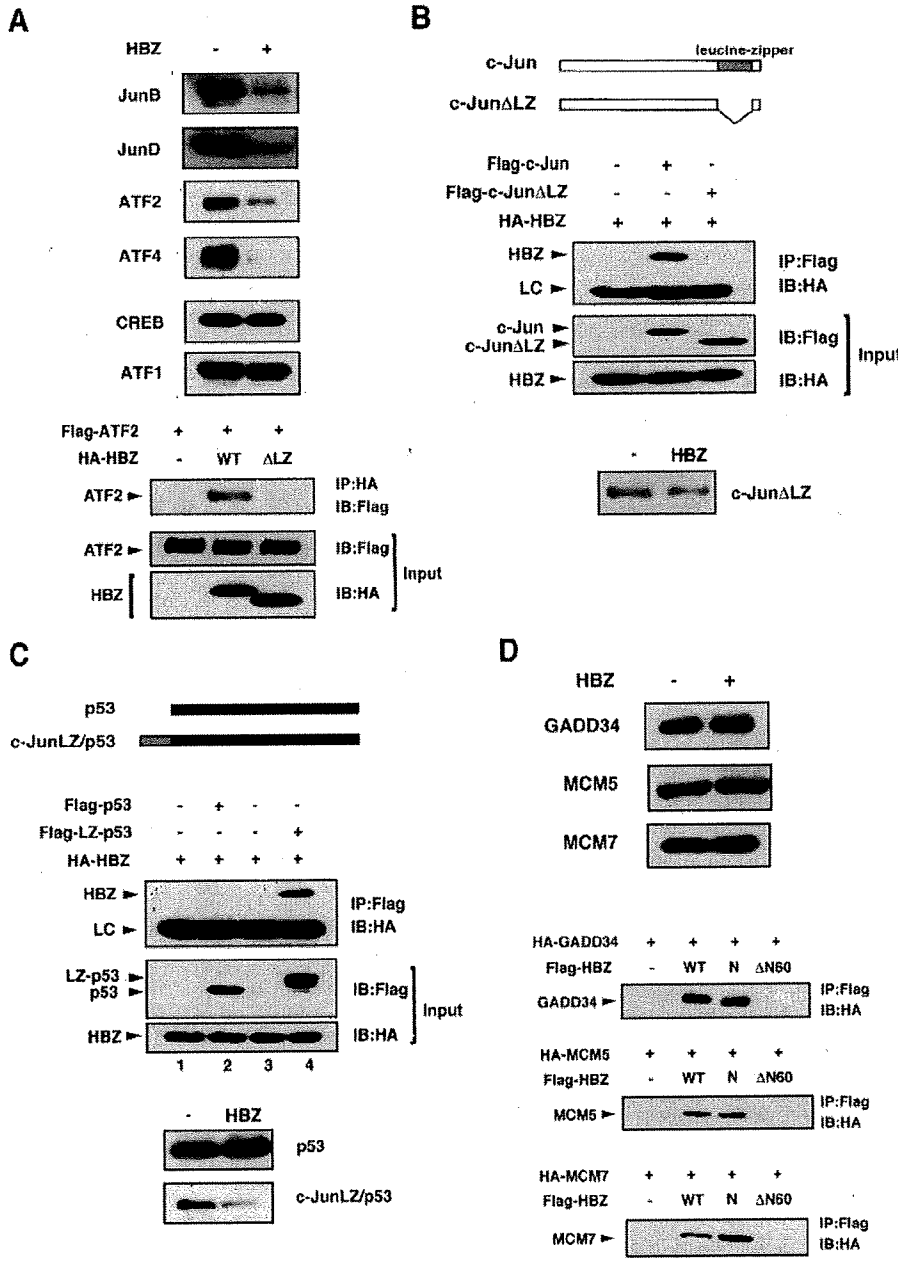

FIGURE 4. **HBZ promotes degradation of proteins that bind its bZIP domain but not its N terminus.** *A*, HBZ promotes the degradation of proteins that bind its bZIP domain. *Upper panel*: HEK-293T cells were transfected with pcDNA3-Myc-JunB, pcDNA3-HA-JunD, pCAG-FLAG-ATF1, pCAG-FLAG-ATF2, pCAG-FLAG-ATF4, or pcDNA3-HA-CREB, with or without HBZ. Cell lysates were immunoblotted with the appropriate epitope-tag antibodies. *Lower panel*: HEK-293T cells were transfected with 2.5 μg of pcDNA3-FLAG-ATF2 together with 2.5 μg of pcDNA3-HA-HBZ or pcDNA3-HA-HBZ-ΔLZ, followed by treatment with 20 μM MG132 for 12 h. Cell lysates were immunoprecipitated with an anti-HA antibody, followed by immunoblotting with an anti-FLAG antibody. Immunoblotting of whole cell lysates was performed using the antibodies indicated. *B*, an interaction with HBZ is responsible for target protein degradation. *Upper panel*: schematic diagram of c-Jun and c-JunΔLZ used in this study. *Middle panel*: HEK-293T cells were transfected as indicated and then treated with MG132 for 12 h. Cell lysates were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with an anti-HA antibody. *LC* indicates the immunoglobulin light chain. *Lower panel*: HEK-293T cells were transfected with pcDNA3-HA-c-JunΔLZ, with or without HBZ. Cell lysates were immunoblotted with an anti-HA antibody. *C*, *upper panel*: schematic diagram of p53 and c-JunLZ/p53. *Middle panel*: HEK-293T cells were transfected as indicated and then treated