, NDRG1 deficiency results in peripheral nervous system defects. Although a brain magnetic resonance imaging study demonstrated subcortical white matter abnormalities in sibling patients with Charcot-Marie-Tooth disease type 4D (39), the lack of NDRG1 exhibited no adverse effects on higher brain functions (9) and on brain anatomy (17), suggesting that other NDRG members may compensate for the NDRG1 deficiency in the central nervous system. Similarly, the mild phenotypes of the *Ndrg4*$^{-/-}$ mice may be due to the compensatory action of the other NDRG members. Further analysis using double-knockout mice such as *Ndrg1*$^{-/-}$*Ndrg4*$^{-/-}$ may reveal the overlapping roles of the NDRG members.

Although the NDRG4 mRNA is abundantly expressed in the human brain and heart (15, 16), Western blotting analysis in the present study could only detect the NDRG4 protein isoforms in the brain but not in the heart of the wild-type mice. This was probably due to the extremely low levels of NDRG4 protein in the heart. This unexpected finding may be caused by the low translational efficiency or the instability of NDRG4 mRNA in the heart. However, recent reports implicate biological roles of NDRG4 in the heart. The knockdown of NDRG4 during embryonic development in zebrafish results in phenotypes such as a hypoplastic heart with pericardial edema, a dilated atrium, looping defects, reduced circulation, and a slower heart rate with weaker contraction (40). Severe ventricular hypoplasia down-regulates NDRG4 expression in the mouse embryonic heart (16). These reports indicate that NDRG4 is necessary for the normal regulation of myocardial proliferation and cardiac growth during early cardiogenesis. In addition, human chromosome 16q21 near *NDRG4* was identified as the locus that influences QT interval duration (41, 42). Although we currently do not find any histological and functional abnormalities for the *Ndrg4*$^{-/-}$ heart, more detailed studies may reveal the roles of NDRG4 on cardiac function.

In conclusion, we found that NDRG4 has an essential role in retaining normal spatial learning and memory, in protecting cerebral neurons against severe ischemic stress, and in maintaining BDNF levels in the brain within the normal range. Although the mechanisms by which NDRG4 influences intracerebral BDNF levels are yet unidentified, the decreased level of cortical BDNF may induce impairments in the central nervous system of *Ndrg4*$^{-/-}$ mice. Further investigation of *Ndrg4*$^{-/-}$ mice, including brain vasculature characterization and neurogenesis, may provide insight into effective therapies for some central nervous system diseases, including Alzheimer's disease and ischemic stroke.

Acknowledgment—We thank Dr. Yuka Eura for experimental materials.

REFERENCES

1. Yao, L., Zhang, J., and Liu, X. (2008) *Acta Biochim. Biophys. Sin.* **40,** 625–635
2. Schilling, S. H., Hjelmeland, A. B., Radiloff, D. R., Liu, I. M., Wakeman, T. P., Fielhauer, J. R., Foster, E. H., Lathia, J. D., Rich, J. N., Wang, X. F., and Datto, M. B. (2009) *J. Biol. Chem.* **284,** 25160–25169
3. Wang, W., Li, Y., Li, Y., Hong, A., Wang, J., Lin, B., and Li, R. (2009) *Int. J. Cancer* **124,** 521–530
4. Kitowska, A., and Pawelczyk, T. (2010) *Acta Biochim. Pol.* **57,** 15–21
5. Melotte, V., Qu, X., Ongenaert, M., van Criekinge, W., de Bruïne, A. P., Baldwin, H. S., and van Engeland, M. (2010) *FASEB J.* **24,** 4153–4166
6. Kokame, K., Kato, H., and Miyata, T. (1996) *J. Biol. Chem.* **271,** 29659–29665
7. Taketomi, Y., Sunaga, K., Tanaka, S., Nakamura, M., Arata, S., Okuda, T., Moon, T. C., Chang, H. W., Sugimoto, Y., Kokame, K., Miyata, T., Murakami, M., and Kudo, I. (2007) *J. Immunol.* **178,** 7042–7053
8. Kalaydjieva, L., Gresham, D., Gooding, R., Heather, L., Baas, F., de Jonge, R., Blechschmidt, K., Angelicheva, D., Chandler, D., Worsley, P., Rosenthal, A., King, R. H., and Thomas, P. K. (2000) *Am. J. Hum. Genet.* **67,** 47–58
9. Okuda, T., Higashi, Y., Kokame, K., Tanaka, C., Kondoh, H., and Miyata, T. (2004) *Mol. Cell. Biol.* **24,** 3949–3956
10. Drögemüller, C., Becker, D., Kessler, B., Kemter, E., Tetens, J., Jurina, K., Jäderlund, K. H., Flagstad, A., Perloski, M., Lindblad-Toh, K., and Matiasek, K. (2010) *PLoS ONE* **5,** e11258
11. Takahashi, K., Yamada, M., Ohata, H., Momose, K., Higuchi, T., Honda, K., and Yamada, M. (2005) *Int. J. Neuropsychopharmacol.* **8,** 381–389
12. Boulkroun, S., Fay, M., Zennaro, M. C., Escoubet, B., Jaisser, F., Blot-Chabaud, M., Farman, N., and Courtois-Coutry, N. (2002) *J. Biol. Chem.* **277,** 31506–31515
13. Choi, S. C., Kim, K. D., Kim, J. T., Kim, J. W., Yoon, D. Y., Choe, Y. K., Chang, Y. S., Paik, S. G., and Lim, J. S. (2003) *FEBS Lett.* **553,** 413–418
14. Burchfield, J. G., Lennard, A. J., Narasimhan, S., Hughes, W. E., Wasinger, V. C., Corthals, G. L., Okuda, T., Kondoh, H., Biden, T. J., and Schmitz-Peiffer, C. (2004) *J. Biol. Chem.* **279,** 18623–18632
15. Zhou, R. H., Kokame, K., Tsukamoto, Y., Yutani, C., Kato, H., and Miyata, T. (2001) *Genomics* **73,** 86–97
16. Dupays, L., Kotecha, S., Angst, B., and Mohun, T. J. (2009) *Dev. Biol.* **333,** 121–131
17. Okuda, T., Kokame, K., and Miyata, T. (2008) *J. Histochem. Cytochem.* **56,** 175–182
18. Ohki, T., Hongo, S., Nakada, N., Maeda, A., and Takeda, M. (2002) *Brain Res. Dev. Brain Res.* **135,** 55–63
19. Nishimoto, S., Tawara, J., Toyoda, H., Kitamura, K., and Komurasaki, T. (2003) *Eur. J. Biochem.* **270,** 2521–2531
20. Mattson, M. P. (2008) *Ann. N.Y. Acad. Sci.* **1144,** 97–112
21. Gorski, J. A., Balogh, S. A., Wehner, J. M., and Jones, K. R. (2003) *Neuroscience* **121,** 341–354
22. Nagahara, A. H., Merrill, D. A., Coppola, G., Tsukada, S., Schroeder, B. E., Shaked, G. M., Wang, L., Blesch, A., Kim, A., Conner, J. M., Rockenstein, E., Chao, M. V., Koo, E. H., Geschwind, D., Masliah, E., Chiba, A. A., and Tuszynski, M. H. (2009) *Nat. Med.* **15,** 331–337
23. Schäbitz, W. R., Schwab, S., Spranger, M., and Hacke, W. (1997) *J. Cereb. Blood Flow Metab.* **17,** 500–506
24. Yanamoto, H., Nagata, I., Sakata, M., Zhang, Z., Tohnai, N., Sakai, H., and Kikuchi, H. (2000) *Brain Res.* **859,** 240–248
25. Endres, M., Fan, G., Hirt, L., Fujii, M., Matsushita, K., Liu, X., Jaenisch, R., and Moskowitz, M. A. (2000) *J. Cereb. Blood Flow. Metab.* **20,** 139–144
26. Yanamoto, H., Xue, J. H., Miyamoto, S., Nagata, I., Nakano, Y., Murao, K., and Kikuchi, H. (2004) *Brain Res.* **1019,** 178–188
27. Agarwala, K. L., Kokame, K., Kato, H., and Miyata, T. (2000) *Biochem. Biophys. Res. Commun.* **272,** 641–647
28. Takagi, T., Moribe, H., Kondoh, H., and Higashi, Y. (1998) *Development*

