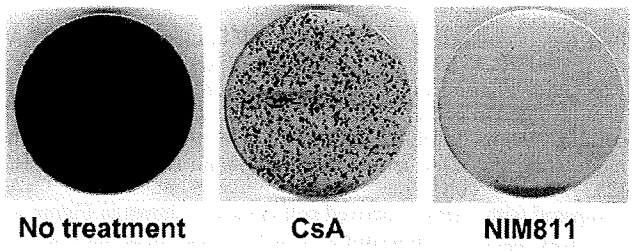

Fig. 2. Cyclophilin (CyP) inhibitor (CPI)-resistant colony emergence. MH14#12 cells were treated either without (left panel) or with 2 μg/mL CPIs, cyclosporin A (CsA) (middle panel), and NIM811 (right panel), in the presence of 700 μg/mL G418. Following 4 weeks in culture, cells were fixed and stained with crystal violet.

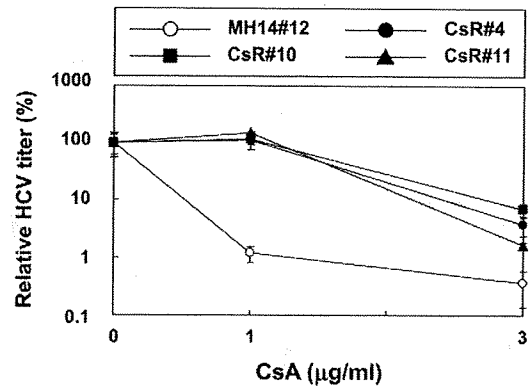

Fig. 3. Cyclosporin A (CsA) responses of the hepatitis C virus (HCV) replicon clones surviving the selection with CsA and G418. MH14#12 cells and three MH14#12-derived cell clones that survived double selection with G418 and CsA, CsR#4, CsR#10, and CsR#11, were treated with 1 and 3 μg/mL CsA for 7 days, and the HCV-RNA titers were measured by real-time RT-PCR. The data represent the percentage of HCV-RNA level in cells either untreated or treated with CsA, and the dots represent the means of three independent experiments.

CCAGGACCCGUAUGCUU-3′; siCyPA459, 5′-CUUCUUG CUGGUCUUGCCAUU-3′) were synthesized (Yahima Pure Chemicals, Osaka, Japan). SiRNAs against CyP40 were purchased from Invitrogen (siCyP40-3) and from Ambion (siCyP40-4). Pre-designed siRNAs, siCyPC, siCyPE, siCyPF, and siCyPG were obtained from Ambion. Transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) with 20 nM siRNAs in the absence of CsA according to the manufacturer's protocol.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis. RT-PCR was performed as described previously[4] using the following primer sets: 5′-TGTTCTTCGACATTGCCGTC-3′ and 5′-CAGTCTTGGCAGTGCAGATG-3′ to detect mRNA for CyPA, 5′-TCTCCGAACGCAACATGAAG-3′ and 5′-CTGCGA TGATCACATCCTTC-3′to detect mRNA for CyPB, 5′-GGCGCA CTTGTGTTTTCTTC-3′ and 5′-TGCCATAGTGCTTCAGCTTG-3′ to detect mRNA for CyPC, 5′-TTTCGTGCACTGTGTACAGG-3′ and 5′-TTGGCTCTATCTGCTGTCTC-3′ to detect mRNA for CyP40, 5′-AGAGGAAGTGGACGACAAAG-3′ and 5′-GATGTCCATGTACACCTGAG-3′ to detect mRNA for CyPE, 5′-TGGAGCTGAAGGCAGATGTC-3′ and 5′-ACGTGACCG AACACAACATG-3′ to detect mRNA for CyPF, 5′-GAGTTGT CTCTTTCACAGAG-3′ and 5′-AACTGAGTATCCGTACCTCC-3′ to detect mRNA for CyPG, and 5′-ATGGGGAAGGTGAA GGTCGG-3′ and 5′-TGGAGGGATCTCGCTCCTGG-3′ to detect glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Results

Resistance emergence against individual CPIs. We have previously demonstrated the robust anti-HCV activities of CPIs, and it was reported that CPI significantly decreased HCV viral load in HCV-infected patients.[14,15] The problem of the drug-resistant HCV variants, hence, should be assessed *in vitro*, considering that practical efficacies of these inhibitors with long-term effectiveness are required in patients. In the first step of this study, we investigated the emergence of drug resistant replicon against CPIs. We treated MH14#12 cells, Huh7 cells carrying wild-type MH14 replicon with 2 μg/mL CsA, or the non-immunosuppressive analog NIM811 in the presence of 700 μg/mL G418 for 4 weeks. To visualize the appearance of drug-resistant clones, we stained cells after the selection. We observed colonies resistant to CsA, while we obtained few colonies under the treatment with the same concentration of NIM811 (Fig. 2).

Isolation and characterization of replicon cells resistant to CsA. To characterize the CsA-resistant HCV, we isolated the resistant clones following selection with 2 μg/mL CsA and 1000 μg/mL G418 for 4 weeks. We obtained several clones (named CsR cells), and examined their CsA responses. In contrast to the wild-type MH14#12 replicon cell, which showed an approximately 2-log reduction of HCV-RNA level by treatment with 1 μg/mL CsA for 7 days, all the clones isolated (the results of three representative clones, CsR#4, CsR#10, and CsR#11 cells are shown here) demonstrated resistant phenotypes against CsA with no significant reduction of HCV-RNA by CsA treatment at 1 μg/mL (Fig. 3). The resistance of these clones was thought to arise as a result of (1) mutations on the HCV-RNA genome or (2) alterations in cellular factors. To test the first possibility, we investigated whether HCV-RNA itself in CsR#11 could induce the CsA resistance to naïve cells. Fresh Huh7 cells were transfected with total RNA, including HCV replicon RNA, extracted from CsR#11 cells or MH14#12 cells as controls and cultured for 3 weeks in the presence of G418 (Fig. 1). The resulting colonies were isolated and propagated individually (named cell clones from total RNA of wild-type MH14#12, MH14#12-1, MH14#12-4, and MH14#12-5 cells, and those from CsR#11, CsR#11-2, CsR#11-3, and CsR#11-5 cells). The HCV-RNA titer in MH14#12-derived cells was reduced approximately to 100th by treatment with 1 μg/mL CsA for 7 days (Fig. 4). In contrast, cell clones generated from CsR#11 cells retained a normal HCV titer level after treatment with CsA, indicating that they had lost their sensitivity to CsA. Thus, it was suggested that the CsA-resistant profile in CsR#11 cells was attributed to its HCV-RNA.

D320E mutation in NS5A confers HCV replicon resistance to CsA. In order to identify the mutation in the HCV genome that resulted in the resistance to CsA, HCV subgenomic RNA isolated from CsR#11 cells was sequenced across the subgenomic region encoding non-structural proteins. We found three specific base changes that resulted in amino acid alteration including changes from glutamine to arginine, and isoleucine to threonine at positions 86 (Q86R) and 252 (I252T) in NS3, respectively, and a change from aspartic acid to glutamic acid at position 320 (D320E) in NS5A. Given that all these three mutations, Q86R, I252T, and D320E, were retained in every replicon in CsR#11-2, CsR#11-3, and CsR#11-5 cells, it is likely that they are inherited from CsR#11 cells and are associated with the acquired CsA-resistant phenotype. To examine this possibility, we synthesized replicon RNA carrying all these three mutations and established cells carrying these replicons. The resultant cell clones were named Q86R/I252T/D320E-1 and -3 cells. Treatment of these cells with 1 μg/mL CsA decreased the HCV titer only

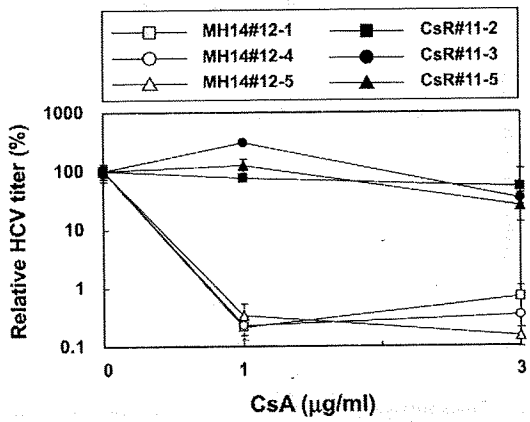

Fig. 4. Hepatitis C virus (HCV) RNA alteration contributed to cyclosporin A (CsA)-resistance. Total RNA extracted from CsA-resistant CsR#11 cells or that from wild-type MH14#12 cells as a control was transfected into Huh7 cells. Colonies established after 4-week selection with G418 were isolated, propagated individually, and tested for CsA response. Three cell clones derived from MH14#12 cells, MH14#12-1, MH14#12-4, and MH14#12-5 cells, and three cell clones from CsR#11 cells, CsR#11-2, CsR#11-3, and CsR#11-5 cells, were treated with 1 and 3 μg/mL CsA for 7 days. HCV-RNA titers were quantified by real-time RT-PCR analysis. The dots represent the means of three independent experiments.

by 1 log, in contrast to the wild-type MH14 clone, in which CsA decreased HCV-RNA by more than 2 logs under the same experimental condition (Fig. 5b). Thus, these mutations were demonstrated to confer CsA resistance; in addition to this, some cellular factors in Huh7 cells may also play minor roles in modulating the CsA sensitivities, given the result that Q86R/I252T/D320E cell clones were relatively sensitive to CsA compared with CsR#11-derived cell clones as shown in Figure 4. We next aimed to determine which of the three mutations, Q86R/I252T/D320E, was responsible for the CsA resistant phenotype, and individual mutations were engineered back into the wild-type MH14 replicon and stable replicon cells were produced as described above. Among three single amino acid mutations, the I252T mutation in NS3 resulted in a significant reduction in replication fitness (Fig. 5a), and almost failed to produce cell colonies. Cell clones harboring MH14 with both Q86R and D320E mutations, Q86R/D320E-2 and Q86R/D320E-3 cells, showed reduced sensitivity to CsA that was comparable to the levels in Q86R/I252T/D320E cells, suggesting Q86R and/or D320E mutation(s) was enough to confer the resistance. Subsequently, we treated the replicon cell clones carrying MH14 with either Q86R or D320E mutation alone, Q86R (Q86R-1 and -4 cells) and D320E (D320E-1 and -2 cells) cells, with CsA for 1 week. The titer of Q86R replicons was reduced to less than 100th by CsA treatment at a concentration of 1 μg/mL, similar to the wild type. In contrast, HCV replicon with D320E mutation in NS5A exhibited reduced sensitivity to CsA, resulting in little reduction of HCV-RNA by the treatment with 1 μg/mL CsA (Fig. 5b). Q86R mutation considerably enhanced colony