with MG132 for 12 h. Cell lysates were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with an anti-HA antibody. *Lower panel*: HEK-293T cells were transfected with pcDNA3-FLAG-p53 or pcDNA3-FLAG-c-JunLZ/p53, with or without HBZ. Cell lysates were immunoblotted with an anti-FLAG antibody. *D*, HBZ does not promote degradation of proteins that bind its N terminus. *Upper panel*: HEK-293T cells were transfected with pcDNA3-HA-GADD34, pcDNA3-HA-MCM5, or pcDNA3-HA-MCM7, with or without HBZ. Cell lysates were immunoblotted with an anti-HA antibody. *Lower panel*: HEK-293T cells were transfected with 2.5 μg of pcDNA3-HA-GADD34, pcDNA3-HA-MCM5, or pcDNA3-HA-MCM7, together with 2.5 μg of pcDNA3-FLAG-HBZ, pcDNA3-FLAG-HBZ-N, or pcDNA3-FLAG-HBZ-ΔN60. Cell lysates were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with an anti-HA antibody.

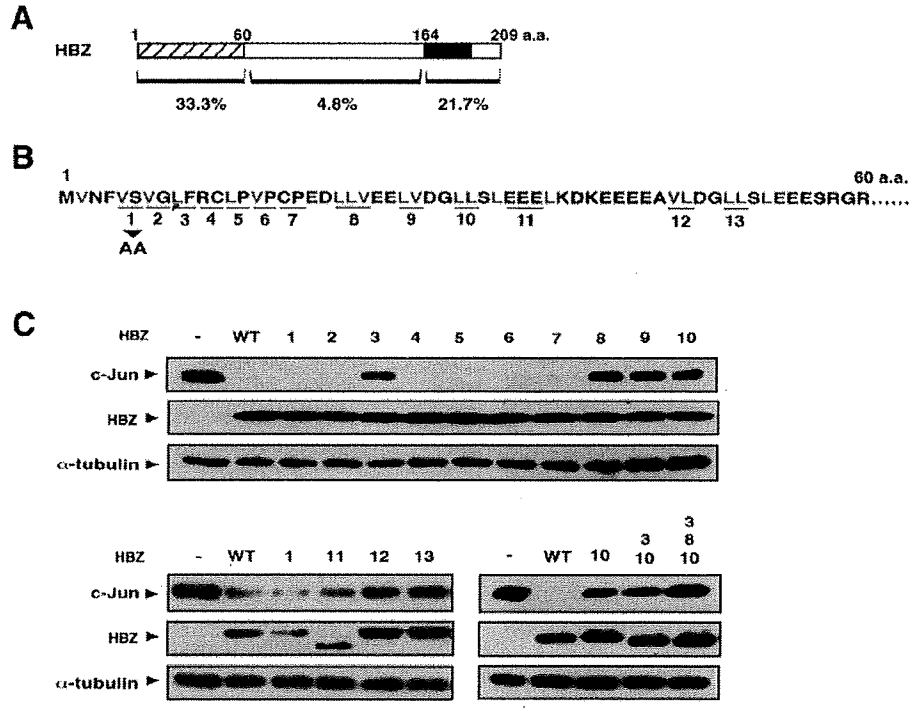

FIGURE 5. **The N-terminal region of HBZ (especially some hydrophobic residues) is responsible for destabilization of c-Jun.** *A*, the N-terminal region of HBZ is rich in hydrophobic residues. The percentage of hydrophobic residues (Leu/Ile/Val) in the respective regions is indicated below. *B*, alanine-scanning substitutions in the N-terminal region of HBZ. The *gray letters* indicate hydrophobic residues (Leu/Ile/Val). *C*, some alanine mutants promoted c-Jun degradation less effectively than wild-type. HEK-293T cells were transfected with c-Jun and either pcDNA3-FLAG-HBZ or its mutants. Cell lysates were analyzed by immunoblotting.