125, 21–31
29. Yagi, T., Nada, S., Watanabe, N., Tamemoto, H., Kohmura, N., Ikawa, Y., and Aizawa, S. (1993) *Anal. Biochem.* **214,** 77–86
30. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 8424–8428
31. Kokame, K., Agarwala, K. L., Kato, H., and Miyata, T. (2000) *J. Biol. Chem.* **275,** 32846–32853
32. Nakajo, Y., Miyamoto, S., Nakano, Y., Xue, J. H., Hori, T., and Yanamoto, H. (2008) *Brain Res.* **1241,** 103–109
33. Morris, R. (1984) *J. Neurosci. Methods* **11,** 47–60
34. Yanamoto, H., Nagata, I., Niitsu, Y., Xue, J. H., Zhang, Z., and Kikuchi, H. (2003) *Exp. Neurol.* **182,** 261–274
35. Yanamoto, H., Nagata, I., Niitsu, Y., Zhang, Z., Xue, J. H., Sakai, N., and Kikuchi, H. (2001) *Stroke* **32,** 232–239
36. Santhanam, A. V., Smith, L. A., and Katusic, Z. S. (2010) *Stroke* **41,** 350–356
37. Lin, H., Lin, T. N., Cheung, W. M., Nian, G. M., Tseng, P. H., Chen, S. F., Chen, J. J., Shyue, S. K., Liou, J. Y., Wu, C. W., and Wu, K. K. (2002) *Circulation* **105,** 1962–1969
38. Kolarow, R., Brigadski, T., and Lessmann, V. (2007) *J. Neurosci.* **27,** 10350–10364
39. Echaniz-Laguna, A., Degos, B., Bonnet, C., Latour, P., Hamadouche, T., Lévy, N., and Leheup, B. (2007) *Neuromuscul. Disord.* **17,** 163–168
40. Qu, X., Jia, H., Garrity, D. M., Tompkins, K., Batts, L., Appel, B., Zhong, T. P., and Baldwin, H. S. (2008) *Dev. Biol.* **317,** 486–496
41. Newton-Cheh, C., Eijgelsheim, M., Rice, K. M., de Bakker, P. I., Yin, X., Estrada, K., Bis, J. C., Marciante, K., Rivadeneira, F., Noseworthy, P. A., Sotoodehnia, N., Smith, N. L., Rotter, J. I., Kors, J. A., Witteman, J. C., Hofman, A., Heckbert, S. R., O'Donnell, C. J., Uitterlinden, A. G., Psaty, B. M., Lumley, T., Larson, M. G., and Stricker, B. H. (2009) *Nat. Genet.* **41,** 399–406
42. Pfeufer, A., Sanna, S., Arking, D. E., Müller, M., Gateva, V., Fuchsberger, C., Ehret, G. B., Orrú, M., Pattaro, C., Köttgen, A., Perz, S., Usala, G., Barbalic, M., Li, M., Pütz, B., Scuteri, A., Prineas, R. J., Sinner, M. F., Gieger, C., Najjar, S. S., Kao, W. H., Mühleisen, T. W., Dei, M., Happle, C., Möhlenkamp, S., Crisponi, L., Erbel, R., Jöckel, K. H., Naitza, S., Steinbeck, G., Marroni, F., Hicks, A. A., Lakatta, E., Müller-Myhsok, B., Pramstaller, P. P., Wichmann, H. E., Schlessinger, D., Boerwinkle, E., Meitinger, T., Uda, M., Coresh, J., Kääb, S., Abecasis, G. R., and Chakravarti, A. (2009) *Nat. Genet.* **41,** 407–414

Thrombosis Research 128 (2011) 307–316

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Review Article

Pharmacogenomics of clopidogrel: Evidence and perspectives

Tong Yin [a], Toshiyuki Miyata [b,*]

[a] Institute of Geriatric Cardiology, General Hospital of People's Liberation Army, Beijing, China
[b] Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Osaka, Japan

ARTICLE INFO

Article history:
Received 20 January 2011
Received in revised form 13 April 2011
Accepted 15 April 2011
Available online 18 May 2011

Keywords:
Antiplatelet therapy
Clopidogrel
CYP2C19
Pharmacogenomics
PON1,$P2Y_{12}$ receptor

ABSTRACT

Clopidogrel has become the mainstay oral antiplatelet regimen to prevent recurrent ischemic events after acute coronary syndromes or stent placement. However, there is marked interindividual variability in the antiplatelet effects of clopidogrel, and a reduced response to this drug may be a risk factor for ischemic complications. Pharmacogenomic analyses, including candidate-gene and genome-wide association studies, have confirmed that genetic polymorphisms in the hepatic cytochrome P450 (CYP) 2C19 dominantly affect the antiplatelet effects of clopidogrel. *CYP2C19* reduced-function alleles have been associated with a significant decrease in clopidogrel responsiveness and a higher risk of adverse cardiac events including stent thrombosis, myocardial infarction, and death in several prospective studies, although these effects were not reproduced in a recent large randomized study that included a randomized control group. The US Food and Drug Administration addressed this issue by adding a boxed warning to the clopidogrel label and suggesting that adjusting the clopidogrel dose or using alternative antiplatelet agents should be potentially implemented for high-risk individuals who are identified based on the *CYP2C19* genotype. Although it is promising that *CYP2C19* genotyping could be used to guide personalized antiplatelet clopidogrel therapy, currently there is insufficient evidence to recommend routine genetic testing. Prospective randomized clinical trials are necessary to validate this pharmacogenomic approach to clopidogrel therapy. In the most recent trial, paraoxonase-1 (PON1) was identified as a crucial new enzyme for clopidogrel bioactivation, with its common Q192R polymorphism determining the rate of active metabolite and the clinical activity of clopidogrel. Further studies are needed to investigate the comprehensive influence of a number of different polymorphisms of *CYP2C19* and *PON1* variant alleles or other genetic variants on clopidogrel in various ethnic populations.

Contents

Abbreviations: CYP, cytochrome P450; paraoxonase-1, PON1; PCI, percutaneous coronary intervention; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; VASP, vasodilator-stimulated phosphoprotein; OR, odds ratio; CI, confidence interval; PPI, proton-pump inhibitors.

* Corresponding author at: Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 5658565, Japan. Tel.: +81 66833 5012; fax: +81 66835 1176.
E-mail address: miyata@ri.ncvc.go.jp (T. Miyata).

0049-3848/$ – see front matter
doi:10.1016/j.thromres.2011.04.010

Introduction

The antiplatelet drug clopidogrel is an important therapeutic agent that is used on top of aspirin to provide additive efficacy and overcome aspirin resistance in patients who are at risk for cardiovascular events [1]. Clopidogrel plus aspirin is recommended in the American College of Cardiology/American Heart Association guidelines and has become the standard of care for patients with acute coronary syndrome [2–5]. In combination with aspirin, clopidogrel is also the gold standard to prevent stent thrombosis in patients undergoing percutaneous coronary intervention (PCI) [2,6]. However, the antiplatelet effects of clopidogrel are not consistent in all patients [7–9]. Despite adequate antiplatelet therapy, up to 15% of high-risk patients with acute coronary syndrome continue to suffer from ischemic events, and up to 30% of the patient populations have marked interindividual variability in the extent of platelet inhibition [10]. Individual differences in the rate of platelet activation and reactivity markedly influence normal hemostasis and the pathological outcome of thrombosis. Clopidogrel resistance has been recognized in patients that exhibited less platelet aggregatory inhibition, and the prevalence of this resistance varied widely from 4% to 30%, with the value depending in part on the loading dose of clopidogrel and the methods used to assess non-responsiveness [11,12]. The inter-patient variability in the response to clopidogrel is multi-factorial and largely influenced by environmental, clinical and genetic factors. Among the environmental and clinical factors, age, smoking, diet, drug—drug interactions, diabetes, acute coronary syndrome, patient compliance, triglycerides, high-density lipoprotein cholesterol and body mass index may cause variability in the response to clopidogrel [13–16].

Clopidogrel is a prodrug and therefore must be metabolized before it can inhibit adenosine diphosphate (ADP)-induced platelet aggregation. The conversion of clopidogrel to its active metabolite is catalyzed by several different cytochromes P450s (CYPs) and an esterase paraoxonase-1 (PON1). Recent data demonstrated that individuals with *CYP2C19* and *PON1* gene variants have low active metabolite levels of clopidogrel [17–20]. Increasing evidence suggests that *CYP2C19* variants greatly influence the marked interindividual heterogeneity of the clopidogrel response and are linked to an increased risk of adverse cardiovascular outcomes, including stent thrombosis, myocardial infarction, and death [13,19,21–26]. Data are limited for the influence of *PON1* genotypes on the pharmacokinetics and pharmacodynamics of clopidogrel. Based on the vital pharmacogenomic information, personalized clopidogrel antiplatelet therapy is becoming a promising treatment approach.

In this review, we focus mainly on outlining the genetic polymorphisms associated with the variability in antiplatelet response to clopidogrel and the difference in clinical outcomes among these genotypes. Additionally, we review the perspectives on pharmacogenomic findings that relate to personalized antiplatelet clopidogrel therapy in the clinical setting.

Search methodology

We searched PUBMED and MEDLINE of the English-language literature from January 1989 to January 2011 using the following search items in different combinations: "clopidogrel," "thienopyridine," "resistance," "platelet responsiveness," "genetic polymorphism," "pharmacogenomics," "pharmacogenetics," "clinical outcome" and "primary endpoints." After reviewing the abstracts, we obtained and reviewed the full text of the relevant articles and their reference lists. Additional citations were obtained from the articles retrieved from the literature search.