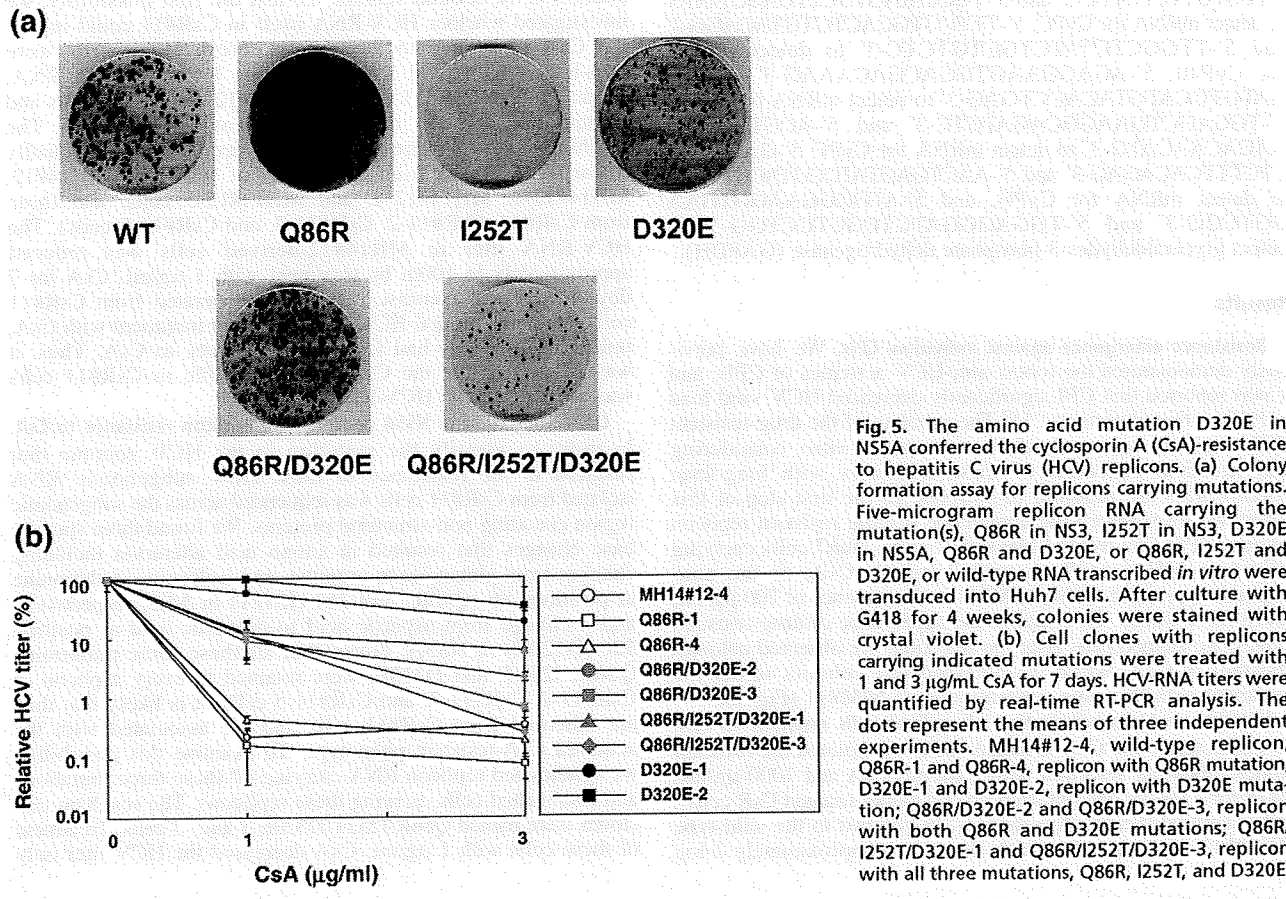

Fig. 5. The amino acid mutation D320E in NS5A conferred the cyclosporin A (CsA)-resistance to hepatitis C virus (HCV) replicons. (a) Colony formation assay for replicons carrying mutations. Five-microgram replicon RNA carrying the mutation(s), Q86R in NS3, I252T in NS3, D320E in NS5A, Q86R and D320E, or Q86R, I252T and D320E, or wild-type RNA transcribed *in vitro* were transduced into Huh7 cells. After culture with G418 for 4 weeks, colonies were stained with crystal violet. (b) Cell clones with replicons carrying indicated mutations were treated with 1 and 3 μg/mL CsA for 7 days. HCV-RNA titers were quantified by real-time RT-PCR analysis. The dots represent the means of three independent experiments. MH14#12-4, wild-type replicon; Q86R-1 and Q86R-4, replicon with Q86R mutation; D320E-1 and D320E-2, replicon with D320E mutation; Q86R/D320E-2 and Q86R/D320E-3, replicon with both Q86R and D320E mutations; Q86R/I252T/D320E-1 and Q86R/I252T/D320E-3, replicon with all three mutations, Q86R, I252T, and D320E.

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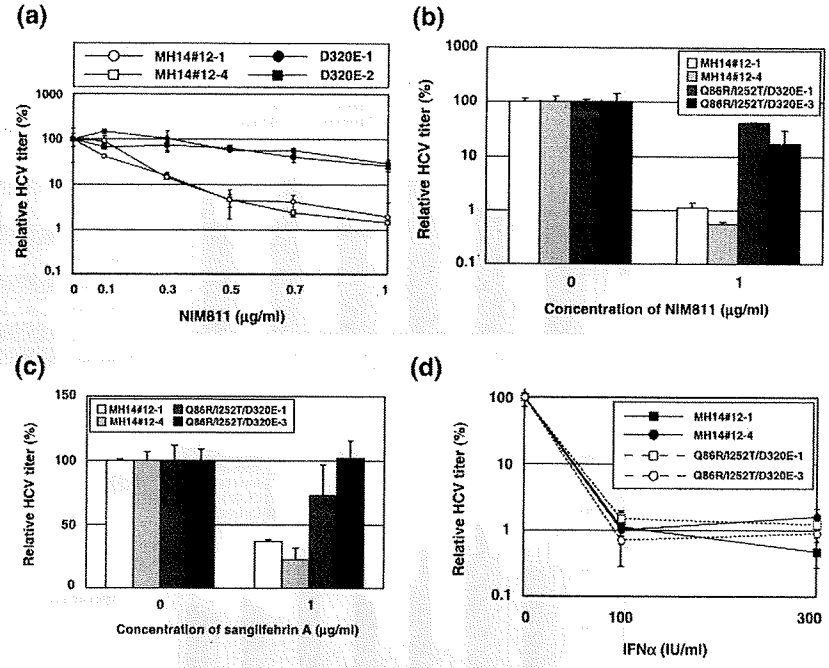

Fig. 6. Cyclosporin A (CsA)-resistant replicons demonstrated cross resistance to additional cyclophilin (CyP) inhibitors, NIM811 and sanglifehrin A (SFA), but not interferon (IFN)-α. MH14#12-1, MH14#12-4, D320E-1 and D320E-2 cells were treated with NIM811 at 0.1, 0.3, 0.5, 0.7 and 1 μg/mL (a), and MH14#12-1, MH14#12-4, Q86R/I252T/D320E-1, and Q86R/I252T/D320E-3 cells were treated with 1 μg/mL NIM811 (b), 1 μg/mL SFA (c), and 100 and 300 IU/mL IFN-α (d) for 7 days. The amounts of hepatitis C virus (HCV) RNA were quantified by real-time RT-PCR analysis. The data represent the means of three independent experiments.

formation efficiency and the D320E mutation showed little significant effect on the efficiency (Fig. 5a). Thus, the D320E mutation in NS5A was suggested as a sufficient factor to induce HCV replicon resistance to CsA, while the Q86R mutation was likely not to contribute to the resistance but to augment the efficiency of HCV replication itself.

The point mutation in NS5A conferred resistance to CPIs. Next, we examined cross-resistance between CsA and other CPIs or IFNα using the CsA-resistant replicon we produced above. Treatment with 0.1–1 μg/mL NIM811 for 7 days showed that the response to NIM811 of D320E-1 and -2 cells was less compared with that of MH14#12-1 and -4 cells, indicating that a CsA-resistant clone also acquired NIM811 resistance (Fig. 6a). A similar result was seen using Q86R/I252T/D320E cells (Fig. 6b). We then tested the anti-HCV activity of SFA, an additional CPI possessing distinct chemical backbone from those of cyclosporins.[16,17] Treatment with 1 μg/mL SFA reduced HCV replication in the wild-type cells, MH14#12-1, and -4 cells; however, it did not significantly reduce replication in Q86R/I252T/D320E cells (Fig. 6c). These results demonstrate that the CsA-resistant cells described in this study were also resistant to additional CPIs, confirming that these two compounds exerted anti-HCV effects via targeting CyP. Finally, we treated Q86R/I252T/D320E cell clones with 100 and 300 IU/mL IFNα for 7 days, and HCV-RNA titers were reduced by 2 logs in both clonal cell lines examined, Q86R/I252T/D320E-1 and Q86R/I252T/D320E-3 cells, as well as in wild-type MH14#12-derived cells, MH14#12-1, and MH14#12-4 cells (Fig. 6d). These results suggested no cross-resistance between CsA and IFNα, consistent with the previous report that the anti-HCV activity of CsA was independent of the IFNα signaling pathway.[18]

The role of CyP subtypes in HCV replication. We have previously reported that CyPB played a significant role in the efficient replication of HCV and CsA inhibited CyPB-mediated regulation of HCV replication. We have also suggested the involvement of other CyP subtypes in HCV replication.[19] To gain further insight into mechanisms underlying the anti-HCV properties of CPIs, we examined the roles of individual CyP subtypes in HCV replication in the wild-type MH14#12-1 and -4 replicon cells. In order to achieve this we knocked down CyPB with siRNAs (Fig. 7d), siCyPB-1 and -2, and found that this procedure reduced the amount of replicons to approximately half the initial level (Fig. 7c), a result consistent with the previous reports. Knockdown of CyPC, CyPE, CyPF, and CyPG (Fig. 7b) did not significantly affect the viral replication under these experimental conditions (Fig. 7a). Some groups have also suggested a role of CyPA in HCV replication.[20,21] Then, we synthesized individual siRNAs reported so far to be effective against CyPA, siCyPA-161, siCyPA-285, and siCyPA-459, and transfected them using a reagent with low cytotoxic activity to knock down endogenous CyPA (Fig. 7d). As shown in Figure 7c, the siRNAs directed against CyPA reduced HCV titers in MH14#12-1, and -4 cells. We previously observed that knockdown of CyPA little affected HCV replication in MH14 cells.[5] Here, by using a new transfection reagent with less cytotoxicity and higher knockdown efficiency, we observed the effect of CyPA knockdown on HCV replication, which suggests that CyPA-mediated regulation of HCV replication is strictly influenced by CyPA's expression level and cellular condition. Under this experimental condition, our RNAi experiments also displayed that knockdown of CyP40 (Fig. 7g), alternatively known as peptidylprolyl isomerase D (NM_005038), decreased the HCV titer (Fig. 7f) without significant cytotoxic effects, presenting CyP40 as additional cellular factor required for HCV replication.

CyPA was related to the CsA-resistant phenotype. We next asked which CyP subtype among CyPA, B, and 40 was related to the CsA resistance observed in our clones. To answer this question, we performed RNAi experiments in the CsA-resistant cell lines, CsR#11-2 and CsR#11-3 cells. Transfection of these cells with specific CyPB or CyP40 siRNAs resulted in the reduction of each subtype (Fig. 7d,g) and decreased the amount of HCV-RNA in CsR#11-derived cells and wild-type MH14#12-derived cells by approximately 50% (Fig. 7c,f). Thus, CyPB and CyP40 were likely to play roles in viral replication, even in the CsA-resistant cells. However, relative HCV titers were not reduced by CyPA knockdown in these CsA-resistant cells in contrast to the case with the wild-type replicon cells (Fig. 7c). A similar resistant phenotype to CyPA knockdown was observed in D320E