not associate with HBZ (Fig. 4*C*, *middle panel*, *lane* 2). However, an artificial fusion protein that contained the c-Jun leucine-zipper region and p53 (c-JunLZ/p53) could interact with HBZ (Fig. 4*C*, *middle panel*, *lane 4*) and was degraded by HBZ (Fig. 4*C*, *lower panel*). These results suggest that the association with HBZ is essential for target protein degradation.

Does HBZ promote the degradation of all its binding partners? To further investigate this question, we examined whether HBZ promotes degradation of proteins that bind its N terminus. We identified growth arrest and DNA damage-inducible transcript 34 (GADD34), minichromosome maintenance protein 5 (MCM5) and MCM7 as cellular proteins that bind the HBZ N terminus using a yeast two-hybrid screen with the HBZ N-terminal 120 amino acids as bait. These interactions were confirmed *in vivo* (Fig. 4*D*, *lower panel*) and *in vitro* (data not shown). Interestingly, HBZ did not promote degradation of these N-terminal-binding proteins (Fig. 4*D*, *upper panel*).

The N-terminal Region of HBZ (Especially Some Hydrophobic Residues) Is Essential for c-Jun Destabilization—In our previous report, we identified the regions of HBZ responsible for the destabilization of c-Jun (26). We have shown that not only the leucine-zipper region, as an interaction domain with c-Jun, but also the N-terminal 60 amino acids of HBZ are responsible for the destabilization of c-Jun. Notably, several hydrophobic residues (especially Leu, Ile, and Val) are concentrated in the N-terminal 60 amino acids of HBZ (Fig. 5*A*). We performed alanine-scanning mutagenesis on this region to identify point mutants that were unable to destabilize c-Jun. Alanine substitutions were focused primarily on Leu, Ile, and Val residues (Fig. 5*B*). These mutants associated with c-Jun comparably to wild-type HBZ (data not shown). As shown in Fig. 5*C*, some of these alanine mutants promoted c-Jun degradation less effectively than wild-type. In particular, the triple mutant (HBZ-3.8.10) had little ability to degrade c-Jun. These results suggest that the N-terminal 60 amino acids of HBZ (particularly certain hydrophobic residues) are essential for the destabilization of c-Jun.

The N-terminal Region of HBZ Directly Interacts with the 26 S Proteasome—It is plausible that the N-terminal region of HBZ recruits cellular factors that regulate c-Jun degradation. We performed a yeast two-hybrid screen to identify these factors. We used a fusion protein that combined the GAL4 DNA-binding domain with the HBZ N-terminal 120 amino acids as bait, because the HBZ N-terminal 60 amino acids alone possessed intensive transactivation ability. Some positive clones were found to correspond to Rpn5 (also known as PSMD12 or p55), a 19 S proteasome subunit. As shown in Fig. 6*A* (*upper panel*), the HBZ N-terminal 120 amino acids (HBZ-N) interacted with Rpn5 in yeast, whereas the mutant HBZ-N-3.8.10, which lacked the ability to destabilize c-Jun (Fig. 5*C*), did not associate with Rpn5. On the other hand, WD40 protein, another target identified in the screen, interacted with both HBZ-N and HBZ-N-3.8.10 (Fig. 6*A*, *lower panel*).

We next confirmed the interaction between HBZ and Rpn5 using an *in vitro* GST pull-down assay. Recombinant HBZ-N and HBZ-N-3.8.10 fused to GST (GST-HBZ-N and GST-HBZ-N-3.8.10) were incubated with ^{35}S-labeled Rpn5 that was translated *in vitro*. The ^{35}S-labeled Rpn5 copurified with GST-HBZ-N but not with GST-HBZ-N-3.8.10 (Fig. 6*B*). To further confirm the interaction between HBZ and Rpn5, we performed a coimmunoprecipitation assay in HEK-293T cells transiently expressing HA-tagged Rpn5 and FLAG-tagged HBZ wild-type (-WT) or mutants (-ΔN60, -3.8.10). As shown in Fig. 6*C*, Rpn5 coimmunoprecipitated with HBZ-WT, but not with either the HBZ-ΔN60 or -3.8.10 mutants. These results indicate that the N-terminal region of HBZ associates with the proteasomal subunit Rpn5 and that this association correlates with the destabilization of c-Jun.