Antiplatelet mechanism and metabolism of clopidogrel

Platelets have a central role in the development of atherothrombotic events, and therefore antiplatelet therapy has become the cornerstone for cardiovascular disease management [27]. Clopidogrel is used to treat arterial thrombosis because this drug pharmacologically targets the platelet receptor systems. The key mediator of platelet activation and aggregation is ADP, which binds and activates platelets through two G-protein coupled receptors, $P2Y_1$ and $P2Y_{12}$ (Fig. 1). The $P2Y_{12}$ receptor is coupled to Gi and is responsible for platelet aggregation, especially its stabilization. The $P2Y_{12}$ receptor amplifies cytoplasmic Ca^{2+} mobilization that is mediated by the $P2Y_1$ receptor and induces the secretion of dense granules via stimulation of secretion-inducing agonists such as thromboxane A2 and thrombin. Several studies have suggested that phosphatidylinositol 3-kinase plays an important role in ADP-dependent, $P2Y_{12}$ receptor-mediated potentiation of platelet activation [28]. However, the signal transduction pathways that are mediated through the $P2Y_{12}$ receptor are not well understood. Since clopidogrel reduces the cyclic adenosine monophosphate (cAMP) levels by inhibiting adenylate cyclase by Gi, the effectiveness of clopidogrel can be directly monitored based on flow cytometric analyses of the phosphorylated vasodilator-stimulated phosphoprotein (VASP-P) levels [29].

Clopidogrel is a prodrug that must be converted into an active thiol-containing metabolite before it can express antiplatelet function [30]. The active metabolite covalently binds to the platelet $P2Y_{12}$ receptor and irreversibly inhibits ADP-stimulated platelet aggregation [30–32]. Pharmacokinetic studies indicate that clopidogrel is converted into its active metabolite by hepatic CYPs in a two-step oxidation process (Fig. 1). The first oxidative step is catalyzed by CYP2C19, CYP1A2 and CYP2B6, where each enzyme is responsible for 45%, 36% and 19% of the conversion, respectively. The second step is mediated by CYP3A4, CYP2B6, CYP2C19 and CYP2C9, where each enzyme is responsible for 40%, 33%, 21% and 7% of the conversion, respectively [33]. Thus, CYP2C19 substantially contributes to both oxidative steps that generate the active clopidogrel metabolite. However, in a recent study using recombinant metabolizing enzymes in a microsomal fraction expressed in HEK293 cells, Bouman *et al.* found that CYP3A4, CYP3A5, CYP2B6, and CYP1A2 were the first oxidative step enzymes, and PON1, an esterase synthesized in the liver and associated with high density lipoprotein in the blood, emerged as the rate-limiting enzyme for the second step of hydrolytic cleavage of the γ-thiobutyrolactone ring of 2-oxo-clopidogrel [20]. Approximately 15% of clopidogrel is available as an active metabolite and the remaining 85% is hydrolyzed to an inactive carboxylic acid derivative compound by esterases [34].

Genetic polymorphisms in *CYP2C19* that are relevant to clopidogrel

Deleterious genetic variants of CYP2C19

Although the active metabolite of clopidogrel arises from complex biochemical reactions that involve a number of different hepatic CYP enzymes, there is accumulating evidence that CYP2C19 plays a dominant role in clopidogrel activation [19,33,35]. The *CYP2C19* gene is located on chromosome 10 (10q24.1-q24.3) and consists of 490 amino acid residues. *CYP2C19* is one of the most polymorphic CYP genes among diverse racial groups. At least 25 genetic variants in *CYP2C19* have been identified [36]. Among these, two loss-of-function variant alleles, *CYP2C19*2* and **3*, account for the majority of the defective genotypes. *CYP2C19*2* (rs4244285) carries a 681G>A change in exon 5, which produces an aberrant splice site and leads to a truncated nonfunctional protein [37]. *CYP2C19*3* (rs4986893) has a 636G>A change in exon 4 that produces a premature stop codon [38]. Recently, a mutation in the regulatory region, *CYP2C19*17*, characterized by a -806C>T change in the 5'-flanking region of the gene was reported to be associated with increased CYP2C19 expression and enzymatic activity [39]. Additional missense mutations in *CYP2C19* that are relevant to defective enzyme function are summarized in

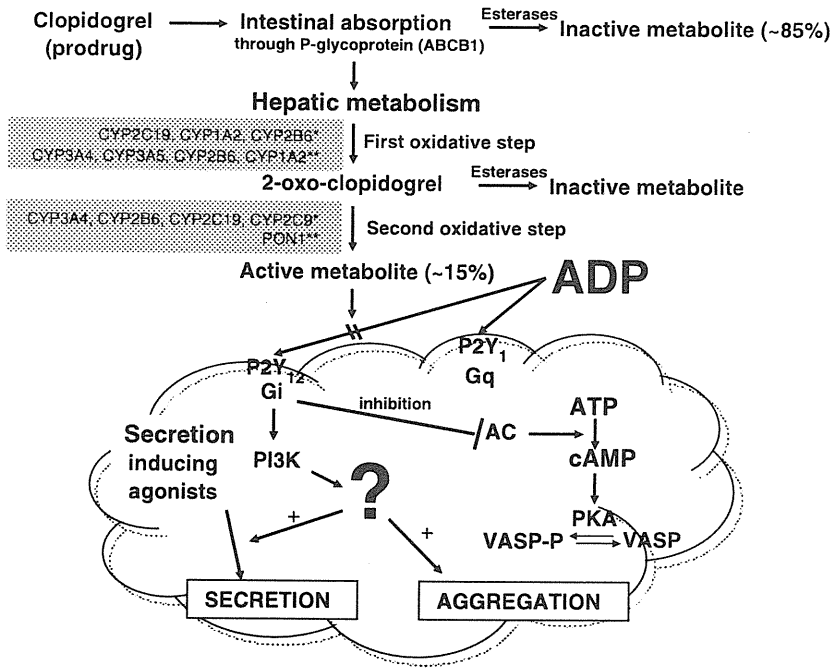

Fig. 1. Two-step activation of clopidogrel and its antiplatelet function. After being absorbed through the intestine via P-glycoprotein, clopidogrel can be converted into a thiol-containing active metabolite through two-step oxidations that involve several cytochrome P450 isoforms and paraosonase-1 (PON1). The first oxidative step is catalyzed by CYP enzymes, and the second step is mediated by CYP enzymes or PON1. Approximately 15% of clopidogrel is converted into active metabolites and the remaining 85% is converted into inactive metabolites by ubiquitous esterases. The thiol-containing active metabolite covalently binds to Cys97 in the $P2Y_{12}$ receptor to irreversibly inhibit ADP-stimulated platelet aggregation. ADP, adenosine diphosphate; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; VASP-P, phosphorylated form of vasodilator-stimulated phosphoprotein; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A. *CYP2C19, CYP1A2 and CYP2B6 are involved in the first step and CYP3A4, CYP2B6, CYP2C19, and CYP2C9 are involved in the second step [33]. **CYP3A4, CYP3A5, CYP2B6 and CYP1A2 are involved in the first step and PON1 is involved in the second step [20].

Table 1. Based on the *CYP2C19* *2, *3, *4, *5, *8 and *17 alleles, individuals can be grouped into the following phenotypes: ultra-rapid metabolizers (*17/*17 and *1/*17), extensive metabolizers (*1/*1), intermediate metabolizers (*1/*2, *1/*3, *1/*4, and *1/*8), and poor metabolizers (*2/*2, *2/*3, *2/*4, *2/*5, *2/*8 and *3/*3) [19].

Ethnic differences in CYP2C19 variants

Significant interethnic differences exist in the allele frequencies of *CYP2C19* variants. The *CYP2C19*2* allele is the most frequent variant in Caucasian, African-American, and Asian populations. However, the allelic frequency is significantly higher in Asian populations (~30%) than in Caucasians (~13%) and African-Americans (~18%). The *CYP2C19*3* allele also occurs more frequently in Asian populations (~10%) compared with other racial groups (< 1%) [47,48]. The *CYP2C19*17* allele is fairly common among Caucasians and Ethiopians (18%), but relatively rare among Asians (4%) [39]. Individuals carrying *CYP2C19*2* or *3 as homozygotes (*2/*2, *3/*3) or compound heterozygotes (*2/*3) are defined as poor metabolizers. Different distributions of poor metabolizers have been observed among different racial groups, with much greater prevalence (10–25%) in Asian populations compared to only 2% to 3% in Caucasians and 4% in Africans [49].

Effects of CYP2C19 variants on the pharmacokinetics and pharmacodynamics of clopidogrel

It has been hypothesized that reduced-function alleles of CYP enzymes contribute to decreased formation of the active metabolite of clopidogrel, leading to a reduced antiplatelet response. Several studies have demonstrated that different *CYP2C19* genotypes affect the pharmacokinetics and pharmacodynamics of clopidogrel, and the most widely analyzed genotype is *CYP2C19*2* (Table 2) [13,17–19,35,50–54].

Hulot et al. conducted a pharmacogenomic study using young healthy volunteers who were treated with clopidogrel and provided novel evidence that the *CYP2C19*2* loss-of-function allele was associated with a marked decrease in platelet responsiveness to clopidogrel [35]. Therefore, they proposed that the *CYP2C19*2* allele is an important genetic contributor to clopidogrel resistance in the clinical setting. This fundamental observation has been validated by similar studies [13,17–19,50,52], and was further confirmed in cardiovascular patients [13,51,53,54]. Moreover, the *CYP2C19*2* allele was significantly associated with reduced levels of the clopidogrel active metabolite, which corresponded to a diminished pharmacodynamic response to clopidogrel [17–19]. In healthy volunteers, a 32.4% relative reduction in plasma exposure to the active clopidogrel metabolite and a relative reduction of approximately 25% in maximal platelet aggregation were observed in carriers with at least one *CYP2C19* reduced-function allele compared with non-carriers [19]. These findings suggest that a low level or complete absence of responsiveness to clopidogrel is mainly caused by lower levels of the active clopidogrel metabolite. The pharmacogenomic status of *CYP2C19* is a determinant for the formation of the active metabolite of clopidogrel and its antiplatelet effects.