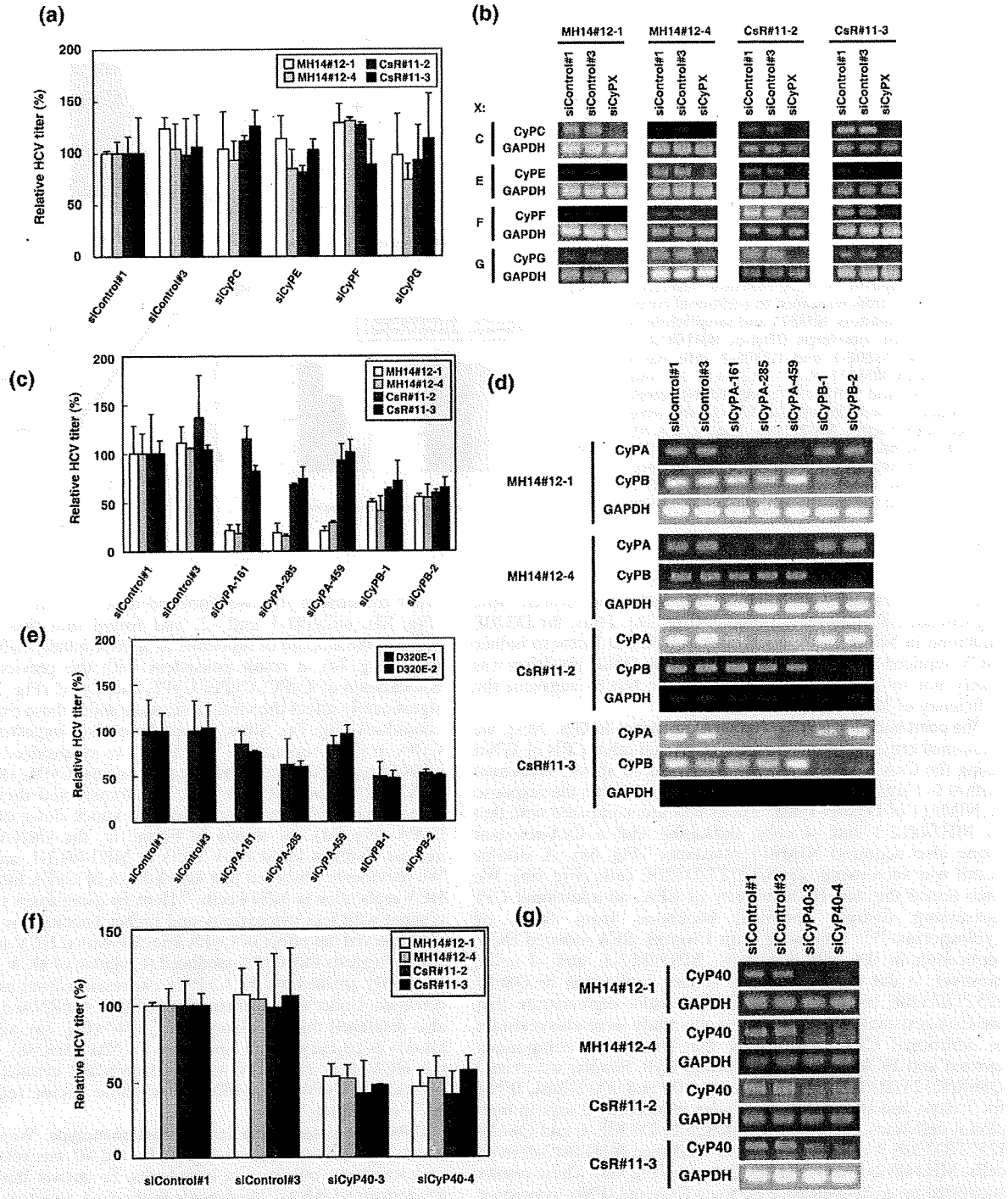

Fig. 7. Cyclophilin (CyP) subtypes related to anti-hepatitis C virus (HCV) effect of CyP inhibitor. MH14#12-derived cells, MH14#12-1 and MH14#12-4 cells, and CsR#11-2 and CsR#11-3 cells, were transfected with siRNAs specific for CyPC (siCyPC), CyPE (siCyPE), CyPF (siCyPF), and CyPG (siCyPG) (a); or those specific for CyPA (siCyPA-161, siCyPA-285, and siCyPA-459) and CyPB (siCyPB-1 and siCyPB-2) (c); or those specific for CyP40 (siCyP40-3 and siCyP40-4) (f); or randomized siRNA controls (siControl#1 and siControl#3). D320E cells were also transfected with the above siRNAs specific for either CyPA or CyPB (e). At 5 days post-transfection, the levels of HCV-RNA were quantified by real-time RT-PCR analysis. The mRNA levels of individual CyP subtypes, CyPC, CyPE, CyPF, and CyPG (CyPX corresponds to each CyP subtype indicated on the left side of the panels) (b), or CyPA and CyPB (d), or CyP40 (g) were measured using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as internal controls by RT-PCR analysis at 5 days post-transfection. The data represent the means of three independent experiments.

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cell clones (Fig. 7c), showing that CyPA was related to the CsA-resistance conferred by D320E mutation. The CsA-resistant clones obtained in this study were likely to have acquired CyPA independence for efficient HCV replication.

Discussion

Given that CPIs suppressed HCV viral load in cell culture and in patients with chronic hepatitis C,[14,15] CPIs are expected to be new anti-HCV agents. It is important to further reveal the factors related to CPI's anti-HCV activities, thinking over the practical use of CPIs with maximized efficacy and high specificity facing challenges such as side effects and the emergence of resistance to them in clinical settings. Here, we isolated and characterized a variant resistant to CPIs using a HCV subgenomic replicon system. A mutation in NS5A, D320E, was shown to confer the CPI-resistance to HCV replicon, resulting in CyPA independence for efficient viral replication. In addition, assessment of a wide range of CyP subtype knockdown experiments found CyP40 to be a new contributor to HCV replication.

Of the mutations identified, Q86R substitution in NS3 dramatically enhanced the capacity of replication. This mutation was observed as compensatory mutation[22] following the selection of replicons resistant to protease inhibitors SCH503034[23] and SCH6.[24] In addition, this mutation also appeared following the passaging of replicon cells in the absence of drug pressure.[25,26] In actuality, this mutation did not contribute to CsA resistance in the replicon cells (Fig. 5b), and thus was thought to be an adaptive mutation similar to that suggested in previous reports. I252T mutation in NS3, on the other hand, severely reduced the replicative fitness of HCV. The significance of I252T mutation under CsA treatment remains to be studied. The alteration of amino acid residue in NS5A, D320E, resulted in the conversion of HCV replicon to that of the CsA-resistant phenotype. There have been no reports of a link between NS5A and individual CyP subtype in the context of HCV replication, though mutations in NS5A were found to be keys for the acquisition of CsA resistance.[27] We have previously reported that CyPB was important for viral replication, but NS5A did not interact with CyPB in MH14 cells.[5] Indeed, in cells harboring replicons with D320E mutation, CyPB was found to contribute to viral replication but was not related to CsA resistance, as knockdown of CyPB diminished the viral titer to approximately half, similar to the case of the wild type. Therefore, other CyP molecules crucial for viral replication were suggested to be involved in the phenomenon of the CsA resistance. CyPA is another CyP subtype recently published to be critical for HCV replication in connection with viral polymerase.[20,21] Our CsA-resistant replicon cells displayed resistance to CyPA knockdown when compared to wild-type replicon, suggesting that CyPA participated in the replication process and the CsA resistance was due in part to resistance to CyPA inhibition. Therefore, it might be possible that NS5A functions coordinated with CyPA for viral replication and D320E mutation could contribute to enhancement of the relation. But NS5A was unable to bind CyPA *in vitro*.[5] NS5A might be regulated by CyPA associated with other cellular or viral factors during HCV replication. The fact that the D320 falls upon one of the two discontinuous domains needed for the interaction with NS5B to functionally modulate it[28,29] lead us to presume influence of NS5A on the reported NS5B–CyPA interaction.[21] In addition to CyPA and CyPB, which have been published to be cellular factors required for HCV replication, the results suggested that another CyP subtype, CyP40, contributed to viral replication. Acting as a molecular chaperone, it is conceivable that CyP40 directly interacts with viral proteins to boost their functions, similar to CyPA and CyPB. Heat shock protein (Hsp) 90 is a well-known chaperone forming complex with CyP40. Recently, Hsp90 was shown to be harnessed by HCV NS5A via the FK-506 binding protein 8 (FKBP8) bridge. FKBP8 is a homologous immunophilin of CyP40 that is required for viral replication.[30] This result led to the hypothesis that CyP40 serves as a linker between viral proteins and Hsp90. CyP40 is also known to associate with estrogen receptor (ESR) and we have published that ESRα escorted NS5B to replication complex (RC).[31] We also speculate CyP40 connected to ESRα may be important for the recruitment or functional reinforcement of viral and cellular factors for HCV replication in RC. Among these CyP subtypes, CyPA dependency was suggested to be one of the determinants of CsA sensitivity. Interestingly, CyPB and CyP40 play significant role in HCV replication even in CsA-resistant replicon cells. Another CPI, NIM811, is also likely to target CyPA, at least in part, to suppress HCV replication, given the cross-resistance of CsA-resistant replicon to MIN811. However, there is still also the possibility that other CyPs mediate anti-HCV effect of NIM811, which needs to be elucidated in future study.

Understanding the profile of CPI-resistance mutations in the HCV genome and the viral and cellular factors involved will aid in the progression of CPI-centered strategies preparing for the problem of drug resistance. In addition, the cells harboring CPI-resistant replicons established here may prove beneficial for further characterization of resistance mechanisms and for the screening of novel compounds with the potential of clinical application to defeat CPI-resistant variants. Also, CyP40 as a contributor to HCV replication could become another specific antiviral target. The information arising from this study is expected to contribute to the successful use of CPIs against a liver carcinogen, HCV.

Acknowledgments

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References

1 Sarbah SA, Younossi ZM. Hepatitis C: an update on the silent epidemic. *J Clin Gastroenterol* 2000; **30**: 125–43.
2 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958–65.
3 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975–82.
4 Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 2003; **38**: 1282–8.
5 Watashi K, Ishii N, Hijikata M *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 2005; **19**: 111–22.
6 Manns MP, Foster GR, Rockstroh JK, Zeuzem S, Zoulim F, Houghton M. The way forward in HCV treatment – finding the right path. *Nat Rev Drug Discov* 2007; **6**: 991–1000.
7 McGovern BH, Abu Dayyeh BK, Chung RT. Avoiding therapeutic pitfalls: the rational use of specifically targeted agents against hepatitis C infection. *Hepatology* 2008; **48**: 1700–12.
8 Melnikova I. Hepatitis C therapies. *Nat Rev Drug Discov* 2008; **7**: 799–800.

9 Cordes F, Kaiser R, Selbig J. Bioinformatics approach to predicting HIV drug resistance. *Expert Rev Mol Diagn* 2006; **6**: 207–15.
10 Mo H, Lu L, Dekhtyar T *et al.* Characterization of resistant HIV variants generated by *in vitro* passage with lopinavir/ritonavir. *Antiviral Res* 2003; **59**: 173–80.
11 Molla A, Korneyeva M, Gao Q *et al.* Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. *Nat Med* 1996; **2**: 760–6.
12 Shulman N, Winters M. A review of HIV-1 resistance to the nucleoside and nucleotide inhibitors. *Curr Drug Targets Infect Disord* 2003; **3**: 273–81.
13 Watashi K, Hijikata M, Tagawa A, Doi T, Marusawa H, Shimotohno K. Modulation of retinoid signaling by a cytoplasmic viral protein via sequestration of Sp110b, a potent transcriptional corepressor of retinoic acid receptor, from the nucleus. *Mol Cell Biol* 2003; **23**: 7498–509.
14 Flisiak R, Dumont JM, Crabbe R. Cyclophilin inhibitors in hepatitis C viral infection. *Expert Opin Invest Drugs* 2007; **16**: 1345–54.
15 Flisiak R, Horban A, Gallay P *et al.* The cyclophilin inhibitor Debio-025 shows potent anti-hepatitis C effect in patients coinfected with hepatitis C and human immunodeficiency virus. *Hepatology* 2008; **47**: 817–26.
16 Zhang LH, Liu JO. Sanglifehrin A, a novel cyclophilin-binding immunosuppressant, inhibits IL-2-dependent T cell proliferation at the G1 phase of the cell cycle. *J Immunol* 2001; **166**: 5611–8.
17 Zenke G, Strittmatter U, Fuchs S *et al.* Sanglifehrin A, a novel cyclophilin-binding compound showing immunosuppressive activity with a new mechanism of action. *J Immunol* 2001; **166**: 7165–71.
18 Goto K, Watashi K, Murata T, Hishiki T, Hijikata M, Shimotohno K. Evaluation of the anti-hepatitis C virus effects of cyclophilin inhibitors, cyclosporin A, and NIM811. *Biochem Biophys Res Commun* 2006; **343**: 879–84.
19 Ishii N, Watashi K, Hishiki T *et al.* Diverse effects of cyclosporine on hepatitis C virus strain replication. *J Virol* 2006; **80**: 4510–20.
20 Nakagawa M, Sakamoto N, Tanabe Y *et al.* Suppression of hepatitis C virus replication by cyclosporin A is mediated by blockade of cyclophilins. *Gastroenterology* 2005; **129**: 1031–41.
21 Yang F, Robotham JM, Nelson HB, Irsigler A, Kenworthy R, Tang H. Cyclophilin a is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance *in vitro*. *J Virol* 2008; **82**: 5269–78.
22 Xavier LL, Moya A, Gonzalez-Candelas F. Mapping natural polymorphisms of hepatitis C virus NS3/4A protease and antiviral resistance to inhibitors in worldwide isolates. *Antivir Ther* 2008; **13**: 481–94.
23 Tong X, Chase R, Skelton A, Chen T, Wright-Minogue J, Malcolm BA. Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034. *Antiviral Res* 2006; **70**: 28–38.
24 Yi M, Tong X, Skelton A *et al.* Mutations conferring resistance to SCH6, a novel hepatitis C virus NS3/4A protease inhibitor. Reduced RNA replication fitness and partial rescue by second-site mutations. *J Biol Chem* 2006; **281**: 8205–15.
25 Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000; **290**: 1972–4.
26 Krieger N, Lohmann V, Bartenschlager R. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J Virol* 2001; **75**: 4614–24.
27 Fernandes F, Poole DS, Hoover S *et al.* Sensitivity of hepatitis C virus to cyclosporine A depends on nonstructural proteins NS5A and NS5B. *Hepatology* 2007; **46**: 1026–33.
28 Shirota Y, Luo H, Qin W *et al.* Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. *J Biol Chem* 2002; **277**: 11149–55.
29 Shimakami T, Hijikata M, Luo H *et al.* Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. *J Virol* 2004; **78**: 2738–48.
30 Okamoto T, Nishimura Y, Ichimura T *et al.* Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *Embo J* 2006; **25**: 5015–25.
31 Watashi K, Inoue D, Hijikata M, Goto K, Aly HH, Shimotohno K. Anti-hepatitis C virus activity of tamoxifen reveals the functional association of estrogen receptor with viral RNA polymerase NS5B. *J Biol Chem* 2007; **282**: 32765–72.