Although HBZ interacted with ectopically expressed Rpn5, it was not distinguishable whether HBZ interacts with free Rpn5 or complex form of proteasome. To investigate this point, we examined whether other subunits of the proteasome were coimmunoprecipitated with HBZ. As shown in Fig. 6*D*, all of

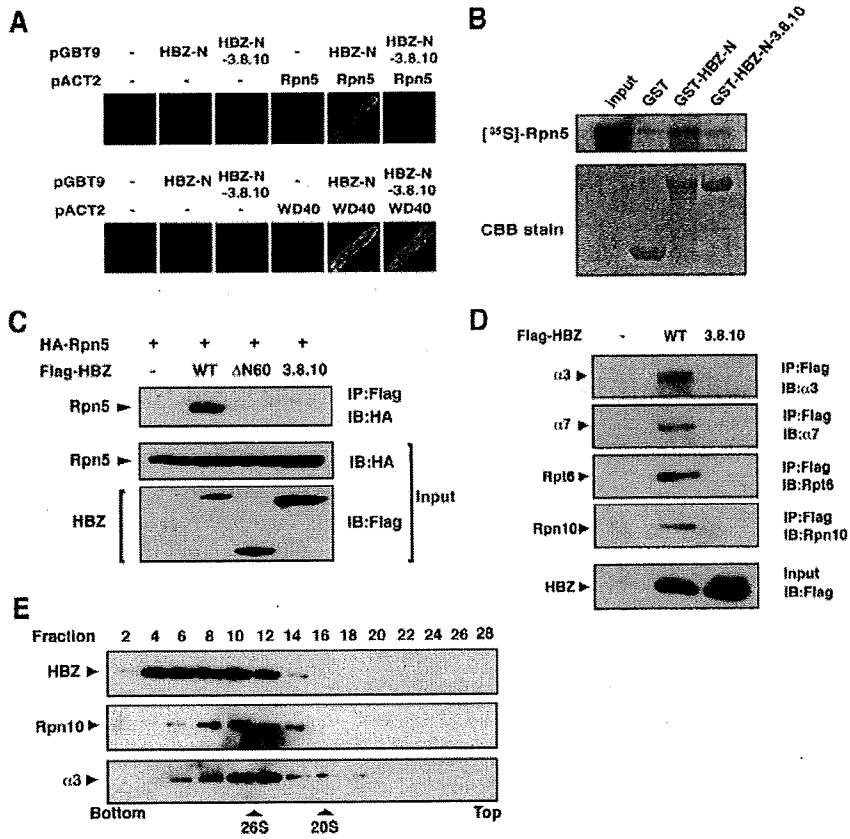

FIGURE 6. **The N-terminal region of HBZ directly interacts with the 26 S proteasome.** *A*, identification of Rpn5 as a target for the N-terminal region of HBZ using a yeast two-hybrid screen. The yeast strain Y190 was transformed as indicated. Transformants were grown in -Leu/-Trp/-His medium and selected for histidine prototrophy. *B*, HBZ associates with Rpn5 *in vitro*. GST, GST-HBZ-N, or GST-HBZ-N-3.8.10 fusion proteins were incubated with ^{35}S-labeled Rpn5. Bound proteins were detected by autoradiography. The abundance of GST proteins is shown by CBB staining. *C*, HBZ associates with Rpn5 in cells. HEK-293T cells were transfected as indicated. Cell lysates were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with an anti-HA antibody. *D*, HBZ associates with the 26 S proteasome. HEK-293T cells were transfected with pcDNA3-FLAG-HBZ or pcDNA3-FLAG-HBZ-3.8.10 and then treated with MG132 for 12 h. Cell lysates were immunoprecipitated with an anti-FLAG antibody, followed by immunoblotting with the antibodies indicated. *E*, cells transiently expressing HA-tagged HBZ were fractionated by 10–40% glycerol density gradient centrifugation. Each fraction was analyzed by immunoblotting with anti-HA, anti-Rpn10, or anti-α3 antibodies. The *peaks* corresponding to the 26 S and 20 S proteasomes are indicated.