There are limited data investigating the influence of other *CYP2C19* genotypes on the pharmacokinetics and pharmacodynamics of clopidogrel. Recently, the novel allelic variant *CYP2C19*17* has been associated with ultrarapid enzymatic activity and increased medication metabolism [39]. Frére et al. evaluated the impact of the *CYP2C19*17* gain-of-function allele on the platelet response to clopidogrel in a retrospective study of 598 patients with non-ST elevation acute coronary syndrome who had been administered a 600-mg clopidogrel loading dose and found a significant association between the gain-of-function *CYP2C19*17* genotype and the platelet

Table 1
Missense mutations in *CYP2C19* with functional effects.

Allele	Nucleotide change in cDNA	Amino acid change	Enzymatic activity	References
*CYP2C19*2*	681G>A	Frame shift	An aberrant splice resulting in a truncated, non-functional, catalytically inactive protein	de Morais *et al.*, 1994 [37]
*CYP2C19*3*	636G>A	Trp212X	A premature stop codon resulting in a truncated, non-functional, catalytically inactive protein	de Morais *et al.*, 1994 [38]
*CYP2C19*4*	1A>G	Met1Val	An A–>G mutation in the initiation codon resulting in a translation defect and a catalytically inactive protein	Ferguson *et al.*, 1998 [40]
*CYP2C19*5*	1297C>T	Arg433Trp	Decrease: abolished activity toward S-mephenytoin and tolbutamide	Xiao *et al.*, 1997 [41] Ibeanu *et al.*, 1998 [42]
*CYP2C19*6*	395G>A	Arg132Gln	Decrease: negligible catalytic activity toward both mephenytoin and tolbutamide	Ibeanu *et al.*, 1999 [43]
*CYP2C19*8*	358T>C	Trp120Arg	Decrease: approximately 90% and 70% reduction in the metabolism of S-mephenytoin and tolbutamide, respectively	Ibeanu *et al.*, 1999 [43]
*CYP2C19*9*	431G>A	Arg114 His	Decrease: a modest decrease in the V(max) for 4'-hydroxylation of mephenytoin	Blaisdell *et al.*, 2002 [44]
*CYP2C19*10*	680C>T	Pro227Leu	Decrease: a dramatically higher K(m) and lower V (max) for mephenytoin	Blaisdell *et al.*, 2002 [44]
*CYP2C19*12*	1473A>C	X 491Cys	Unstable activity for mephenytoin	Blaisdell *et al.*, 2002 [44]
*CYP2C9*17*	No change	No change	Increase: increased transcriptional activity (−806C>T) with extensive metabolization of omeprazole, escitalopram, and clopidogrel	Sim *et al.*, 2006 [39] Rudberg *et al.*, 2007 [45] Sibbing *et al.*, 2010 [26]
*CYP2C19*26*	766G>A	Asp256Asn	Decrease: 76% reduction in intrinsic clearance of S-mephenytoin	Lee *et al.*, 2009 [46]

Missense mutations with functional activity are listed. Mutations where the functional activity has not been examined in vivo or in vitro are not listed.

reactivity index, which was evaluated by VASP phosphorylation [55]. In a subsequent study of a cohort of 1,524 patients who underwent PCI, Sibbing et al. demonstrated that *CYP2C19*17* gain-of-function allele carriers had significantly lower ADP-induced platelet aggregation and a higher risk for bleeding during clopidogrel treatment compared to wild-type carriers (Table 2) [26]. However, platelet function in patients with bleeding was not reported. Very little is known about the less common loss-of-function *CYP2C19* variants (e.g., **3*, **4*, **5*, **6*, **7*, **8*).

Effects of CYP2C19 genotypes on the cardiovascular outcomes of clopidogrel-treated patients

The loss-of-function *CYP2C19*2* allele has been associated with reduced generation of the clopidogrel active metabolite and higher ex vivo platelet reactivity to ADP. Independent post hoc analyses further showed that *CYP2C19*2* was associated with worse clinical outcomes during clopidogrel therapy. Recently, several large cohort studies indicated that *CYP2C19*2* and other *CYP2C19* loss-of-function alleles play an important role in clopidogrel non-responsiveness and adverse clinical outcomes (Table 3).

The role of the *CYP2C19* genotype in clopidogrel responsiveness was assessed initially in the Low Responsiveness to Clopidogrel and Sirolimus- or Paclitaxel-Eluting Stent Thrombosis (RECLOSE) trial [21]. This study included 772 consecutive patients who received drug-eluting stents and dual antiplatelet therapy with aspirin and clopidogrel. Patients carrying at least one variant *CYP2C19*2* allele were at increased risk for stent thrombosis (Table 3). In addition, the *CYP2C19* genotype was an independent predictor of stent thrombosis after several clinical risk factors were included in a multivariable logistic regression model.

Another large cohort study by Sibbing et al. assessed 2,485 consecutive patients undergoing PCI who were uniformly pretreated with a clopidogrel 600-mg loading dose and demonstrated that *CYP2C19*2* carriers had a significantly higher cumulative 30-day incidence of stent thrombosis compared with wild-type *CYP2C19* carriers (Table 3) [22]. These findings provided additional support that patients undergoing PCI who possess at least one *CYP2C19*2* allele are at increased risk for stent thrombosis.

To date, most of the investigations assessing *CYP2C19* genotypes and clopidogrel responsiveness have been cohort studies. One group of investigators used data from a randomized controlled trial. Among patients with acute coronary syndrome who were undergoing stenting and were treated with clopidogrel in the TRITON–TIMI 38 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing

Table 2
Main effects of *CYP2C19* variants on platelet responsiveness to clopidogrel.

Population	Subject number	Clopidogrel dose per day	Platelet functional methods/parameters	Allelic variants	Results	Reference
Healthy subjects	28	MD: 75 mg	Optical aggregometry (10 μmol/L ADP)/Reduction in ADP-induced platelet aggregation from baseline (%), Flow cytometry /VASP-P	*CYP2C19*2, *3, *4, *5, *6*	The *CYP2C19*2* allele was associated with a marked decrease in ADP-induced platelet aggregation ($p<0.003$). The PRI measured by flow cytometry in individuals with *1/*2 was significantly different from those with *1/*1.	Hulot *et al.*, 2006 [35]
Healthy subjects	74	LD: 300 mg	Optical aggregometry (20 μmol/L ADP)/ Inhibition of platelet aggregation	*CYP2C19*2,*3, *4, *5*	The *CYP2C19*2* allele was associated with a poor-responder status to clopidogrel ($p=0.030$) †.	Brandt *et al.*, 2007 [17]
Healthy subjects	94	LD: 300 mg MD: 75 mg	Optical aggregometry (20 μmol/L ADP)/ Reduction in ADP-induced platelet aggregation from baseline (%)	*CYP2C19*2*	The *CYP2C19*2* allele explained 10% of the variability in clopidogrel responsiveness (beta coefficient 0.32; 95% CI 0.11–0.47, $p=0.02$).	Fontana *et al.*, 2007 [50]
ACS patients	1,419	LD: 600 mg MD: 75 mg	Optical aggregometry (2, 10 μmol/L ADP)/Residual platelet reactivity	*CYP2C19*2*	The *CYP2C19*2* allele was associated with higher platelet aggregability in high-risk vascular patients ($p=0.001$).	Giusti *et al.*, 2007 [51]
Healthy subjects	24	LD: 300 mg MD: 75 mg	Optical aggregometry (5 μmol/L ADP) /Inhibition of platelet aggregation Plasma concentration of clopidogrel	*CYP2C19*2, *3*	The *CYP2C19* genotype affects the plasma levels of clopidogrel and modulates the antiplatelet effects of clopidogrel.	Kim *et al.*, 2008 [52]
Non-ST elevation ACS patients	603	LD: 600 mg	Optical aggregometry (10 μmol/L ADP)/ Residual platelet reactivity Flow cytometry/VASP-P Flow cytometry/P-selectin expression	*CYP2C19*2*	The *CYP2C19*2* allele was associated with ADP-induced platelet aggregation, VASP-P index, and ADP-induced P-selectin expression. The *CYP2C19*2* allele carriers exhibited the highest platelet index levels in a multivariate analysis ($p=0.03$).	Frere C *et al.*, 2008 [53]
Patients undergoing PCI	797	LD: 600 mg MD: 75 mg	Optical aggregometry (5 μmol/L, 20 μmol/L ADP)/ Residual platelet reactivity	*CYP2C19*2*	Patients carrying at least one *CYP2C19*2* allele showed high residual platelet reactivity.	Trenk *et al.*, 2008 [54]
Healthy subjects	47	LD: 300 mg	Optical aggregometry (20 μmol/L ADP)/ Inhibition of platelet aggregation Flow cytometry/VASP-P Plasma concentration of the active metabolite	*CYP2C19*2, *3*	In the PMs (**2/*2* or **2/*3*) and the IMs (**1/*2* or **1/*3*), the PRI at 4 h after dosing was significantly higher compared to that in the EMs (*CYP2C19*1/*1*) ($p<0.01$ and $p<0.05$). The *CYP2C19* genotype is a determinant for the formation of the active clopidogrel metabolite.	Umemura *et al.*, 2008 [18]
Healthy subjects	162	LD: 300 mg or 600 mg MD: 75 mg	Optical aggregometry (20 μmol/L ADP)/ΔMPA	*CYP2C19*2, *3, *4, *5, *8*	Carriers of at least one *CYP2C19* reduced-function allele had 9 percentage points less ΔMPA compared to non-carriers ($p<0.001$).	Mega *et al.*, 2009 [19]
Non-ST elevation ACS patients	598	LD: 600 mg	Optical aggregometry (10 μmol/L ADP)/Maximal intensity of platelet aggregation Flow cytometry/VASP-P	*CYP2C19*4, *5, *6 , *17*	The *CYP2C19*17* genotype was significantly associated with lower PRI VASP values (adjusted $p=0.0005$). The *CYP2C19*17* genotype was not significantly associated with ADP-induced platelet aggregation.	Frere *et al.*, 2009 [55]
Healthy subjects and PCI patients	656 (429 and 227)	LD: 300 mg MD: 75 mg	Optical aggregometry (20 μmol/L ADP)/ Reduction in ADP-induced platelet aggregation from baseline (%)	Genome-wide association study	The *CYP2C19*2* allele was associated with a reduced clopidogrel response, accounting for 12% of the variation in platelet aggregation to ADP ($p=4.3\times10^{-11}$).	Shuldiner *et al.*, 2009 [13]
Patients undergoing PCI	1,524	LD: 600 mg MD: 75 mg	Optical aggregometry (6.4 μmol/L ADP)/ Residual platelet reactivity	*CYP2C19*17*	The *CYP2C19*17* allele was associated with decreased ADP-induced platelet aggregation ($p=0.039$).	Sibbling *et al.*, 2010 [26]