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Gene-expression profiles in human nasal polyp tissues and identification of genetic susceptibility in aspirin-intolerant asthma

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Clinical & Experimental Allergy

Summary

Background Aspirin-intolerant asthma (AIA) is a subtype of asthma induced by non-steroidal anti-inflammatory drugs and characterized by an aggressive mucosal inflammation of the lower airway (asthma) and the upper airways (rhinitis and nasal polyp). The lower airway lesion and the nasal polyp in AIA are postulated to have common pathogenic features involving aspirin sensitivity that would be reflected in the gene expression profile of AIA polyps.

Objective This study was conducted to clarify the pathogenesis of AIA using gene expression analysis in nasal polyps, and identify genetic susceptibilities underlying AIA in a case–control association study.

Methods Global gene expression of nasal polyps from nine AIA patients was examined using microarray technology in comparison with nasal polyps from five eosinophilic sinusitis (ES) patients, a related disease lacking aspirin sensitivity. Based on the AIA-specific gene expression profile of nasal polyp, candidate genes for AIA susceptibility were selected and screened by a case–control design of 219 AIA patients, 374 non-asthmatic control (CTR), and 282 aspirin-tolerant asthmatic (ATA) subjects.

Results One hundred and forty-three elevated and three decreased genes were identified as AIA-specific genes that were enriched in immune response according to Gene Ontology analysis. In addition, a *k*-means-based algorithm was applied to cluster the genes, and a subclass characteristic of AIA comprising 18 genes that were also enriched in immune response was identified. By examining the allelic associations of single nucleotide polymorphisms (SNPs) of AIA candidate genes relevant to an immune response with AIA, two SNPs, one each of *INDO* and *IL1R2*, showed significant associations with AIA ($P = 0.011$ and 0.026 after Bonferroni's correction, respectively, in AIA vs. CTR). In AIA–ATA association analysis, modest associations of the two SNPs with AIA were observed.

Conclusion These results indicate that *INDO* and *IL1R2*, which were identified from gene expression analyses of nasal polyps in AIA, represent susceptibility genes for AIA.

Keywords aspirin-intolerant asthma, candidate genes, genetic association, genome-wide gene expression, single nucleotide polymorphism

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Introduction

In some asthmatic patients, aspirin and several other non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase enzymes (COXs) induce a severe asthmatic attack, a disease known as aspirin-intolerant asthma (AIA) [1, 2]. Several large surveys have concluded that the incidence of AIA in adult asthmatic patients is 5–15% based on patients' histories alone, but the frequency becomes two to three times higher when adult asthmatic

patients are challenged with aspirin. In women, AIA is overrepresented in a ratio of 2.3 : 1 and is more severe and has an earlier onset. AIA patients have typical clinical features including asthma, aspirin sensitivity, and bilateral nasal polyps, known as Samter's triad. Despite the well-defined pharmacological trigger, the molecular pathogenesis of AIA is still unclear. The usual hypothesis is a disturbance in the metabolism of arachidonic acid, because aspirin and NSAIDs target COXs, key enzymes of the prostaglandin biosynthetic pathway. However, the precise pathogenesis requires further investigation.

There is a moderate genetic background in AIA: the European Network on Aspirin-Induced Asthma found that 5.8% of 500 AIA patients had a family history of aspirin sensitivity [3]. First, a polymorphism in the promoter of leukotriene C_4 synthase, A-444C, was reported to be associated with AIA in Polish patients [4, 5]. A recent report showed that a haplotype of the 5-lipoxygenase gene was weakly associated with AIA in a Korean population [6]. With an extensive candidate gene analysis related to arachidonic acid metabolism, our group reported that single nucleotide polymorphisms (SNPs) in the prostaglandin E_2 receptor subtype 2 gene were significantly associated with AIA, and the functional impact of a promoter variant was further demonstrated [7]. Most recently, SNPs in prostaglandin E_2 receptor subtype 3 gene were associated in Korean population [8].

In the past few years, microarray techniques for gene expression profiling have been applied to a wide range of biological problems and have contributed to the discoveries of complex networks of biochemical processes underlying complex diseases. Microarray techniques have also helped to identify novel biomarkers, disease subtypes, and discrepancies of gene expression in human populations. Despite the advances in microarray techniques, application of the technology to identify susceptibility genes underlying complex diseases appears to be unsuccessful so far, with some exceptions [9, 10].

AIA is characterized by an aggressive mucosal inflammation of the lower airway (asthma) and the upper airways (rhinitis and nasal polyp). Rhinitis symptoms first occur in most AIA patients before the development of asthmatic intolerance to aspirin and other NSAIDs, whereas nasal polyps in AIA patients are first diagnosed at almost the same time aspirin intolerance appears [3]. We postulated that the lower airway lesion and the polyp in AIA have a common pathophysiology of aspirin intolerance, suggesting the nasal polyp as a pleiotropic genetic model of the bronchial inflammation of AIA. Global gene expression of the nasal polyps of AIA patients was examined using microarray technology for comparison with nasal polyps of eosinophilic sinusitis (ES) patients: ES is typically characterized by a nasal polyp with an inflammatory cell infiltration similar to that in an AIA polyp but without aspirin sensitivity, thus being an appropriate reference for the selection of AIA-specific genes.

Materials and methods

Nasal polyp tissues and Aspirin-Intolerant Asthma Subjects

Nasal polyp tissues for microarray analysis were obtained from nine Japanese patients (aged from 35 to 76 years, five males/four females) with AIA, five (aged from 34 to 73 years, three males/two females) with ES, and two (aged 61 and 71 years, both males) with only chronic sinusitis (CS) (Table 1). These patients had not been exposed to preoperative treatment with steroids for at least 1 year before surgery. According to the definition of rhinosinusitis, CS with nasal polyps with eosinophilic inflammatory features without fungal hyphae includes aspirin-sensitive and aspirin-tolerant types [11]. Thus, three groups of patients with nasal polyps were sequentially defined as follows: first, CS with nasal polyps was diagnosed based on clinical symptoms, such as nasal discharge, postnasal drip, headache, hyposmia, and nasal obstruction, and endonasal findings of muco-purulent secretion and nasal polyps with a paranasal shadow observed by CT examination [12]. Among CS patients with nasal polyps, ES patients were identified histologically by counting the number of eosinophils at ×200 magnification under light microscopy. Five fields were examined for each section,

Table 1. Clinical characteristics of patients with nasal polyps for microarray analysis

ID	Age/ gender	Parameters in peripheral blood: WBC (/mm^3)	Parameters in peripheral blood: Eosinophil (%)	Allergic rhinitis	Asthma	AIA episode
AIA#1	76/M	8000	3	–	+	+
AIA#2	48/M	5500	13	–	+	+
AIA#3	73/M	6500	3	–	+	+
AIA#4	59/F	9500	28	–	+	+
AIA#5	50/F	5720	14	–	+	+
AIA#6	40/M	9100	4	–	+	+
AIA#7	35/M	8800	6	–	+	+
AIA#8	50/F	6000	9	+	+	+
AIA#9	66/F	7000	8	–	+	+
ES#1	73/F	7200	2	–	+	–
ES#2	64/F	6400	23	–	+	–
ES#3	69/M	7700	4	+	–	–
ES#4	61/M	4900	5	–	+	–
ES#5	34/M	6300	3	+	+	–
CS#1	61/M	7400	10	–	+	–
CS#2	67/M	9700	10	–	–	–

M, male; F, female; WBC, white blood cell; –, no allergic rhinitis, no asthma, or no AIA episode; AIA, aspirin-intolerant asthma; CS, chronic sinusitis; ES, eosinophilic sinusitis.

and the average was considered to be the number of eosinophils infiltrating the sample. Nasal polyps having more than 100 eosinophils were classified as ES [12]. Among ES patients, those who had had apparent episodes of asthma attacks in response to aspirin and other NSAIDs were classified as AIA patients (AIA#1–9). The remaining five ES patients without AIA episodes (ES#1–5) had no troubles even after taking NSAIDs in postoperative courses during hospitalization. The oral provocation test for AIA patients was not performed in most of the patients due to potential risk, although severe reactions against the provocation were improbable [13], and only verbal history has yielded some false positives [14]. The ethics committees of Kagoshima University approved the study protocols, and each participant gave written informed consent.

DNA samples from 219 unrelated individuals with AIA (age: 55.7±13.5 years; 70 males/149 females) and 374 non-asthmatic controls (CTR) (age: 44.5±23.2 years; 181 males/193 females) were obtained as described previously [7]. For AIA-associated SNPs, 282 unrelated individuals with aspirin-tolerant asthma (ATA) (age: 56.0±16.1 years; 132 males/150 females) [7] were also genotyped, and used as asthmatic controls. The subjects were recruited at Niigata University Hospital, University of Tokyo Hospital, Nagoya University Hospital, Doai Memorial Hospital, and Kyushu University Hospital, with Institutional Review Board approvals. The diagnosis of AIA was based on a self-reported history due to the potential risk of a provocation test. ATA was defined as adult asthma diagnosed by expert physicians according to the American Thoracic Society criteria [15] and no history of aspirin or NSAID-induced asthmatic attack, and comprised of 154 atopic asthmatic (age: 48.0±15.6 years; 80 male/74 female) and 128 non-atopic asthmatic (age: 65.9±10.0 years; 52 male/76 female) subjects. CTR were outpatients with diseases (e.g., hypertension) other than respiratory diseases including asthma, and who self-reported no history of aspirin sensitivity. The patients and controls were all of Japanese ethnicity. Although the Japanese population is thought to be genetically homogenous, nearly identical numbers of patients and controls from the various locations were recruited to avoid geographical differences in allelic frequencies.

RNA extraction

The nasal polyp tissue was removed during endoscopic sinus surgery, submerged in RNA*later* regent (Ambion Inc., Austin, TX, USA) to avoid RNA degradation, and used for RNA extraction within 48 h after resection. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were analysed using the Agilent 2100 bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) with an RNA6000 Nano LabChip Kit (Agilent Technologies). RNAs from two CS patients were equally pooled, and used as a common reference in the two-colour microarray experiments, where a single microarray was used to compare each test sample from an AIA or an ES patient with the reference sample.

cRNA synthesis, labelling, hybridization, and expression profiling

For fluorescent cRNA synthesis, high-quality total RNA (150 ng) was labelled with the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions. In this procedure, cyanine 5-CTP (Cy5) and cyanine 3-CTP (Cy3) (PerkinElmer, Boston, MA, USA) were used to generate labelled cRNA from the individual AIA or ES RNA and the pooled CS RNA as a reference, respectively. Labelled cRNAs (0.75 μg each) from the AIA, ES, or CS patients were fragmented in a hybridization mixture with the In Situ Hybridization Kit Plus (Agilent Technologies) according to the manufacturer's instructions. The mixture was hybridized for 17 h at 65 °C to an Agilent Human 1A(v2) Oligo Microarray. After hybridization, the microarray was washed with SSC buffer, and then scanned in Cy3 and Cy5 channels with the Agilent DNA Microarray Scanner, model G2565AA. Signal intensity per spot was generated from the scanned image with Feature Extraction Software ver7.5 (Agilent Technologies) in default settings. Spots that did not pass quality control procedures with the software were flagged and removed for further analysis.

GeneSpring software GX 7.3 (Agilent Technologies) was used for the Lowess (locally weighted linear regression curve fit) normalization of the ratio (Cy5/Cy3) of the signal intensities generated in each microarray and the subsequent data analysis. To determine the AIA-specific expression profile of nasal polyps, ES transcripts with ratios ranging from 0.5 to 2 were extracted, and the AIA transcripts with expression undergoing a twofold change or more were extracted as decreased or elevated genes. Of the transcripts overlapping the two groups, only those with statistically significant differences in expression between the AIA and CS nasal polyps (Benjamini and Hochberg false discovery rate (FDR)<0.01; [16]) were counted as AIA-specific genes. To identify novel expression patterns in nasal polyps from AIA patients, the *k*-means method [17], a well-known unsupervised partitional clustering approach, was applied to the AIA-specific genes. For functional subclassification of the AIA-specific genes, we applied the Gene Ontology (GO) classification for biological processes with DAVID 2.1 (http://david.abcc.ncifcrf.gov/), a web-accessible program [18]. A permutation test with 10 000 iterations was used for multiple test correction when nasal polyps from AIA

and ES patients were compared at the transcriptome level [19]. $P<0.05$ was considered significant in every statistical analysis.

Quantitative real-time reverse transcription polymerase chain reaction analysis

Two transcripts, *INDO* and *IL1R2*, that were differentially expressed between AIA and CS nasal polyps were subjected to real-time reverse transcription polymerase chain reaction (RT-PCR) for verification of the microarray data, using a validation set of total RNAs from AIA ($n = 10$) and CS ($n = 4$) nasal polyps including nine AIA and two CS samples for the present microarray experiment. Total RNA from each nasal polyp was used as a template in first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, Tokyo, Japan) with TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The relative quantification method [20] was used to measure the amounts of the respective genes in nasal polyps, normalized to *GAPDH* as an endogenous control. The statistical significance in gene expression between the AIA and the CS samples was determined by the Welch *t*-test; $P<0.05$ was considered significant.