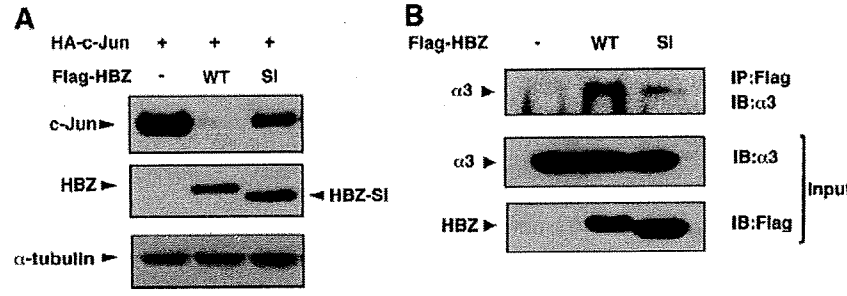

FIGURE 7. **HBZ splicing isoform has less ability to destabilize c-Jun than wild-type.** *A*, HBZ-SI promotes degradation of c-Jun less effectively than wild-type. HEK-293T cells were transfected with 0.5 μg of pcDNA3-HA-c-Jun and 3 μg of either pcDNA3-FLAG-HBZ or pcDNA3-FLAG-HBZ-SI. Cell lysates were immunoblotted with anti-HA, anti-FLAG, or anti-α-tubulin antibodies. *B*, HBZ-SI associates with the proteasome less effectively than wild type. HEK-293T cells were transfected with 8 μg of pcDNA3-FLAG-HBZ or pcDNA3-FLAG-HBZ-SI and then treated with MG132. Cell lysates were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with an anti-α3 antibody. The abundance of α3 and HBZ proteins in whole cell lysates are shown (*middle panel* and *lower panel*).

the proteasomal subunits tested here coimmunoprecipitated with HBZ, but not with its mutant. α3 and α7 are α-subunits that form the outer rings of the 20 S proteasome, whereas Rpt6 and Rpn10 are subunits of the 19 S regulatory complex. The components of both the 19 S and 20 S proteasomes coprecipitated with HBZ, suggesting that HBZ interacts with the 26 S proteasome complex. This notion was supported by the cosedimentation of HBZ and the 26 S proteasome in a glycerol density gradient (Fig. 6*E*).

Our findings indicate that HBZ interacts with the 26 S proteasome and c-Jun via its N and C termini, respectively. This suggests that HBZ acts as a tethering factor between c-Jun and the 26 S proteasome, thereby facilitating delivery of c-Jun to the proteasome in a ubiquitin-independent manner.

Recently, it has been reported that the HTLV-1 genome encodes an alternative splicing isoform of HBZ (35–37), termed HBZ-SI by Murata's group. We investigated whether the two HBZ isoforms differed in their ability to destabilize c-Jun. Interestingly, HBZ-SI promoted c-Jun degradation less efficiently than wild-type (Fig. 7*A*). As expected from the above results, HBZ-SI interacted with the proteasome less efficiently than wild-type (Fig. 7*B*). This correlation also supports the significance of the association between HBZ and the 26 S proteasome in c-Jun destabilization.

DISCUSSION

Viruses have evolved sophisticated strategies to utilize or manipulate the host ubiquitin-proteasome system for their propagation. For example, human papillomavirus type-16 E6 protein and adenovirus E1B55k-E4orf6 proteins act as part of E3 ubiquitin ligase complexes to promote ubiquitination and subsequent degradation of the tumor suppressor, p53 (14, 38). The human immunodeficiency virus type-1 Vif protein also acts as an E3 ubiquitin ligase that targets APOBEC3G, a potent cellular anti-

viral factor, for proteasomal degradation in a ubiquitin-dependent manner (39). There are an increasing number of viral proteins that utilize cellular ubiquitination machinery to dysregulate cellular functions (15–17). On the other hand, the human cytomegalovirus (CMV) pp71 protein has been shown to promote proteasomal degradation of Rb protein without promoting its ubiquitination (12), the molecular mechanism of which, however, is unknown. Recently, a number of eukaryotic proteins, including ODC (8), p53 (9), $p21^{waf1/cip1}$ (10, 11), and IκBα (40), have been shown to be degraded through a proteasome-dependent, ubiquitin-independent pathway, suggesting the significance of this alternative pathway in various cellular events (7). Here, we demonstrate that the HTLV-1 HBZ protein promotes degradation of c-Jun, a cellular transcription factor, using a proteasome-dependent but ubiquitin-independent mechanism. First, although HBZ-mediated degradation of c-Jun was prevented by proteasome inhibitor treatment, HBZ failed to promote c-Jun ubiquitination. Second, HBZ destabilized a c-Jun mutant lacking all lysine targets for ubiquitination. Third, a dominant-negative ubiquitin (K48R) mutant failed to abrogate HBZ-mediated c-Jun degradation. Fourth, c-Jun degradation mediated by HBZ was unaffected in ts20 cells at the restrictive temperature. Taken together, these results indicate that HBZ promotes c-Jun degradation in a ubiquitin-independent manner.