MD, maintenance dose; LD, loading dose; ADP, adenosine diphosphate; FCM, flow cytometry; VASP-P, vasodilator-stimulated phosphoprotein phosphorylation; PRI, platelet reactivity index; RPR, residual platelet reactivity; PM, Poor metabolizer; EM, Extensive metabolizer; IM, Intermediate metabolizer; ACS, acute coronary syndromes; PCI, percutaneous coronary intervention; ΔMPA, an absolute reduction in maximal platelet aggregation from baseline.
†A pharmacodynamic poor-responder was defined as a subject with less than 20% inhibition of platelet aggregation to 20 μmol/L ADP at either 4 h or 24 h.

Table 3
Effects of *CYP2C19* genetic variants on the efficacy and safety of clopidogrel.

Design	Parent study	Population	Subject number	Dose of clopidogrel	Follow-up	Allele variants	Effects of CYP2C19 alleles on outcomes: Primary endpoint [a]HR or OR (95% confidence interval)	Effects of CYP2C19 alleles on outcomes: Secondary endpoint [a]HR or OR (95% confidence interval)	Reference
Prospective cohort study	RECLOSE trial	PCI patients	772	LD 600 mg MD 75 mg	6 months	*CYP2C19*2*	6-month incidence of ST OR 3.43 (1.01–12.78), $p=0.047$	Composite feature of cardiac mortality and ST OR 2.70 (1.00–8.42), $p=0.049$	Giusti et al., 2009 [21]
Prospective randomized study	ISAR studies	PCI patients	2,485	LD 600 mg MD 75 mg	30 days	*CYP2C19*2*	30-day incidence of ST HR 3.81 (1.45–10.02), $p=0.007$	ND	Sibbling et al., 2009 [22]
Prospective randomized study	TRITON–TIMI 38 study	PCI in ACS patients	1,477	LD 300 mg MD 75 mg	15 months	*CYP2C19*2, *3, *4, *5, *8*	Composite of death from cardiovascular causes, MI, or stroke For at least one reduced function allele: HR 1.53 (1.07−2.19), $p=0.01$ For *CYP2C219*2*: HR 1.42 (0.98−2.05), $p=0.04$	ST For at least one reduced function allele: HR 3.09 (1.19−8.00), $p=0.02$ For *CYP2C19*2*: HR 3.33 (1.28−8.62), $p=0.004$	Mega et al., 2009 [19]
Prospective cohort study	FAST-MI study	AMI patients	2,208	LD 300 mg MD 75 mg	1 year	*CYP2C19*2, *3, *4, *5*	Composite of death from any cause, nonfatal MI, or stroke For any two *CYP2C19* loss-of-function alleles: HR 1.98 (1.10–3.58), $p=0.003$ [b]HR 3.58 (1.71–7.51), $p=0.005$	ND	Simon et al., 2009 [23]
Prospective cohort study	AFIJI multicentre registry	AMI patients (<45 years)	259	MD 75 mg	The median clopidogrel exposure time was 1.07 years.	*CYP2C19*2*	Composite of death, MI, and urgent coronary revascularization HR 3.69 (1.69–8.05), $p=0.0005$	ST HR 6.02 (1.81–20.04), $p=0.0009$	Collet et al., 2009 [24]
Prospective study	ND	PCI patients	227	LD 600 mg or 300 mg MD 75 mg	1 year	*CYP2C19*2*	Cardiovascular ischemic event or death HR 2.42 (1.18−4.99), $p=0.02$	ND	Shuldiner et al., 2009 [13]
Prospective study	ND	PCI patients	1,524	LD 600 mg MD 75 mg	30 days	*CYP2C19*17*	Efficacy outcome: 30-day incidence of ST No significant influence ($p=0.79$) Safety outcome: 30-day incidence of bleeding For *CYP2C19*17* allele: OR 1.85 (1.19−2.86), $p=0.006$ For homozygous *CYP2C19*17* allele: OR, 3.41 (1.42−8.17), $p=0.006$	ND	Sibbling et al., 2010 [26]
Prospective randomized, placebo controlled study	CURE study and ACTIVE A study	ACS or AF patients	For ACS patients in the CURE study: Clopidogrel: 2549 Placebo: 2510 For AF patients in the ACTIVE A study: Clopidogrel: 570 Placebo: 586	MD 75 mg	CURE study: 1 year ACTIVE A study: median duration, 3.6 years	*CYP2C19*2, *3* and **17* alleles	For ACS patients: (the CURE study) Efficacy outcomes: composite of death from cardiovascular causes, nonfatal MI, or stroke For *CYP2C19*2* and **3*: Carriers: 0.69 (0.49−0.98); Non-carriers: 0.72 (0.59−0.87) $p=0.84$ for interaction For *CYP2C19*17*: Carriers: 0.55 (0.42−0.73); Non-carriers: 0.85 (0.68−1.05). $p=0.02$ for the interaction Safety outcomes: major bleeding The effects were consistent in genotypic subgroups. For AF patients (ACTIVE A study) There was no interaction with respect to either efficacy or bleeding between genotypic carriers.	For ACS patients: (the CURE study) Efficacy outcomes: Composite of the first primary outcome, recurrent ischemia, or hospitalization for unstable angina. For *CYP2C19*2* and **3*: The effects were the same as the first primary outcomes. For *CYP2C19*17*: Carriers 0.66 (0.54−0.82); Non-carriers 0.90 (0.76−1.06) $p=0.03$ for the interaction	Paré et al., 2010 [25]

HR, hazard ratio; OR, odds ratio; PCI, percutaneous coronary intervention; LD, loading dose; MD, maintenance dose; ST, stent thrombosis; ND, not determined; ACS, acute coronary syndrome; AF, atrial fibrillation; AMI, acute myocardial infarction; MI, myocardial infarction. RECLOSE, the Low Responsiveness to Clopidogrel and Sirolimus- or Paclitaxel- Eluting Stent Thrombosis; TRITON–TIMI, the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction; FAST-MI, the French Registry of Acute ST-Elevation and Non-ST-Elevation Myocardial Infarction; AFIJI, Appraisal of risk Factors in young Ischemic patients Justifying aggressive Intervention; ISAR, the Intracoronary Stenting and Antithrombotic Regimen study; CURE, Clopidogrel in Unstable Angina to Prevent Recurrent Events; ACTIVE A, the Atrial Fibrillation Clopidogrel Trial with Irbesartan for Prevention of Vascular Events.

[a] HR in the placebo controlled studies of Paré et al. reflects the effects of clopidogrel compared with the placebo in subgroups that were defined according to metabolizer phenotype or functional allele carrier status.

[b] In 1535 patients who underwent PCI.

Platelet Inhibition With Prasugrel–Thrombolysis In Myocardial Infarction 38) study, *CYP2C19* loss-of-function allele carriers had a higher rate of recurrent ischemic events, including stent thrombosis, compared with non-carriers (Table 3) [19]. Genetic variants in other examined CYP enzymes were not associated with the primary outcome, and no association was observed between bleeding risk and CYP genotypes. This study further confirms that patients carrying at least one variant *CYP2C19* allele have an increased risk for adverse cardiovascular events after PCI.