Single nucleotide polymorphism genotyping

For gene-based association analysis, SNPs of AIA candidate genes were obtained from the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) using SNPbrowser Software (Applied Biosystems), to cover the entire regions of the genes positionally and genetically. SNPs were genotyped using the TaqMan SNP Genotyping assay (Applied Biosystems) with the allelic discrimination software SDS version 2.1 (Applied Biosystems) on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis of association study

Differences in allelic frequencies were evaluated by a case–control design with a χ^2 test. Haplotype frequencies for multiple loci were estimated using the expectation-maximization method with SNPAlyze v6.0 software (DYNACOM, Mobara, Japan). Bonferroni's correction was adopted for each gene and haplotype for multiple test correction.

Pairwise LD was estimated as $D = x_{11} - p_1 q_1$ where x_{11} is the frequency of haplotype A_1B_1, and p_1 and q_1 are the frequencies of alleles A1 and B1 at loci A and B, respectively. A standardized LD coefficient, r, is given by $D/(p_1 p_2 q_1 q_2)^{1/2}$, where p_2 and q_2 are the frequencies of the other alleles at loci A and B, respectively [21]. Lewontin's coefficient, D', is given by $D' = D/D_{max}$, where $D_{max} = \min(p_1 q_2, p_2 q_1)$ when $D > 0$ or $D_{max} = \min(p_1 q_1, p_2 q_2)$ when $D < 0$ [22].

The power of the present association analysis was calculated using 'Genetic Power Calculator [23] (http://pngu.mgh.harvard.edu/~purcell/gpc/)'. Using our sample sizes in the AIA–CTR comparison, the study has had 80% power to detect common alleles (risk allele frequency = 0.1) with a relative risk of 1.65, and 50% power to detect the alleles with a relative risk of 1.44 at a threshold of nominal *P*-value = 0.05 under an additive model in the log-odds scale.

Results and discussion

Microarray analysis of nasal polyp tissues of Aspirin-Intolerant Asthma patients

Bronchial biopsy specimens from AIA patients exhibit a fourfold increase in eosinophils compared with those from ATA patients [24]. The increased influx of eosinophils into the airway mucosa of AIA patients is likely a result of an inflammatory rather than an atopic mechanism. It is noteworthy that the nasal polyps of AIA patients show very similar pathological characteristics such as infiltration of eosinophils into the bronchial mucosa [12, 13]. These observations led us to postulate a common molecular mechanism in the development of a polyp and AIA. In such a case, genes related to nasal polyp development in AIA patients might suggest both potential susceptibility genes and pathways involved in aspirin hypersensitivity and the development of AIA. Because it is not practical to apply bronchial tissues for microarray analysis, we used nasal polyp tissues from AIA patients that were under resection for therapeutic purpose and monitored global gene expressions to demonstrate AIA-specific gene expression profiles. ES is known to be a related disorder of AIA; ES is typically characterized by a nasal polyp with inflammatory cell infiltration similar to that in an AIA polyp but without aspirin sensitivity, thus being an appropriate reference for the selection of AIA-specific genes.

The global gene expression profiles of AIA nasal polyps and those of ES nasal polyps were then compared. Similar expression profiles were expected in polyps of AIA and ES patients due to the similar histological and biochemical characteristics such as extensive infiltration of eosinophils. Figure 1a shows a hierarchical clustering (HC) dendrogram for the profiles of nasal polyps from nine AIA and five ES patients. Unexpectedly, two discrete clusters appeared, representing AIA and ES nasal polyps, respectively, with the exception of one (ES#5) of the ES tissues, from a patient who was aspirin tolerant and had clinical characteristics similar to those of other ES patients

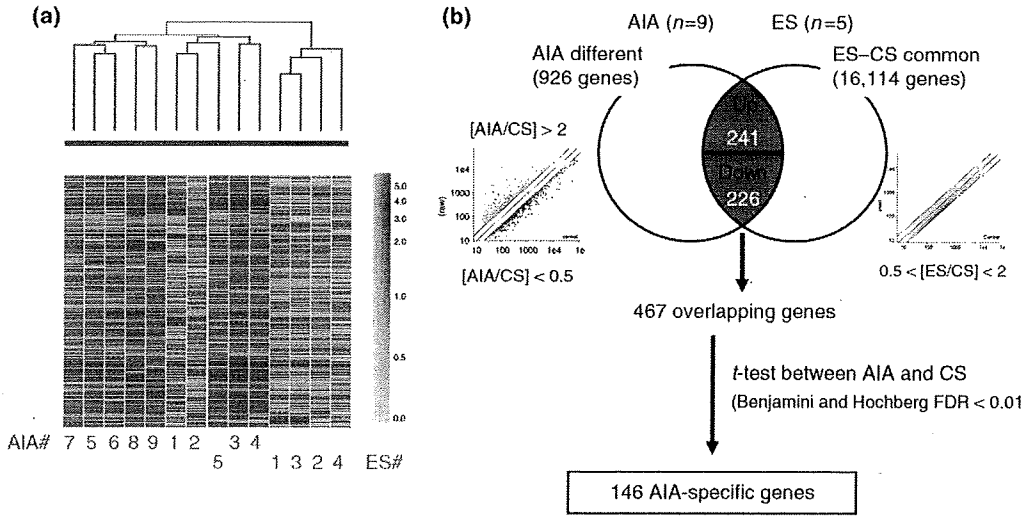

Fig. 1. Experimental design to extract aspirin-intolerant asthma (AIA)-specific genes with microarray analysis. (a) Hierarchical clustering (HC)-based classification of nasal polyps from AIA and eosinophilic sinusitis (ES) patients. Based on the gene expression in nasal polyps, HC clustering shows distinct expression profiles in AIA and ES patients. The clinical characteristics of the patients for the expression analysis are summarized in Table 1. (b) Strategy for discovering AIA-specific gene expression profiles, referred to as 'AIA-specific genes'. From 16,114 genes representing no change (less than twofold) in gene expression between ES and chronic sinusitis (CS) polyps, 146 AIA candidate genes were extracted at the threshold of twofold differences in expression with statistical significance (FDR < 0.01) between AIA and CS polyps.

(Table 1). Thus, AIA and ES nasal polyps appear to exhibit distinct expression profiles. The HC analysis was supported statistically in that 4012 of 18716 transcripts surveyed by microarray displayed significant differences in expression between the AIA and the ES polyps using a permutation test, followed by Student's *t*-test at a significance level of 0.05. While the expression differences between the two groups could be due to an inter-group variation in cell composition within the nasal polyp tissues, they could not have been due to aspirin sensitivity, and so a two-step selection process was used to extract an AIA-specific expression profile (Fig. 1b). We first obtained genes (16114 genes) common to polyp formation, i.e., genes showing no difference (less than twofold) between ES and chronic sinusitis (CS) polyps, in which a difference in the pathological state of the polyps such as infiltration of inflammatory cells would be minimized. We then selected 926 genes differentially expressed between AIA and CS polyps showing twofold differences, which could be related to the pathophysiology of aspirin sensitivity. From the overlapping genes (467 genes) between the two gene lists, 146 genes were statistically extracted including 143 elevated and three decreased transcripts that were defined as AIA-specific genes.

To examine the biological features of these AIA-specific genes, we assigned 146 genes to the GO classification using the web-accessible DAVID program. As shown in Table 2, nine GO terms were highly associated with the AIA-specific genes. It is notable that the genes involved in cell proliferation and immune response were enriched in the AIA candidate genes, indicating successful extraction of the genes related to nasal polyp formation, because both nasal cell growth and acute inflammation in the respiratory tract are clinical characteristics during the development of nasal polyp in AIA patients.

We then applied the *k*-means algorithm [17], an unsupervised partitional clustering approach, to organize AIA-specific genes into functionally meaningful groups. The *k*-means method has been efficient in showing a significant enrichment of genes belonging to given functional categories in the *k*-means-based clusters [25]. In this analysis, we selected an optimal number of clusters (*k*) in which the number of unclassified genes was minimized. As shown in Table 2, four distinct clusters, subsets 1, 2, 3, and 4, in gene expression were generated for the AIA-specific genes using the *k*-means method (figure not shown). Thus, four types of distinct expression patterns across samples were observed using the dataset of 146 AIA-specific genes. The three genes with decreased expression in AIA nasal polyps were categorized into subset 4, and the 143 elevated genes were classified into three subsets, 1, 2, and 3 (Table 2). Interestingly, genes involved in immune response (18/21 genes) and response to external signal (11/15 genes) were highly enriched in subset 2, while another enrichment of cell proliferation-related genes (17/24 genes) was observed in subset 1 (Table 2). These features of the gene enrichments indicate the biological significance of the present *k*-means-based clusters for AIA candidate genes. According to the cluster–GO correlation, the most notable functional patterning occurred for genes relevant to an immune response owing to the highest concentration (85.7% of the genes

Table 2. Enrichements of genes involved in GO-functional categories within *k*-means-based clusters

			k-means clusters (no. of genes assigned)			
GO TERM (biological process; level 3)	Count	*P*-value	Subset 1 ($n=57$)	Subset 2 ($n=57$)	Subset 3 ($n=29$)	Subset 4 ($n=3$)
Cell proliferation	24	0.0000028	17	7		
Immune response	21	0.068		18	3	
Biopolymer metabolism	20	0.034	9	7	3	
Response to stress	17	0.00097	4	11	2	
Response to external stimulus	15	0.083		11	4	
Catabolism	14	0.017	5	5	4	
Cell organization and biogenesis	12	0.031	2	8		1
Cell motility	6	0.012	2	4		
Cellular defense response	4	0.024		3	1	

DAVID v2.1 (http://david.abcc.ncifcrf.gov/) was used to classify 146 AIA-specific genes functionally according to Gene ontology (GO) classification for biological process. Genes in the respective GO categories were mapped to four *k*-means-based clusters for gene expression.

extracted) in one subset (subset 2) of clusters, indicating that their expression might be highly coordinated in nasal polyps.

Association study with candidate genes for AIA

Based on functional clustering of the AIA-specific genes in the *k*-means clustering, immune response-related genes might serve as candidate genes for susceptibility underlying AIA because the AIA-specific changes in gene expression reflect elevated immune and inflammatory reactions in the nasal polyps of AIA patients. Table 3 shows 21 immune response-related genes in descending order based on the expression ratios in the microarray analysis. We focused on the three top-ranked genes, *INDO*, *IL1R2*, and *CLECSF6*, and screened 17 SNPs of these three genes (three SNPs for *INDO*, 11 SNPs for *IL1R2*, and three SNPs for *CLECSF6*) for an allelic association study between 219 AIA patients and 178 non-asthmatic controls (CTR) in the first screening. One SNP of *INDO* and four SNPs of *IL1R2* were significantly associated with AIA evaluated by a simple χ^2 test (data not shown) based on nominal *P*-values. Differential expressions of the two genes, *INDO* and *IL1R2*, in AIA nasal polyps were confirmed by real-time RT-PCR (Fig. 2). Because the three SNPs of *CLECSF6* examined were not associated with AIA, the gene was not pursued, and other ranked genes were also not screened further.