How, in the absence of polyubiquitination, does HBZ target c-Jun for proteasomal degradation? ODC was the first protein shown to be degraded in a ubiquitin-independent manner. Attachment of antizyme causes conformational changes in ODC, thereby exposing its C-terminal degradation signal for recognition by the 26 S proteasome (8, 41). Thus, antizyme promotes ODC degradation by enhancing the direct association of ODC with the 26 S proteasome. In contrast, degradation of $p21^{waf1/cip1}$ and Rb proteins is shown to be mediated by the 20 S proteasome. These proteins are directly recognized by the α7 subunit of the 20 S proteasome (11, 13). Notably, the MDM2 oncoprotein has been shown to promote degradation of $p21^{waf1/cip1}$ and Rb in a ubiquitin-independent manner (13, 42, 43). MDM2 directly associates with both these proteins and the 20 S proteasome and facilitates p21-20 S or Rb-20 S interactions. This tethering function of MDM2 is similar to HBZ-mediated c-Jun degradation. In this study, we have shown that HBZ interacts with the 26 S proteasome and c-Jun via its N and C termini, respectively. Moreover, we found that these associations are essential for c-Jun destabilization. These observations suggest that HBZ acts as a tethering factor between c-Jun and the 26 S proteasome, thereby facilitating the delivery of c-Jun to the proteasome without ubiquitination.

What is the physiological significance of HBZ-mediated c-Jun repression in the viral life cycle? In HTLV-1-infected cells, AP-1 is constitutively activated predominantly by Tax (44, 45). Although AP-1 contributes to T-cell transformation, aberrantly up-regulated AP-1 often accelerates cell death (46). HBZ may protect infected cells from cell death by preventing aberrant AP-1 activation. In addition, in this study we have shown that HBZ promotes protein degradation of various cellular bZIP proteins (*e.g.* ATF4, JunB, JunD, and c-Jun) that are involved in transcription from the HTLV-1 LTR. Because viral products (in particular Tax) are major targets of the host immune response (20), their overexpression is unfavorable for viral propagation. Therefore, as a negative regulator of viral expression, HBZ may play a key role in establishing and maintaining long, latent HTLV-1 infection.

In this study, we disclose a new viral strategy to utilize the host proteolytic apparatus for viral propagation. HBZ acts as a tethering factor between the 26 S proteasome and its substrate, thereby mimicking the targeting function of ubiquitination. Our findings also provide significant insight into the cellular proteasomal degradation pathway. It is possible that this direct tethering model between the proteasome and its substrate is a common cellular proteolytic event. We assume that in the future accumulating reports on ubiquitin-independent degradation will show the physiological significance of this alternative degradation pathway in various cellular events.

Acknowledgments—We thank Dr. Harvey Ozer for providing the mouse ts20 cells, and Dr. H. Miyoshi for providing the lentivirus-based transfection system.