Another study assessed a cohort of 2,208 patients with myocardial infarction from the prospective, observational French Registry of Acute ST-Elevation and Non-ST-Elevation Myocardial Infarction (FASTMI) registry [23]. This study also used composite primary outcomes, which included death from any cause, nonfatal myocardial infarction, or stroke. In this study, the *CYP2C19*2*, **3*, **4*, and **5* alleles and the *ABCB1* variant were genotyped. The *ABCB1* gene encodes an intestinal efflux pump P-glycoprotein that is involved in clopidogrel absorption. Patients with two variant *CYP2C19* alleles were more likely to experience a primary outcome event compared with non-carriers (Table 3). Those patients who possessed two *CYP2C19* loss-of-function alleles and at least one *ABCB1* variant allele were at the highest risk for a primary outcome event compared to both *CYP2C19* and *ABCB1* wild-type homozygous patients.

The association between the *CYP2C19* genotype and a composite end point of cardiovascular death, nonfatal myocardial infarction, and urgent revascularization was studied in a prospective cohort study that consisted of 259 young patients aged <45 years who had survived a first myocardial infarction (Table 3) [24]. Compared to wild-type *CYP2C19* homozygotes (72%), patients with the *CYP2C19*2* allele (28%) were at significantly higher risk for the primary end point at one year. This increased risk was demonstrated soon after clopidogrel treatment was initiated and persisted throughout the study period. An increased risk for stent thrombosis was also found in *CYP2C19*2* allele carriers. A multivariable analysis suggested that the *CYP2C19* genotype was the only independent predictor of cardiovascular events in this population.

In a recent systematic meta-analysis of 10 studies involving 11,959 patients, Hulot et al. demonstrated that carriers of the loss-of-function *CYP2C19*2* allele were at 30% higher risk for major adverse clinical events compared to non-carriers (9.7% vs. 7.8%; odds ratio (OR): 1.29; 95% confidence interval (CI): 1.12 to 1.49; $p<0.001$). *CYP2C19*2* alone was also associated with increased mortality (1.8% vs. 1.0%; OR: 1.79; 95% CI: 1.10 to 2.91; $p=0.019$; n = 6,225) and stent thrombosis (2.9% vs. 0.9%; OR: 3.45; 95% CI: 2.14 to 5.57; $p<0.001$; n = 4,905). This increased risk was apparent in both heterozygotes and homozygotes and was independent of the baseline cardiovascular risk [56].

The above-mentioned studies that showed carriers of loss-of-function alleles (such as the *CYP2C19*2* and *CYP2C19*3* alleles) had reduced benefits from clopidogrel did not include a randomized control group. Therefore, the potential pleiotropic effects of loss-of-function alleles on the outcomes of clopidogrel treatment cannot be excluded. A more recent study included two large randomized placebo controlled trials (the CURE and ACTIVE A studies), and the results suggested that the efficacy and safety of clopidogrel compared to the placebo were not affected by *CYP2C19* loss-of-function alleles (Table 3) [25]. There was no significant difference in the effects of clopidogrel treatment on the clinical outcomes when these patients were stratified based on the metabolizer phenotype. As for reason why the greatest benefit of clopidogrel was the reduction in the rate of stent thrombosis, one possible explanation for this difference from previous studies might be that it was partially due to the interaction between study treatment and genotype with respect to cardiovascular events among patients. A relatively large difference in the rates of PCI patients with stenting could be found, with 14.5% in the CURE study and more than 70% in previous studies [13,19,23,24], and thus an interaction in the subgroup of patients who receive stents, particularly drug-eluting stents which were not in use at the time of the CURE trial, could not be excluded. The finding of the previous studies that the greatest effect of loss-of-function alleles on the clinical efficacy of clopidogrel was the effect on stent thrombosis, supports the possible influence of such an interaction on the opposite conclusion [19,21,22,24].

In a recent cohort study of 1,524 patients who underwent PCI, the gain-of-function allele *CYP2C19*17* in the regulatory region of the gene was associated with a higher risk of bleeding among clopidogrel-treated patients [26]. Among patients who were homozygous for the *CYP2C19*17* variant, there was an approximately 4-fold increase in bleeding events. Despite the significant impact of this allele on bleeding events, the protective effects of the *CYP2C19*17* allele on the occurrence of stent thrombosis or other ischemic events were not observed (Table 3) [23,26]. On the other hand, a recent prospective randomized, placebo controlled study found that clopidogrel was associated with an enhanced ability to reduce ischemic events in patients with acute coronary syndromes who had the *CYP2C19*17* allele, although this effect was not observed in patients with atrial fibrillation who had the *CYP2C19*17* allele (Table 3) [25]. The increased benefit of clopidogrel in *CYP2C19*17* carriers is consistent with pharmacodynamic data that associated this genetic variant with increased enzymatic activity and an enhanced platelet response to clopidogrel [26,39,55]. However, the effects of clopidogrel compared to the placebo on major bleeding were consistent among the subgroups that were defined according to the metabolizer phenotype or their functional allele carrier status [25].

Genome-wide association study of clopidogrel responsiveness

To further reconcile and solidify the existing data on the genetic determinants of clopidogrel responsiveness, a genome-wide association study examined the marked heterogeneity in ADP-stimulated platelet aggregation in response to clopidogrel among healthy Amish subjects [13]. Remarkably, a cluster of 13 single nucleotide polymorphisms within and flanking the *CYP2C18-2C19-2C9-2C8* cluster on chromosome 10q24 (out of about 400,000 single nucleotide polymorphisms analyzed genome-wide) was strongly associated with a clopidogrel response ($p=10^{-12}$ to 10^{-7}). Further mapping identified the *CYP2C19*2* variant, which accounted for most or all of the signals that were associated with 10q24. In a replication study involving patients undergoing PCI, carriers of the *CYP2C19*2* allele had higher rates of cardiovascular events at the 1-year follow-up assessment compared to non-carriers, which was consistent with the increased risk for major adverse events in previous studies (Table 3). In this study, the *CYP2C19*2* genotype was estimated to account for approximately 12% of the variation in the response to clopidogrel. With age, body mass index, and lipid levels, approximately 22% of the variation in the response to clopidogrel could be explained.

Contribution of *PON1* to clopidogrel interindividual variability

Following the identification of *PON1* as a new mediator that drives the conversion of the clopidogrel into the active metabolite, Bouman *et al.* found that the common *PON1* Q192R gene polymorphism was functional in which the *PON1* Q192 variant had a lower and less efficient conversion of 2-oxo-clopidogrel to the active metabolite than the *PON1* R192 variant [20].

With respect to the biological mechanism underlying this effect, a case-cohort study in individuals with coronary artery disease who underwent PCI and received clopidogrel therapy found that, compared with individuals with either the *PON1* RR192 or the QR192 genotype, QQ192 homozygous individuals were more frequent in the stent thrombosis group (66%, 27 out of 41 patients) than in the thrombosis-free group (35%, 25 out of 71 patients) (OR: 3.6; 95% CI: 1.6–7.9; $p=0.003$). This finding was confirmed and extended in an independently replicated study in a prospective cohort of 1,982 individuals with acute coronary syndromes [20]. Overall, the *PON1* Q192R variant accounted for 72.5% of the variability in response to

ADP-induced platelet aggregation after clopidogrel treatment. Therefore, genotyping of *PON1* might be used to identify clopidogrel responders and help to predict the clinical efficacy. However, studies will be needed to replicate the influence of *PON1* polymorphisms on the platelet responsiveness and clinical outcomes with clopidogrel therapy.

Contribution of other factors to clopidogrel interindividual variability

Although the heritability of the response to clopidogrel is approximately 70% [13], the majority of factors, including genetic and non-genetic, that influence the interindividual variation in the platelet response to clopidogrel are still unexplained. Additional genetic factors, including the genetic variants of *CYP3A4*[50,53,57] and *CYP3A5*[53,58–60] encoding CYP enzymes that produce the active clopidogrel metabolite, *P2RY12* encoding the platelet ADP receptor $P2Y_{12}$[23,60–63], and *ABCB1*[23,64] encoding P-glycoprotein for clopidogrel absorption, might be responsible for the interindividual variability in the response to clopidogrel. However, most of the studies that have examined these possibilities have produced differing results on the impact of these genetic variants on clopidogrel responsiveness and clinical outcomes. Of note, the *ABCB1 3435TT* genotype (rs1045642), which has been associated with variability in the response to clopidogrel and worse clinical outcomes, might improve the ability to predict clopidogrel non-responsiveness when combined with the *CYP2C19* genotypes [23,64].