After increasing the sample size of CTR to 374 subjects, we further examined the allelic associations of a total of 14 SNPs (three SNPs for *INDO* and 11 SNPs for *IL1R2*) with AIA in a second screening. As shown in Tables 4 and 5, one SNP of *INDO* (INDO-SNP2: rs7820268) and one SNP of *IL1R2* (IL1R2-SNP10: rs11688145) showed significant associations with AIA after a multiple test correction using Bonferroni's correction (corrected $P=0.011$ for the INDO-SNP2 and corrected $P=0.026$ for the IL1R2-SNP10). None of the SNPs in the controls showed deviation from Hardy–Weinberg's equilibrium (data not shown). Both the significant SNPs observed were located in non-coding regions of the respective genes, and so the functional impacts of the SNPs were not demonstrated. In order to examine whether *INDO* and *IL1R2* were genetic

Table 3. Twenty-one genes involved in *immune response*

Expression ratio (normalized)	Gene Symbol	Name
1 3.70	INDO	Indoleamine-pyrrole 2,3 dioxygenase
2 3.31	IL1R2	Interleukin 1 receptor, type II
3 2.75	CLECSF6	C-type lectin, superfamily member 6
4 2.68	CCL11	Chemokine (C-C motif) ligand 11
5 2.65	CD163	CD163 antigen
6 2.63	TNFSF10	Tumour necrosis factor (ligand) superfamily, member 10
7 2.53	AIF1	Allograft inflammatory factor 1
8 2.46	NCF2	Neutrophil cytosolic factor 2
9 2.32	ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
10 2.31	FPR1	Formyl peptide receptor 1
11 2.21	TYROBP	TYRO protein tyrosine kinase-binding protein
12 2.21	CTSC	Cathepsin C
13 2.11	IFI30	Interferon, gamma-inducible protein 30
14 2.03	MICB	MHC class I polypeptide-related sequence B
15 1.90	LCP2	Lymphocyte cytosolic protein 2
16 1.86	NCK1	NCK adaptor protein 1
17 1.84	LST1	Leukocyte-specific transcript 1
18 1.83	TLR2	Toll-like receptor 2
19 1.76	PTAFR	Platelet-activating factor receptor
20 1.71	CKLF	Chemokine-like factor
21 1.65	EDG6	Endothelial differentiation, G-protein-coupled receptor 6

susceptibility genes underlying aspirin hypersensitivity, we further genotyped the two significant SNPs in 282 ATA patients for comparison. In the AIA–ATA association study, the INDO-SNP2 also showed a statistically significant association with AIA ($P = 0.038$) (Table 4), whereas an association of the IL1R2-SNP10 with AIA was marginal ($P = 0.073$) with the same direction of genetic effect of the associated allele on AIA susceptibility (Table 5). In contrast, no significant differences in allele frequencies at the two SNPs were observed between CTR and ATA groups (statistical data not shown). These SNP-based association results indicate that the two SNPs in *INDO* and *IL1R2* are associated with the risk of aspirin hypersensitivity rather than an asthmatic reaction in Japanese population.

Figure 3 shows D'- and r^2-based LD block structures in the genomic regions around *INDO* and *IL1R2*, respectively. We observed a strong LD ($|D'| = 0.98$) between INDO-SNP1 and -SNP2 in the *INDO* region. A highly structured LD pattern, a major LD block structure ($|D'| > 0.7$) covered by IL1R2-SNP6 to -SNP11, was observed in *IL1R2* (Fig. 3). Next, we conducted a haplotype-based association study within the respective LD blocks (Tables 6 and 7). We found that one haplotype of *INDO*, m/m (double minor haplotype) at INDO-SNP1 and -SNP2, was underrepresented in AIA with statistical significance after multiple test correction with Bonferroni's correction (Table 6; $\chi^2 = 6.74$, df = 1, corrected $P = 0.038$), indicating a protective effect of the m/m haplotype of *INDO*. One haplotype of *IL1R2*, M/M/M (triple major haplotype) at IL1R2-SNP6, -SNP10, and -SNP11, showed a highly significant difference between AIA and CTR (Table 7; $\chi^2 = 8.94$, df = 1, corrected $P = 0.011$), indicating that the M/M/M haplotype represented a risk for AIA.

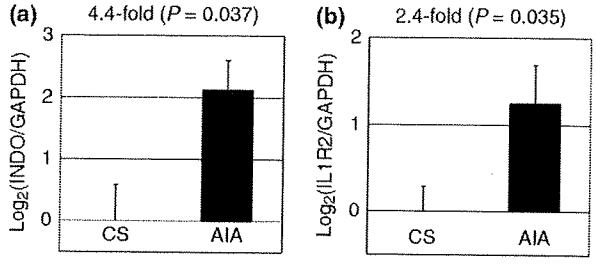

Fig. 2. Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis validates expression differences for two genes, *INDO* and *IL1R2*, in nasal polyps from aspirin-intolerant asthma (AIA) and chronic sinusitis (CS) patients. Relative amounts of the respective genes in nasal polyps (AIA, $n = 10$; CS, $n = 4$) were measured by real-time RT-RCR using TaqMan Gene Expression Assays. *Y*-axes indicate the $\log_2$ relative expression levels, normalized to the amount of *GAPDH* and relative to the averaged expression levels in CS groups. The expression levels of *INDO* and *IL1R2* were significantly higher in AIA than CS nasal polyps by the Welch *t*-test.

INDO encodes indoleamine 2, 3-dioxygenase, which is a rate-limiting enzyme of tryptophan catabolism and is expressed in various cell types such as fibroblasts, macrophages, and dendritic cells [26]. INDO activity is induced by interferons (IFNs) and further enhanced by inflammatory cytokines such as IL-1 [27] but suppressed by anti-inflammatory cytokines such as TGF-β and IL-4 [28, 29]. INDO-induced tryptophan degradation in macrophages results in inhibition of T cell proliferation [30], suggesting that INDO plays an important role in the regulation of T cell-mediated immune responses. Aspirin inhibits INDO activity in stimulated peripheral blood mononuclear cells indirectly, via its inhibitory effect on the production of IFN-γ [31]. Therefore, the functional disturbance of INDO activity due to the INDO-SNP2 might play a role in the pathogenesis of aspirin sensitivity or AIA. A possibility remains that an unknown SNP in tight LD with the INDO-SNP2 or on the m/m haplotype could serve as a *bona fide* causality, which could prevent AIA induction by keeping INDO activity normal. Further studies are needed to resolve the functional significance of the INDO-SNP2 and the m/m haplotype in the genetic aetiology of AIA.

Interleukin 1 receptor type II (IL1R2) acts as a soluble decoy receptor that inhibits IL-1 signalling [32]. The inhibition of IL-1 binding to the receptor in human monocytes results in a reduction of COX-2 activity but not COX-1 activity [33]. As an imbalance in arachidonate

Table 4. Allelic association of *INDO* SNPs with AIA in Japanese population

					MAF		AIA vs. CTR				MAF	AIA vs. ATA	
SNP no.	Position*	Localization	dbSNP ID	Alleles (M/m)†	AIA $n = 219$	CTR $n = 374$	χ^2	Odds ratio (95% CI)	P	Corrected P‡	ATA $n = 282$	χ^2	P
INDO-SNP1	−1953	5′-upstream	rs3808606	T/C	0.414	0.472	3.65	0.79 (0.62–1.01)	0.056	0.17	ND		
INDO-SNP2	6202	intron4	rs7820268	C/T	0.101	0.163	8.47	0.58 (0.40–0.84)	0.0036	0.011	0.145	4.29	0.038
INDO-SNP3	13994	intron9	rs3739319	A/G	0.474	0.436	1.63	1.17 (0.92–1.49)	0.20	0.60	ND		

*Numbers indicate the nucleotide position from the first nucleotide of exon 1.

†M and m denote major and minor alleles, respectively, at each SNP site.

‡Corrected *P* values were obtained using Bonferroni's correction.

MAF, minor allele frequency; AIA, aspirin intolerant asthma; CTR, non-asthmatic control; ATA, aspirin tolerant asthma; ND, not determined; SNP, single nucleotide polymorphism.

Table 5. Allelic association of *IL1R2* SNPs with AIA in Japanese population

SNP No.	Position*	Localization	dbSNP ID	Alleles (M/m)†	MAF AIA $n=219$	MAF CTR $n=374$	AIA vs. CTR χ^2	Odds ratio	P	MAF Corrected P‡	AIA vs. ATA ATA $n=282$	χ^2	P
IL1R2-SNP1	−6913	5′-upstream	rs4851519	C/T	0.272	0.219	4.27	1.34 (1.01-1.76)	0.039	0.43	ND		
IL1R2-SNP2	−4381	5′-upstream	rs35789178	T/G	0.368	0.358	0.12	1.05 (0.81-1.34)	0.73	1	ND		
IL1R2-SNP3	−3657	5′-upstream	rs12467316	A/C	0.299	0.376	7.21	0.71 (0.55-0.91)	0.0072	0.080	ND		
IL1R2-SNP4	−3145	5′-upstream	rs12468239	C/T	0.090	0.093	0.02	0.97 (0.64-1.47)	0.89	1	ND		
IL1R2-SNP5	9147	intron1	rs11691240	C/T	0.439	0.479	1.73	0.85 (0.67-1.08)	0.19	1	ND		
IL1R2-SNP6	14513	intron1	rs3755482	A/G	0.273	0.340	5.58	0.73 (0.56-0.95)	0.018	0.20	ND		
IL1R2-SNP7	15413	intron1	rs719250	G/A	0.381	0.318	4.73	1.32 (1.02-1.69)	0.030	0.33	ND		
IL1R2-SNP8	21335	intron3	rs2110562	C/T	0.179	0.193	0.32	0.91 (0.67-1.25)	0.57	1	ND		
IL1R2-SNP9	40304	3′-downstream	rs4851531	T/C	0.462	0.408	3.16	1.25 (0.98-1.59)	0.075	0.83	ND		
IL1R2-SNP10	42202	3′-downstream	rs11688145	C/A	0.244	0.330	9.23	0.66 (0.50-0.86)	0.0024	0.026	0.296	3.22	0.073
IL1R2-SNP11	54346	3′-downstream	rs7588933	A/G	0.186	0.206	0.70	0.88 (0.65-1.19)	0.40	1	ND		

*Numbers indicate the nucleotide position from the first nucleotide of exon 1.

†M and m denote major and minor alleles, respectively, at each SNP site.

‡Corrected P values were obtained using Bonferroni's correction.

MAF, minor allele frequency; AIA, aspirin intolerant asthma; CTR, non-asthmatic control; ATA, aspirin tolerant asthma; ND, not determined

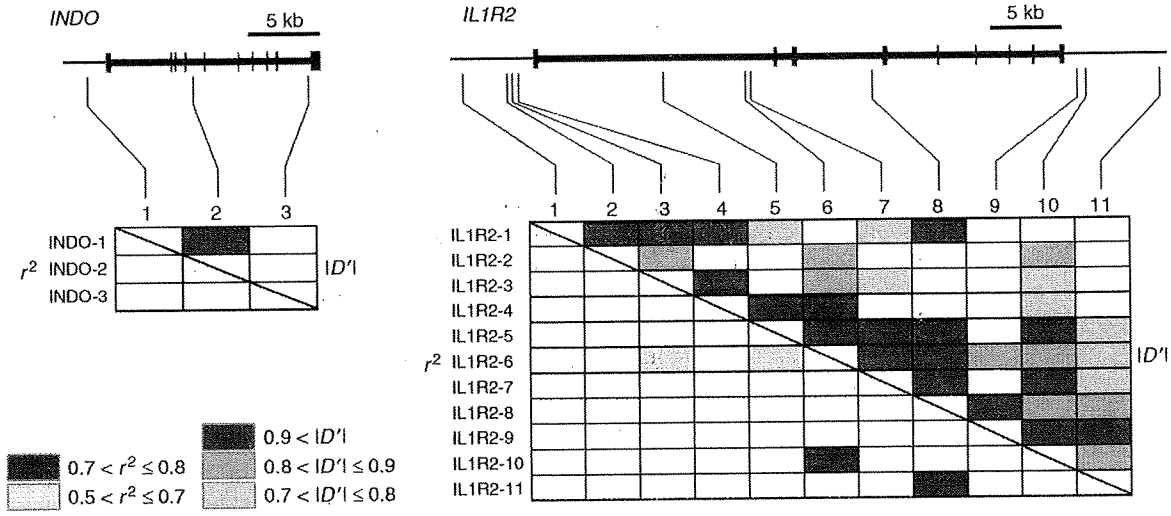

Fig. 3. Linkage disequilibrium pattern of *INDO* and *IL1R2*. The gene structures of *INDO* (left) and *IL1R2* (right), together with positions of the 14 single nucleotide polymorphisms (SNPs) examined, are shown. Pairwise LD coefficients, D' and r^2, were determined and expressed as a block structure. In the schematic block, red boxes indicate a pairwise LD of $|D'|>0.9$, pink $0.9 \geq |D'| > 0.8$, and orange $0.8 \geq |D'| > 0.7$; blue boxes indicate a pairwise LD of $0.8 \geq r^2 > 0.7$ and light blue $0.7 \geq r^2 > 0.5$. Blank boxes represent $|D'| \leq 0.7$ or $r^2 \leq 0.5$.

metabolism is the usual pathogenesis proposed for AIA, the elevated level of IL1R2 that inhibits the inflammatory effects of IL-1α in the respiratory tract might well be involved in the pathogenesis of AIA and the formation of nasal polyps. Although IL1R2-SNP10 showed the strongest allelic association with AIA (Table 5), the functional impact of an SNP locating 3′-downstream of the gene is currently unclear despite its up-regulated expression in the AIA polyp (Table 3). There is also the possibility that an unidentified functional SNP in LD with the IL1R2-SNP10 could be a causality. Further genetic fine mapping in *IL1R2* will be required to fully understand which genetic variant contributes to the risk of AIA. The functional impacts of the SNP and haplotype also require further investigation.