REFERENCES

1. Davies, K. J. (2001) *Biochimie (Paris)* **83,** 301–310
2. DeMartino, G. N., and Slaughter, C. A. (1999) *J. Biol. Chem.* **274,** 22123–22126
3. Glickman, M. H., and Ciechanover, A. (2002) *Physiol. Rev.* **82,** 373–428
4. Goldberg, A. L. (2003) *Nature* **426,** 895–899
5. Prakash, S., Tian, L., Ratliff, K. S., Lehotzky, R. E., and Matouschek, A. (2004) *Nat. Struct. Mol. Biol.* **11,** 830–837
6. Takeuchi, J., Chen, H., and Coffino, P. (2007) *EMBO J.* **26,** 123–131
7. Orlowski, M., and Wilk, S. (2003) *Arch. Biochem. Biophys.* **415,** 1–5
8. Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992) *Nature* **360,** 597–599
9. Asher, G., Lotem, J., Sachs, L., Kahana, C., and Shaul, Y. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 13125–13130
10. Sheaff, R. J., Singer, J. D., Swanger, J., Smitherman, M., Roberts, J. M., and Clurman, B. E. (2000) *Mol. Cell* **5,** 403–410
11. Touitou, R., Richardson, J., Bose, S., Nakanishi, M., Rivett, J., and Allday, M. J. (2001) *EMBO J.* **20,** 2367–2375
12. Kalejta, R. F., and Shenk, T. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 3263–3268
13. Sdek, P., Ying, H., Chang, D. L., Qiu, W., Zheng, H., Touitou, R., Allday, M. J., and Xiao, Z. X. (2005) *Mol. Cell* **20,** 699–708
14. Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993) *Cell* **75,** 495–505
15. Knight, J. S., Sharma, N., and Robertson, E. S. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 18562–18566
16. Gao, G., and Luo, H. (2006) *Can. J. Physiol. Pharmacol.* **84,** 5–14
17. Shackelford, J., and Pagano, J. S. (2005) *Essays Biochem.* **41,** 139–156
18. Takatsuki, K. (2005) *Retrovirology* **2,** 16
19. Yoshida, M. (2001) *Annu. Rev. Immunol.* **19,** 475–496
20. Kannagi, M., Harada, S., Maruyama, I., Inoko, H., Igarashi, H., Kuwashima, G., Sato, S., Morita, M., Kidokoro, M., Sugimoto, M., Funahashi, S., Osame, M., and Shida, H. (1991) *Int. Immunol.* **3,** 761–767
21. Inoue, J., Yoshida, M., and Seiki, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3653–3657
22. Nicot, C., Dundr, M., Johnson, J. M., Fullen, J. R., Alonzo, N., Fukumoto, R., Princler, G. L., Derse, D., Misteli, T., and Franchini, G. (2004) *Nat. Med.* **10,** 197–201
23. Gaudray, G., Gachon, F., Basbous, J., Biard-Piechaczyk, M., Devaux, C., and Mesnard, J. M. (2002) *J. Virol.* **76,** 12813–12822
24. Basbous, J., Arpin, C., Gaudray, G., Piechaczyk, M., Devaux, C., and Mesnard, J. M. (2003) *J. Biol. Chem.* **278,** 43620–43627

25. Thebault, S., Basbous, J., Hivin, P., Devaux, C., and Mesnard, J. M. (2004) *FEBS Lett.* **562,** 165–170
26. Matsumoto, J., Ohshima, T., Isono, O., and Shimotohno, K. (2005) *Oncogene* **24,** 1001–1010
27. Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20,** 1425
28. Ciechanover, A., Orian, A., and Schwartz, A. L. (2000) *BioEssays* **22,** 442–451
29. Wertz, I. E., O'Rourke, K. M., Zhang, Z., Dornan, D., Arnott, D., Deshaies, R. J., and Dixit, V. M. (2004) *Science* **303,** 1371–1374
30. Nateri, A. S., Riera-Sans, L., Da Costa, C., and Behrens, A. (2004) *Science* **303,** 1374–1378
31. Ward, C. L., Omura, S., and Kopito, R. R. (1995) *Cell* **83,** 121–127
32. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) *Cell* **78,** 787–798
33. Chowdary, D. R., Dermody, J. J., Jha, K. K., and Ozer, H. L. (1994) *Mol. Cell. Biol.* **14,** 1997–2003
34. Lemasson, I., Lewis, M. R., Polakowski, N., Hivin, P., Cavanagh, M. H., Thebault, S., Barbeau, B., Nyborg, J. K., and Mesnard, J. M. (2007) *J. Virol.* **81,** 1543–1553
35. Satou, Y., Yasunaga, J., Yoshida, M., and Matsuoka, M. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 720–725
36. Murata, K., Hayashibara, T., Sugahara, K., Uemura, A., Yamaguchi, T., Harasawa, H., Hasegawa, H., Tsuruda, K., Okazaki, T., Koji, T., Miyanishi, T., Yamada, Y., and Kamihira, S. (2006) *J. Virol.* **80,** 2495–2505
37. Cavanagh, M. H., Landry, S., Audet, B., Arpin-Andre, C., Hivin, P., Pare, M. E., Thete, J., Wattel, E., Marriott, S. J., Mesnard, J. M., and Barbeau, B. (2006) *Retrovirology* **3,** 15
38. Querido, E., Blanchette, P., Yan, Q., Kamura, T., Morrison, M., Boivin, D., Kaelin, W. G., Conaway, R. C., Conaway, J. W., and Branton, P. E. (2001) *Genes Dev.* **15,** 3104–3117
39. Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., and Yu, X. F. (2003) *Science* **302,** 1056–1060
40. Krappmann, D., Wulczyn, F. G., and Scheidereit, C. (1996) *EMBO J.* **15,** 6716–6726
41. Li, X., and Coffino, P. (1993) *Mol. Cell. Biol.* **13,** 2377–2383
42. Jin, Y., Lee, H., Zeng, S. X., Dai, M. S., and Lu, H. (2003) *EMBO J.* **22,** 6365–6377
43. Zhang, Z., Wang, H., Li, M., Agrawal, S., Chen, X., and Zhang, R. (2004) *J. Biol. Chem.* **279,** 16000–16006
44. Fujii, M., Niki, T., Mori, T., Matsuda, T., Matsui, M., Nomura, N., and Seiki, M. (1991) *Oncogene* **6,** 1023–1029
45. Mori, N., Fujii, M., Iwai, K., Ikeda, S., Yamasaki, Y., Hata, T., Yamada, Y., Tanaka, Y., Tomonaga, M., and Yamamoto, N. (2000) *Blood* **95,** 3915–3921
46. Shaulian, E., and Karin, M. (2002) *Nat. Cell Biol.* **4,** E131