In addition to genetic factors, the interpatient variability in the response to clopidogrel is influenced by environmental, cellular, and pathophysiological clinical factors. Among the environmental factors, age, smoking, diet, and drug-drug interactions involving CYP2C19, CYP3A4, CYP1A2, and CYP2C9 isoenzymes have been shown to influence the response to clopidogrel. Proton-pump inhibitors (PPIs), lipophilic statins, calcium channel blockers, caffeine, St. John's wort, and warfarin are thought to alter the pharmacodynamic effects of clopidogrel through drug-drug interactions [65–67]. However, currently there are insufficient data to judge the clinical consequences of these interactions. Furthermore, cellular mechanisms such as the life span of platelets, increased platelet sensitivity or reaction to ADP, and upregulation of the $P2Y_1$ and $P2Y_2$ pathways as well as pathophysiological clinical factors such as diabetes, acute coronary syndrome, patient compliance, underdosing or inappropriate clopidogrel dosing, triglycerides, high-density lipoprotein cholesterol and body mass index may cause variability in the response to clopidogrel [13–16]. Therefore, it will be important to further investigate whether these factors affect the response to clopidogrel when optimizing personalized antiplatelet therapy.

Perspectives for personalized clopidogrel dosing

The US Food and Drug Administration (FDA) has added a boxed warning to the clopidogrel label which suggests that individuals with poor metabolizer genotypes (i.e., *CYP2C19*2* and **3* carriers) may be at increased risk for adverse cardiovascular outcomes and should consider other antiplatelet medications or alternative dosing strategies [68]. This notice advocates that it may be beneficial to identify patients at "higher or lower risk of poor clopidogrel responsiveness" defined as carriers or non-carriers of the *CYP2C19* loss-of-function alleles. The combination of genotyping and *ex vivo* platelet function testing may ultimately become the most cost-effective strategy to identify a subset of patients who are most likely to respond to clopidogrel [69,70]. The US FDA notice also advocates implementing strategies that adjust the clopidogrel dose or use alternative antiplatelet agents in high-risk patients. Alternative strategies for antiplatelet treatment, such as a higher clopidogrel maintenance dose and the use of the more potent thienopyridine or direct acting drugs (i.e., prasugrel, cangrelor, ticagrelor and elinogrel) may be beneficial in patients with stent thrombosis and hypo- or non-responsiveness to clopidogrel [71–76]. However, according to late-breaking results of the clinical trial GRAVITAS (Gauging Responsiveness With a VerifyNow Assay-Impact on Thrombosis and Safety), a high dose of clopidogrel did not reduce the incidence of death, myocardial infarction, or in-stent restenosis in certain high-risk patients who had drug-eluting stents implanted [77].

In the future, it is likely that pharmacogenomic findings will be used in clinical practice to individualize medicine. However, before routine genotyping can be used to guide personalized antiplatelet clopidogrel therapy, some challenges should be addressed. For personalized antiplatelet therapy to be optimally applied, it will be necessary to generate a rapid and accurate point-of-care *CYP2C19* genotyping platform because clopidogrel therapy is often immediately initiated when the patient presents at the emergency department, allowing little time for genetic testing before initiating the therapy. On the other hand, there are also cost-effectiveness issues associated with genotyping a population of patients with acute coronary syndrome [78]. In addition, it is still unknown whether the platelet function assay combined with *CYP2C19* genetic testing will be more effective in identifying clopidogrel non-responsive or high-risk patients than either test alone. To date, there have been no studies that have evaluated the safety and efficiency of switching patients to alternative therapies, including higher clopidogrel maintenance or loading doses and other $P2Y_{12}$ receptor inhibitors, when the patients were classified as clopidogrel non-responsive based on their genotypes. Apart from genetic factors, it is still necessary to determine how exogenous modifiers of cytochrome P450 function (such as PPIs) interact with endogenous genetic modulators [79].

There have been marked interethnic differences in the incidence of poor metabolizers among individuals with *CYP2C19* and *PON1* polymorphisms. Further studies are needed to confirm the comprehensive influence of *CYP2C19* and *PON1* variant alleles on the antiplatelet effects of clopidogrel and the clinical outcomes in various ethnic populations, especially among individuals with Asian and African ancestry, who have a much higher prevalence of loss-of-function *CYP2C19* alleles but a lower prevalence of the *PON1* Q192 allele with lower enzyme activity than Caucasians.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgements

We thank Dr. Shigeki Miyata and Dr. Hiroshi Nonogi at the National Cerebral and Cardiovascular Center for critical reading of the manuscript. This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] Gasparyan AY, Watson T, Lip GY. The role of aspirin in cardiovascular prevention: implications of aspirin resistance. J Am Coll Cardiol 2008;51:1829–43.
[2] Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 2001;345:494–502.
[3] Sabatine MS, Cannon CP, Gibson CM, Lopez-Sendon JL, Montalescot G, Theroux P, Lewis BS, Murphy SA, McCabe CH, Braunwald E. Effect of clopidogrel pretreatment before percutaneous coronary intervention in patients with ST-elevation myocardial infarction treated with fibrinolytics: the PCI-CLARITY study. Jama 2005;294:1224–32.
[4] Sabatine MS, Cannon CP, Gibson CM, Lopez-Sendon JL, Montalescot G, Theroux P, Claeys MJ, Cools F, Hill KA, Skene AM, McCabe CH, Braunwald E. Addition of

clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. N Engl J Med 2005;352:1179–89.
[5] Chen ZM, Jiang LX, Chen YP, Xie JX, Pan HC, Peto R, Collins R, Liu LS. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. Lancet 2005;366:1607–21.
[6] Bhatt DL, Bertrand ME, Berger PB, L'Allier PL, Moussa I, Moses JW, Dangas G, Taniuchi M, Lasala JM, Holmes DR, Ellis SG, Topol EJ. Meta-analysis of randomized and registry comparisons of ticlopidine with clopidogrel after stenting. J Am Coll Cardiol 2002;39:9–14.
[7] Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. Nat Rev Drug Discov 2010;9:154–69.
[8] Kolandaivelu K, Bhatt DL. Overcoming 'resistance' to antiplatelet therapy: targeting the issue of nonadherence. Nat Rev Cardiol 2010;7:461–7.
[9] Paikin JS, Eikelboom JW, Cairns JA, Hirsh J. New antithrombotic agents–insights from clinical trials. Nat Rev Cardiol 2010;7:498–509.
[10] Gurbel PA, Bliden KP, Hiatt BL, O'Connor CM. Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. Circulation 2003;107:2908–13.
[11] Nguyen TA, Diodati JG, Pharand C. Resistance to clopidogrel: a review of the evidence. J Am Coll Cardiol 2005;45:1157–64.
[12] Snoep JD, Hovens MM, Eikenboom JC, van der Bom JG, Jukema JW, Huisman MV. Clopidogrel nonresponsiveness in patients undergoing percutaneous coronary intervention with stenting: a systematic review and meta-analysis. Am Heart J 2007;154:221–31.
[13] Shuldiner AR, O'Connell JR, Bliden KP, Gandhi A, Ryan K, Horenstein RB, Damcott CM, Pakyz R, Tantry US, Gibson Q, Pollin TI, Post W, Parsa A, Mitchell BD, Faraday N, Herzog W, Gurbel PA. Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. Jama 2009;302:849–57.
[14] Wiviott SD, Antman EM. Clopidogrel resistance: a new chapter in a fast-moving story. Circulation 2004;109:3064–7.
[15] Sibbing D, von Beckerath O, Schomig A, Kastrati A, von Beckerath N. Impact of body mass index on platelet aggregation after administration of a high loading dose of 600 mg of clopidogrel before percutaneous coronary intervention. Am J Cardiol 2007;100:203–5.
[16] Singla A, Antonino MJ, Bliden KP, Tantry US, Gurbel PA. The relation between platelet reactivity and glycemic control in diabetic patients with cardiovascular disease on maintenance aspirin and clopidogrel therapy. Am Heart J 2009;158:784 e1-6.
[17] Brandt JT, Close SL, Iturria SJ, Payne CD, Farid NA, Ernest 2nd CS, Lachno DR, Salazar D, Winters KJ. Common polymorphisms of CYP2C19 and CYP2C9 affect the pharmacokinetic and pharmacodynamic response to clopidogrel but not prasugrel. J Thromb Haemost 2007;5:2429–36.
[18] Umemura K, Furuta T, Kondo K. The common gene variants of CYP2C19 affect pharmacokinetics and pharmacodynamics in an active metabolite of clopidogrel in healthy subjects. J Thromb Haemost 2008;6:1439–41.
[19] Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, Walker JR, Antman EM, Macias W, Braunwald E, Sabatine MS. Cytochrome p-450 polymorphisms and response to clopidogrel. N Engl J Med 2009;360:354–62.
[20] Bouman HJ, Schomig E, van Werkum JW, Velder J, Hackeng CM, Hirschhauser C, Waldmann C, Schmalz HG, Ten Berg JM, Taubert D. Paraoxonase-1 is a major determinant of clopidogrel efficacy. Nat Med 2011;17:110–6.
[21] Giusti B, Gori AM, Marcucci R, Saracini C, Sestini I, Paniccia R, Buonamici P, Antoniucci D, Abbate R, Gensini GF. Relation of cytochrome P450 2C19 loss-of-function polymorphism to occurrence of drug-eluting coronary stent thrombosis. Am J Cardiol 2009;103:806–11.
[22] Sibbing D, Stegherr J, Latz W, Koch W, Mehilli J, Dorrler K, Morath T, Schomig A, Kastrati A, von Beckerath N. Cytochrome P450 2C19 loss-of-function polymorphism and stent thrombosis following percutaneous coronary intervention. Eur Heart J 2009;30:916–22.
[23] Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Meneveau N, Steg PG, Ferrieres J, Danchin N, Becquemont L. Genetic determinants of response to clopidogrel and cardiovascular events. N Engl J Med 2009;360:363–75.
[24] Collet JP, Hulot JS, Pena A, Villard E, Esteve JB, Silvain J, Payot L, Brugier D, Cayla G, Beygui F, Bensimon G, Funck-Brentano C, Montalescot G. Cytochrome P450 2C19 polymorphism in young patients treated with clopidogrel after myocardial infarction: a cohort study. Lancet 2009;373:309–17.
[25] Paré G, Mehta SR, Yusuf S, Anand SS, Connolly SJ, Hirsh J, Simonsen K, Bhatt DL, Fox KA, Eikelboom JW. Effects of CYP2C19 genotype on outcomes of clopidogrel treatment. N Engl J Med 2010;363:1704–14.
[26] Sibbing D, Koch W, Gebhard D, Schuster T, Braun S, Stegherr J, Morath T, Schomig A, von Beckerath N, Kastrati A. Cytochrome 2C19*17 allelic variant, platelet aggregation, bleeding events, and stent thrombosis in clopidogrel-treated patients with coronary stent placement. Circulation 2010;121:512–8.
[27] Davi G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med 2007;357:2482–94.
[28] Trumel C, Payrastre B, Plantavid M, Hechler B, Viala C, Presek P, Martinson EA, Cazenave JP, Chap H, Gachet C. A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. Blood 1999;94:4156–65.
[29] Schwarz UR, Geiger J, Walter U, Eigenthaler M. Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets–definition and detection of ticlopidine/clopidogrel effects. Thromb Haemost 1999;82:1145–52.
[30] Savi P, Pereillo JM, Uzabiaga MF, Combalbert J, Picard C, Maffrand JP, Pascal M, Herbert JM. Identification and biological activity of the active metabolite of clopidogrel. Thromb Haemost 2000;84:891–6.
[31] Woulfe D, Yang J, Brass L. ADP and platelets: the end of the beginning. J Clin Invest 2001;107:1503–5.
[32] Stein B, Fuster V, Israel DH, Cohen M, Badimon L, Badimon JJ, Chesebro JH. Platelet inhibitor agents in cardiovascular disease: an update. J Am Coll Cardiol 1989;14: 813–36.
[33] Kazui M, Nishiya Y, Ishizuka T, Hagihara K, Farid NA, Okazaki O, Ikeda T, Kurihara A. Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. Drug Metab Dispos 2010;38:92–9.
[34] Caplain H, Donat F, Gaud C, Necciari J. Pharmacokinetics of clopidogrel. Semin Thromb Hemost 1999;25(Suppl 2):25–8.
[35] Hulot JS, Bura A, Villard E, Azizi M, Remones V, Goyenvalle C, Aiach M, Lechat P, Gaussem P. Cytochrome P450 2C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects. Blood 2006;108: 2244–7.
[36] Home Page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee. Available from:http://www.cypalleles.ki.se/index.htm Mar. 7, 2011 Accessed.
[37] de Morais SM, Wilkinson GR, Blaisdell J, Nakamura K, Meyer UA, Goldstein JA. The major genetic defect responsible for the polymorphism of S-mephenytoin metabolism in humans. J Biol Chem 1994;269:15419–22.
[38] De Morais SM, Wilkinson GR, Blaisdell J, Meyer UA, Nakamura K, Goldstein JA. Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin metabolism in Japanese. Mol Pharmacol 1994;46:594–8.
[39] Sim SC, Risinger C, Dahl ML, Aklillu E, Christensen M, Bertilsson L, Ingelman-Sundberg M. A common novel CYP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. Clin Pharmacol Ther 2006;79:103–13.
[40] Ferguson RJ, De Morais SM, Benhamou S, Bouchardy C, Blaisdell J, Ibeanu G, Wilkinson GR, Sarich TC, Wright JM, Dayer P, Goldstein JA. A new genetic defect in human CYP2C19: mutation of the initiation codon is responsible for poor metabolism of S-mephenytoin. J Pharmacol Exp Ther 1998;284:356–61.
[41] Xiao ZS, Goldstein JA, Xie HG, Blaisdell J, Wang W, Jiang CH, Yan FX, He N, Huang SL, Xu ZH, Zhou HH. Differences in the incidence of the CYP2C19 polymorphism affecting the S-mephenytoin phenotype in Chinese Han and Bai populations and identification of a new rare CYP2C19 mutant allele. J Pharmacol Exp Ther 1997;281:604–9.
[42] Ibeanu GC, Blaisdell J, Ghanayem BI, Beyeler C, Benhamou S, Bouchardy C, Wilkinson GR, Dayer P, Daly AK, Goldstein JA. An additional defective allele, CYP2C19*5, contributes to the S-mephenytoin poor metabolizer phenotype in Caucasians. Pharmacogenetics 1998;8:129–35.
[43] Ibeanu GC, Blaisdell J, Ferguson RJ, Ghanayem BI, Brosen K, Benhamou S, Bouchardy C, Wilkinson GR, Dayer P, Goldstein JA. A novel transversion in the intron 5 donor splice junction of CYP2C19 and a sequence polymorphism in exon 3 contribute to the poor metabolizer phenotype for the anticonvulsant drug S-mephenytoin. J Pharmacol Exp Ther 1999;290:635–40.
[44] Blaisdell J, Mohrenweiser H, Jackson J, Ferguson S, Coulter S, Chanas B, Xi T, Ghanayem B, Goldstein JA. Identification and functional characterization of new potentially defective alleles of human CYP2C19. Pharmacogenetics 2002;12:703–11.
[45] Rudberg I, Mohebi B, Hermann M, Refsum H, Molden E. Impact of the ultrarapid CYP2C19*17 allele on serum concentration of escitalopram in psychiatric patients. Clin Pharmacol Ther 2008;83:322–7.
[46] Lee SJ, Kim WY, Kim H, Shon JH, Lee SS, Shin JG. Identification of new CYP2C19 variants exhibiting decreased enzyme activity in the metabolism of S-mephenytoin and omeprazole. Drug Metab Dispos 2009;37:2262–9.
[47] Desta Z, Zhao X, Shin JG, Flockhart DA. Clinical significance of the cytochrome P450 2C19 genetic polymorphism. Clin Pharmacokinet 2002;41:913–58.
[48] Xie HG, Kim RB, Wood AJ, Stein CM. Molecular basis of ethnic differences in drug disposition and response. Annu Rev Pharmacol Toxicol 2001;41:815–50.
[49] Wedlund PJ. The CYP2C19 enzyme polymorphism. Pharmacology 2000;61:174–83.
[50] Fontana P, Hulot JS, De Moerloose P, Gaussem P. Influence of CYP2C19 and CYP3A4 gene polymorphisms on clopidogrel responsiveness in healthy subjects. J Thromb Haemost 2007;5:2153–5.
[51] Giusti B, Gori AM, Marcucci R, Saracini C, Sestini I, Paniccia R, Valente S, Antoniucci D, Abbate R, Gensini GF. Cytochrome P450 2C19 loss-of-function polymorphism, but not CYP3A4 IVS10 + 12G/A and P2Y12 T744C polymorphisms, is associated with response variability to dual antiplatelet treatment in high-risk vascular patients. Pharmacogenet Genomics 2007;17:1057–64.
[52] Kim KA, Park PW, Hong SJ, Park JY. The effect of CYP2C19 polymorphism on the pharmacokinetics and pharmacodynamics of clopidogrel: a possible mechanism for clopidogrel resistance. Clin Pharmacol Ther 2008;84:236–42.
[53] Frere C, Cuisset T, Morange PE, Quilici J, Camoin-Jau L, Saut N, Faille D, Lambert M, Juhan-Vague I, Bonnet JL, Alessi MC. Effect of cytochrome p450 polymorphisms on platelet reactivity after treatment with clopidogrel in acute coronary syndrome. Am J Cardiol 2008;101:1088–93.
[54] Trenk D, Hochholzer W, Fromm MF, Chialda LE, Pahl A, Valina CM, Stratz C, Schmiebusch P, Bestehorn HP, Buttner HJ, Neumann FJ. Cytochrome P450 2C19 681G>A polymorphism and high on-clopidogrel platelet reactivity associated with adverse 1-year clinical outcome of elective percutaneous coronary intervention with drug-eluting or bare-metal stents. J Am Coll Cardiol 2008;51:1925–34.
[55] Frere C, Cuisset T, Gaborit B, Alessi MC, Hulot JS. The CYP2C19*17 allele is associated with better platelet response to clopidogrel in patients admitted for non-ST acute coronary syndrome. J Thromb Haemost 2009;7:1409–11.
[56] Hulot JS, Collet JP, Silvain J, Pena A, Bellemain-Appaix A, Barthelemy O, Cayla G, Beygui F, Montalescot G. Cardiovascular risk in clopidogrel-treated patients according to cytochrome P450 2C19*2 loss-of-function allele or proton pump inhibitor coadministration: a systematic meta-analysis. J Am Coll Cardiol 2010;56: 134–43.