In conclusion, DNA microarray technology was used to monitor global gene expression patterns specific to AIA nasal polyp tissues to clarify the pathophysiology of AIA. From the gene expression profile, candidate genes underlying AIA were selected and subjected to an association study. We identified SNPs in *INDO* and *IL1R2* that may represent genetic susceptibility to AIA. This genetic study

Table 6. Haplotype-based association of *INDO* with AIA

	Haplotype frequency					
Haplotype (INDO-SNP1/2)*	Total	AIA	CTR	χ^2	*P*	Corrected $P^\dagger$
M/M (T/C)	0.552	0.591	0.531	3.75	0.053	0.21
m/M (C/C)	0.306	0.304	0.307	0.01	0.94	1
m/m (C/T)	0.141	0.105	0.161	6.74	0.0094	0.038
			Global comparison	8.05 (df = 3)	0.045	0.18

*M and m denote major and minor alleles, respectively, at each SNP site. The corresponding nucleotides at the respective sites are shown in parentheses.
†Corrected *P* values were obtained using Bonferroni's correction.
AIA, aspirin intolerant asthma; CTR, non-asthmatic control; SNP, single nucleotide polymorphism.

Table 7. Haplotype-based association of *IL1R2* with AIA

	Haplotype frequency					
Haplotype (IL1R2-SNP6/SNP10/SNP11)*	Total	AIA	CTR	χ^2	*P*	Corrected $P^\dagger$
M/M/M (A/C/A)	0.494	0.552	0.458	8.94	0.0028	0.011
m/m/M (G/A/A)	0.277	0.243	0.299	4.03	0.045	0.18
M/M/m (A/C/G)	0.182	0.170	0.189	0.59	0.44	1
			Global comparison	8.72 (df = 4)	0.069	0.28

*M and m denote major and minor alleles, respectively, at each SNP site. The corresponding nucleotides at the respective sites are shown in parentheses.
†Corrected *P* values were obtained using Bonferroni's correction.
AIA, aspirin intolerant asthma; CTR, non-asthmatic control; SNP, single nucleotide polymorphism.

represents only first-stage evidence of the association because only Japanese individuals were included, and so further replication in independent case–control samples is required to confirm the role of *INDO* and *IL1R2* genotypes in the genetic risk for AIA. A pathophysiological link between the two gene products is unclear and further investigation is evidently needed. In addition, further studies including functional analyses of the SNPs with respect to how genetic variants are responsible for the risk of AIA are also required for a full understanding of the pathogenesis of AIA.

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References

1 Szczeklik A, Stevenson DD. Aspirin-induced asthma: advances in pathogenesis and management. *J Allergy Clin Immunol* 1999; 104:5–13.
2 Stevenson DD, Szczeklik A. Clinical and pathologic perspectives on aspirin sensitivity and asthma. *J Allergy Clin Immunol* 2006; 118:773–86.
3 Szczeklik A, Nizankowska E, Duplaga M. Natural history of aspirin-induced asthma. AIANE Investigators. European Network on aspirin-induced asthma. *Eur Respir J* 2000; 16: 432–6.
4 Sanak M, Simon HU, Szczeklik A. Leukotriene C4 synthase promoter polymorphism and risk of aspirin-induced asthma. *Lancet* 1997; 350:1599–600.
5 Sanak M, Pierzchalska M, Bazan-Socha S, Szczeklik A. Enhanced expression of the leukotriene C_4 synthase due to overactive transcription of an allelic variant associated with aspirin-intolerant asthma. *Am J Respir Cell Mol Biol* 2000; 23:290–6.
6 Choi JH, Park HS, Oh HB *et al*. Leukotriene-related gene polymorphisms in ASA-intolerant asthma: an association with a haplotype of 5-lipoxygenase. *Hum Genet* 2004; 114:337–44.
7 Jinnai N, Sakagami T, Sekigawa T *et al*. Polymorphisms in the prostaglandin E_2 receptor subtype 2 gene confer susceptibility to aspirin-intolerant asthma: a candidate gene approach. *Hum Mol Genet* 2004; 13:3203–17.
8 Park HW, Shin ES, Lee JE *et al*. Association between genetic variations in prostaglandin E_2 receptor subtype EP3 gene (*Ptger3*) and asthma in the Korean population. *Clin Exp Allergy* 2007; 37:1609–15.
9 Nakatani N, Hattori E, Ohnishi T *et al*. Genome-wide expression analysis detects eight genes with robust alterations specific to bipolar I disorder: relevance to neuronal network perturbation. *Hum Mol Genet* 2006; 15:1949–62.
10 Okada H, Tajima A, Shichiri K, Tanaka A, Tanaka K, Inoue I. Genome-wide expression of azoospermia testes demonstrates a

specific profile and implicates *ART3* in genetic susceptibility. *PLoS Genet* 2008; 4:e26.
11 Meltzer EO, Hamilos DL, Hadley JA *et al.* Rhinosinusitis: establishing definitions for clinical research and patient care. *J Allergy Clin Immunol* 2004; 114:S155–212.
12 Yoshifuku K, Matsune S, Ohori J, Sagara Y, Fukuiwa T, Kurono Y. IL-4 and TNF-alpha increased the secretion of eotaxin from cultured fibroblasts of nasal polyps with eosinophil infiltration. *Rhinology* 2007; 45:235–41.
13 Jenkins C, Costello J, Hodge L. Systematic review of prevalence of aspirin induced asthma and its implications for clinical practice. *BMJ* 2004; 328:434.
14 Pleskow WW, Stevenson DD, Mathison DA, Simon RA, Schatz M, Zeiger RS. Aspirin-sensitive rhinosinusitis/asthma: spectrum of adverse reactions to aspirin. *J Allergy Clin Immunol* 1983; 71:574–9.
15 National Heart, Lung and Blood Institute. Guidelines for the diagnosis and management of asthma. National Heart, Lung, and Blood Institute. National Asthma Education Program. Expert panel report. *J Allergy Clin Immunol* 1991; 88:425–534.
16 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testings. *J R Statist Soc B* 1995; 57:289–300.
17 Slonim DK. From patterns to pathways: gene expression data analysis comes of age. *Nat Genet* 2002; 32 (Suppl.):502–8.
18 Dennis G Jr, Sherman BT, Hosack DA *et al.* DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol* 2003; 4:P3.
19 Churchill GA, Doerge RW. Empirical threshold values for quantitative trait mapping. *Genetics* 1994; 138:963–71.
20 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001; 25:402–8.
21 Hill WG, Robertson A. Linkage disequilibrium in finite populations. *Theor Appl Genet* 1968; 38:226–31.
22 Lewontin RC. The interaction of selection and linkage. I. General considerations; Heterotic models. *Genetics* 1964; 49:49–67.
23 Purcell S, Cherny SS, Sham PC. Genetic power calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 2003; 19:149–50.
24 Cowburn AS, Sladek K, Soja J *et al.* Overexpression of leukotriene C_4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998; 101:834–46.
25 Tavazoie S, Hughes JD, Campbell MJ, Cho RJ, Church GM. Systematic determination of genetic network architecture. *Nat Genet* 1999; 22:281–5.
26 Mellor AL, Munn DH. Extinguishing maternal immune responses during pregnancy: implications for immunosuppression. *Semin Immunol* 2001; 13:213–8.
27 Babcock TA, Carlin JM. Transcriptional activation of indoleamine dioxygenase by interleukin 1 and tumor necrosis factor alpha in interferon-treated epithelial cells. *Cytokine* 2000; 12:588–94.
28 Yuan W, Collado-Hidalgo A, Yufit T, Taylor M, Varga J. Modulation of cellular tryptophan metabolism in human fibroblasts by transforming growth factor-beta: selective inhibition of indoleamine 2,3-dioxygenase and tryptophanyl-tRNA synthetase gene expression. *J Cell Physiol* 1998; 177:174–86.
29 MacKenzie CR, González RG, Kniep E, Roch S, Däubener W. Cytokine mediated regulation of interferon-gamma-induced IDO activation. *Adv Exp Med Biol* 1999; 467:533–9.
30 Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J Exp Med* 1999; 189:1363–72.
31 Schroecksnadel K, Winkler C, Wirleitner B, Schennach H, Fuchs D. Aspirin down-regulates tryptophan degradation in stimulated human peripheral blood mononuclear cells in vitro. *Clin Exp Immunol* 2005; 140:41–5.
32 Colotta F, Re F, Muzio M *et al.* Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* 1993; 261:472–5.
33 Porreca E, Reale M, Di Febbo C *et al.* Down-regulation of cyclooxygenase-2 (COX-2) by interleukin-1 receptor antagonist in human monocytes. *Immunology* 1996; 89:424–9.

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Association Analysis of Genes Involved in the Maintenance of the Integrity of the Extracellular Matrix with Intracranial Aneurysms in a Japanese Cohort

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Key Words
Intracranial aneurysm · Single nucleotide polymorphism · Versican · Perlecan · Fibrillin 2 · Collagen 4A1

Abstract
Background: An association between versican *(CSPG2)*, perlecan *(HSPG2)*, fibrillin 2 *(FBN2)* and collagen 4A1 *(COL4A1)* gene variants and intracranial aneurysms (IA) has been reported in 2 studies analyzing Dutch IA patients. The aim of this study was to verify these associations in a Japanese IA population. In addition, a meta-analysis on the association of these genes and IA for the combined Dutch and Japanese populations was performed. ***Methods:*** The associated single nucleotide polymorphisms (SNPs) in these genes identified in the Dutch study were genotyped in 632 Japanese IA patients and 808 healthy control subjects using TaqMan SNP genotyping assays. ***Results:*** A similar association to that previously found in the Dutch population was found for the *CSPG2* (rs251124) and *HSPG2* (rs3767137) SNPs, although both associations were not statistically significant in the Japanese population (*CSPG2* OR 1.18, 95% CI 0.98–1.41, p = 0.08; *HSPG2* OR 1.09, 95% CI 0.90–1.32). Combining the Dutch and Japanese data for a meta-analysis showed an overall association between the *CSPG2* SNP and IA (OR 1.29, 95% CI 1.12–1.48, p = 0.0005) and the *HSPG2* SNP and IA (OR 1.22, 95% CI 1.08–1.39, p = 0.002). No differences in SNP frequency were observed for *FBN2* and *COL4A1* between Japanese patients and controls. ***Conclusions:*** By analyzing *HSPG2*, *CSPG2*, *FBN2* and *COL4A1*, we were able to replicate the association of *CSPG2* and show that there is a trend for HSPG2 towards association in the Japanese IA population by means of a meta-analysis combining the Dutch and Japanese results. The association of *FBN2* and *COL4A1* could not be replicated in the Japanese IA population.

Introduction

Subarachnoid hemorrhage is caused by rupture of an intracranial aneurysm (IA) in 85% of people, while 10% fit into the pattern of the so-called non-aneurysmal perimesencephalic hemorrhage, and the remaining 5%

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are caused by various other causes (e.g. cerebral arteriovenous malformations, cerebral dural arteriovenous fistulae) [1, 2]. IA rupture is most common at the age of 40–60 years, and prognosis after rupture is poor: 50% die and another 20% remain dependent for daily-life activities [3]. A familial predisposition is the strongest risk factor for the development of IA, whereas environmental factors, such as smoking, hypertension and alcohol intake, seem to play an important role in the rupture of IA [4]. The complex inheritance pattern is likely due to the interaction of several genes and environmental factors [5, 6]. Various genome-wide linkage studies in IA have identified several loci for IA [7].

Previously, we hypothesized that a disruption of the extracellular matrix (ECM) of the arterial wall is a likely factor in the pathogenesis of intracranial aneurysms. We studied 45 potential candidate genes involved in the maintenance of the integrity of the ECM in a Dutch Caucasian case-control study analyzing 2 independent populations [8, 9]. The versican *(CSPG2)* and the perlecan *(HSPG2)* genes were identified as susceptibility genes for IA in the Dutch population, with association in 2 independent cohorts [7]. Furthermore, the study showed evidence for association to the fibrillin 2 *(FBN2)* and collagen 4A1 *(COL4A1)* genes [8]. The importance of *CSPG2*, *HSPG2*, *FBN2* and *COL4A1* in the etiology of IA would be strengthened if disease associations could also be demonstrated in other populations. The first, and to our knowledge the only, replication study to date reported a lack of association of the *CSPG2* variants with IA in a Chinese cohort [10].

In this study, the association found between IA and *CSPG2*, *HSPG2*, *FBN2* and *COL4A1* in the Dutch population was verified in a Japanese population. We genotyped the associated SNPs in these genes identified in the Dutch study in Japanese IA patients and healthy control subjects. In addition, a meta-analysis on the association of these genes and IA in the combined Dutch and Japanese population was performed.

Materials and Methods

Patients and Controls

We analyzed 632 Japanese IA patients of the Tokyo Women's Medical University, Chiba University, and their affiliated hospitals. The presence of IA was confirmed by conventional angiography, 3-dimensional CT angiography, MR angiography or surgical findings. The 808 control subjects were recruited at the time of scheduled medical examination of the brain, or recruited as outpatients with diseases other than IA, at the Department of Neurosurgery of the Tokyo Women's Medical University, Chiba University, and their nearby affiliated hospitals. The control samples were outpatients with diseases other than IA, and without any family or medical history of subarachnoid hemorrhage. Most of them underwent neuroradiological imaging, such as 3-D MRI, excluding subjects harboring IA.

Genomic DNA was extracted from peripheral blood or buccal swab according to a standard method. The Ethical Committees of Tokyo Women's Medical University, Chiba University and Tokai University approved the study protocols, and all participants gave written informed consent.

Table 1. SNP rs numbers and the TaqMan assay IDs of the analyzed SNPs

Gene	rs number	TaqMan assay ID
CSPG2	rs251124	C_2387523_10
	rs173686	C_2838882_10
HSPG2	rs3767137	C_1603659_10
FBN2	rs331079	C_1561675_10
COL4A1	rs3783107	C_27500491_10

SNP Genotyping

The associated SNPs of *HSPG2*, *FBN2* and *COL4A1* and the 2 most significant associated SNPs of *CSPG2* identified in the Dutch IA population [8, 9] were genotyped using TaqMan pre-designed assays on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif., USA) according to a standard protocol. Table 1 shows the rs numbers and the TaqMan assay IDs of the analyzed SNPs.

Power of the Study

Using our IA sample size, the study has a good power ranging from 0.731 to 0.995 at the threshold of a nominal p value = 0.05 (Genetic Power Calculator, http://pngu.mgh.harvard.edu/~purcell/gpc; option: case-control for discrete traits).

Statistical Analysis

Differences in allele frequencies between patients and controls were assessed as odds ratios (OR) with corresponding 95% CI, and p values using the allele with the lower frequency in the controls as identified in the previous Dutch association studies [8, 9] as opposed to the allele frequency in patients as the reference allele. In addition, a meta-analysis of the Dutch and Japanese associations was conducted using the Cochrane Mantel-Haenszel test to calculate the pooled OR.

Results

The percentage of women in the IA patient group was 59.3%, and 38.6% in the control group. The mean age was 58.8 years (range 29–89) in the patients and 62.3 years (range 18–92) in the controls. In the patient group, 69.7%

Table 2. The analysis of the previously reported associated SNPs in the versican *(CSPG2)*, perlecan *(HSPG2)*, fibrillin 2 *(FBN2)* and collagen 4A1 *(COL4A1)* identified in the Dutch population, in a cohort of 632 Japanese intracranial aneurysm patients and 808 controls and the meta-analysis of the Dutch and Japanese allele frequencies combined

Gene	rs number	Japanese patients				Dutch patients [4, 5]				Meta-analysis Japanese and Dutch patients	
		cases	controls	OR	p value	cases	controls	OR	p value	OR	p value
CSPG2	rs251124	24.3%	21.4%	1.18 (0.98–1.41)	0.08	17.7%	12.8%	1.47 (1.17–1.84)	0.0006	1.29 (1.12–1.48)	0.0005
HSPG2	rs3767137	81.5%	80.1%	1.09 (0.90–1.32)	0.39	76.9%	71.5%	1.33 (1.13–1.57)	0.0006	1.22 (1.08–1.39)	0.002
FBN2	rs331079	8.0%	9.6%	0.92 (0.69–1.20)	0.52	10.7%	8.0%	1.37 (1.07–1.75)	0.01	1.15 (0.96–1.38)	0.14
COL4A1	rs3783107	41.4%	43.2%	0.93 (0.79–1.09)	0.35	40.1%	35.4%	1.22 (1.05–1.42)	0.007	1.07 (0.96–1.19)	0.20

Values in parentheses are 95% CI.

of the patients had ruptured IAs and 28.2% of the patients had familial IA.

For SNP rs173686 in *CSPG2*, the TaqMan assays did not have an acceptable quality and were therefore not included for further analysis. A total of 4 SNPs were successfully genotyped (call rates >95%). The distribution of the SNP genotypes was consistent with Hardy-Weinberg equilibrium ($p > 0.05$).

The association data of the 4 SNPs in *CSPG2*, *HSPG2*, *FBN2* and *COL4A1* with corresponding ORs and p values are shown in table 2. Comparing the allele frequency of the *CSPG2* rs251124 SNP and the *HSPG2* rs3767137 SNP in the Japanese patient group to the allele frequency of the control group, a similar effect to that previously found in the Dutch population [8, 9] was discovered, although both associations were not statistically significant in the Japanese population (*CSPG2* rs251124 OR 1.18, 95% CI 0.98–1.41, p = 0.08; *HSPG2* rs3767137 OR 1.09, 95% CI 0.90–1.32, p = 0.39). Combining the Dutch and Japanese data for a meta-analysis showed an overall association between the *CSPG2* SNP and IA (OR 1.29, 95% CI 1.12–1.48, p = 0.0005) and between the *HSPG2* SNP and IA (OR 1.22, 95% CI 1.08–1.39, p = 0.002). No statistically significant differences in SNP frequency were observed in *FBN2* and *COL4A1* for the previously reported associated SNPs identified in the Dutch population between Japanese patients and controls. Also, additional meta-analysis showed no overall association between these SNPs and IA.

Discussion

By analyzing *HSPG2*, *CSPG2*, *FBN2* and *COL4A1*, which were previously identified as candidate genes for IA in the Dutch population [8, 9], we were able to replicate the association of both *HSPG2* and *CSPG2* in the Japanese IA population by means of a meta-analysis combining the Dutch and Japanese results. These findings suggest that *HSPG2* and *CSPG2* are common susceptibility markers for IA in the Dutch and Japanese population. The association of *FBN2* and *COL4A1* could not be replicated in the Japanese IA population.

HSPG2 is located in a previously reported locus for intracranial aneurysms on chromosome 1p34.3-p36.13 (ANIB3; HUGO nomenclature committee) identified in both a single North American family [11] and a Dutch family [12]. *CSPG2* is localized close to a previously implicated locus for IA on chromosome 5q in a Japanese cohort [13]. The ECM of the arterial wall is composed of collagen and elastin fibers embedded in glycoproteins and proteoglycans [14]. The 2 largest groups of proteoglycans of the ECM are the chondroitin sulfate proteoglycans, to which versican belongs, and the heparan sulfate proteoglycans, which includes perlecan [15, 16]. Both groups of proteoglycans have various important protein-binding domains with which they are able to interact with diverse molecules, including key components of the ECM [15, 16]. The proteoglycans of the ECM in general may play an important role in the pathogenesis of intracranial aneurysms. Its contribution to the pathogenesis may be explained by the loss of function of the proteoglycans to interact with other ECM components, leading to weakening of the arterial wall and consequently to aneurysm formation.

In this study, we also intended to analyze the association of the serpine 1 (PAI1) gene with IA in the Japanese population since the previous Dutch study also showed evidence for association to this gene [8]. However, we were not able to analyze *PAI1* as a susceptibility gene for IA in the Japanese population as the TaqMan assay of the SNP rs6956010 found to be associated in the Dutch pop-

ulation [8] did not have acceptable quality to permit genotyping analysis. For the *CSPG2* gene, we intended to genotype the 2 most significant associated SNPs of the gene identified in the Dutch IA population: rs173686 and rs251124 [9]. Also, a genotyping problem with the TaqMan assay was found for 1 of these 2 SNPs (rs173686), and this SNP was excluded from further analysis. However, as the 2 SNPs in the *CSPG2* gene associated with IA in the Dutch population were also in linkage disequilibrium with each other, we expect a similar association of SNP rs173686 with IA in the Japanese population as found for SNP rs251124 in this study.

We were unable to detect an association between the tested SNPs in *FBN2* and *COL4A1* and IA in the Japanese cohort. Furthermore, the ORs of the associations of *HSPG2* and *CSPG2* with IA were lower in the Japanese cohort compared to the Dutch cohort. The susceptibility effect of the 4 studied genes may vary between populations, which would influence the power of our current study, especially taking into account that initial association reports often overestimate disease risk [17]. Thus, if the true susceptibility effect of our 4 genes is lower than estimated in the Dutch association studies [8, 9], the power to detect the effect in the studied Japanese cohort decreases. The different results may also be explained by population-specific effects of *HSPG2*, *CSPG2*, *FBN2* and *COL4A1* on IA susceptibility. *FBN2* and *COL4A1* may have no effect while *HSPG2* and *CSPG2* may have a weaker effect in the Japanese population. Population-specific variants may contribute to the risk of subarachnoid hemorrhage and IA. Such variations may for example play a role in the difference in incidence of subarachnoid hemorrhage, which is about 2 times higher in Japan (and in Finland) than in other parts of the world [18].

In our study, we found evidence for *HSPG2* and *CSPG2* as common susceptibility markers for IA in the Dutch and Japanese population. More studies that analyze *HSPG2* and *CSPG2* for an association with IA are needed in different independent populations in order to be able to draw conclusions about the role of these genes as susceptibility genes for IA in different populations.

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References

1 Rinkel GJE, Djibuti M, Algra A, van Gijn J: Prevalence and risk of rupture of intracranial aneurysms. Stroke 1998;29:251–256.
2 van Gijn J, Kerr RS, Rinkel GJE: Subarachnoid haemorrhage. Lancet 2007;369:306–318.
3 Hop JW, Rinkel GJE, Algra A, van Gijn J: Case fatality rates and functional outcome after subarachnoid hemorrhage: a systematic review. Stroke 1997;28:660–664.
4 Feigin VL, Rinkel GJ, Lawes CM, Algra A, Bennett DA, van Gijn J, Anderson S: Risk factors for subarachnoid hemorrhage: an updated systematic review of epidemiological studies. Stroke 2005;36:2773–2780.
5 Krischek B, Tatagiba M: The influence of genetics on intracranial aneurysm formation and rupture: current knowledge and its possible impact on future treatment. Adv Tech Stand Neurosurg 2008;33:137–147.
6 Semmler A, Linnebank M, Krex D, Götz A, Moskau S, Ziegler A, Simon M: Polymorphisms of homocysteine metabolism are associated with intracranial aneurysms. Cerebrovasc Dis 2008;26:425–429.
7 Ruigrok YM, Rinkel GJ: Genetics of intracranial aneurysms. Stroke 2008;39:1049–1055.
8 Ruigrok YM, Rinkel GJ, van't Slot R, Wolfs M, Tang S, Wijmenga C: Evidence in favor of the contribution of genes involved in the maintenance of the extracellular matrix of the arterial wall to the development of intracranial aneurysms. Hum Mol Genet 2006;15:3361–3368.
9 Ruigrok YM, Rinkel GJ, Wijmenga C: The versican gene and the risk of intracranial aneurysms. Stroke 2006;37:2372–2374.
10 Sun H, Zhang D, Zhao J: Chondroitin sulfate proteoglycan 2 (*CSPG2*) gene polymorphisms rs173686 and rs251124 are not associated with intracranial aneurysms in Chinese Han nationality. Ups J Med Sci 2007;112:289–295.
11 Nahed BV, Seker A, Guclu B, Ozturk AK, Finberg K, Hawkins AA, DiLuna ML, State M, Lifton RP, Gunel M: Mapping a Mendelian form of intracranial aneurysm to 1p34.3-p36.13. Am J Hum Genet 2004;76:172–179.
12 Ruigrok YM, Wijmenga C, Rinkel GJ, van't Slot R, Baas F, Westerveld A, Ross YB: Genomewide linkage in a large Dutch family with intracranial aneurysms: replication of 2 loci for intracranial aneurysms to chromosome 1p36.11-p36.13 and Xp22.2-p22.32. Stroke 2008;39:1096–1102.
13 Onda H, Kasuya H, Yoneyama T, Takakura K, Hori T, Takeda J, Nakajima T, Inoue I: Genomewide-linkage and haplotype-association studies map intracranial aneurysm to chromosome 7q11. Am J Hum Genet 2001;69:804–819.
14 Davis GE, Senger DR: Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ Res 2005;97:1093–1107.
15 Wu YJ, La Pierre DP, Wu J, Yee AJ, Yang BB: The interaction of versican with its binding partners. Cell Res 2005;15:483–494.
16 Segev A, Nili N, Strauss BH: The role of perlecan in arterial injury and angiogenesis. Cardiovasc Res 2004;63:603–610.
17 Colhoun HM, McKeigue PM, Davey SG: Problems of reporting genetic associations with complex outcomes. Lancet 2003;361:865–872.
18 de Rooij NK, Linn FH, van der Plas JA, Algra A, Rinkel GJ: Incidence of subarachnoid haemorrhage: a systematic review with emphasis on region, age, gender and time trends. J Neurol Neurosurg Psychiatry 2007;78:1365–1372.