doi: 10.1111/j.1365-2222.2009.03229.x

ORIGINAL ARTICLE Asthma and Rhinitis

Clinical & Experimental Allergy, 39, 972–981

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

Submitted 5 March 2008; revised 11 January 2009; accepted 26 January 2009

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Cite this as: T. Sekigawa, A. Tajima, T. Hasegawa, Y. Hasegawa, H. Inoue, Y. Sano, S. Matsune, Y. Kurono and I. Inoue, *Clinical & Experimental Allergy*, 2009 (39) 972–981.

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

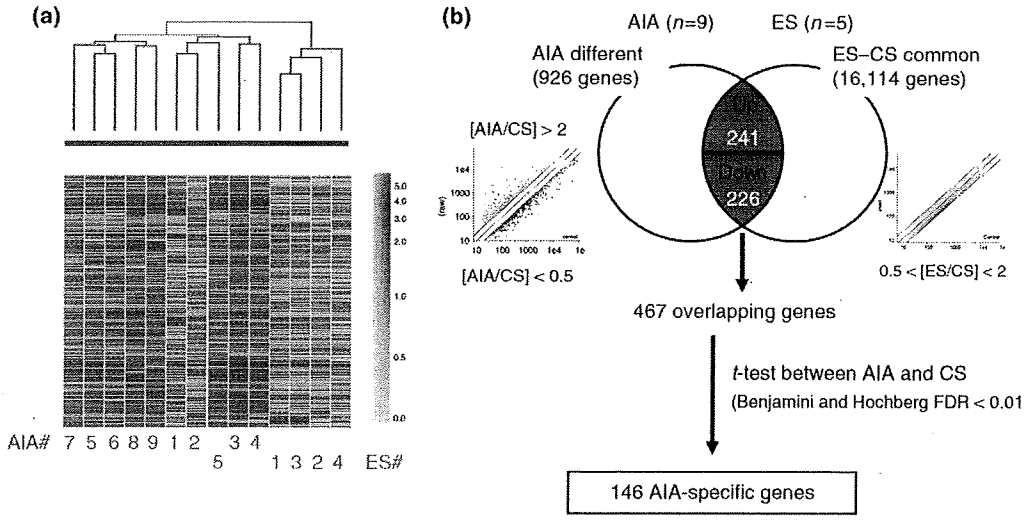

Fig. 1. Experimental design to extract aspirin-intolerant asthma (AIA)-specific genes with microarray analysis. (a) Hierarchical clustering (HC)-based classification of nasal polyps from AIA and eosinophilic sinusitis (ES) patients. Based on the gene expression in nasal polyps, HC clustering shows distinct 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.

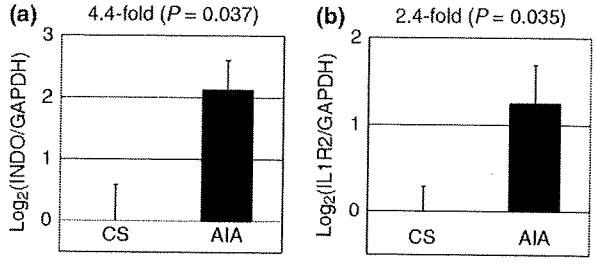

Fig. 2. Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis validates expression differences for two genes, *INDO* and *IL1R2*, in nasal polyps from aspirin-intolerant asthma (AIA) and chronic sinusitis (CS) patients. Relative amounts of the respective genes in nasal polyps (AIA, $n = 10$; 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

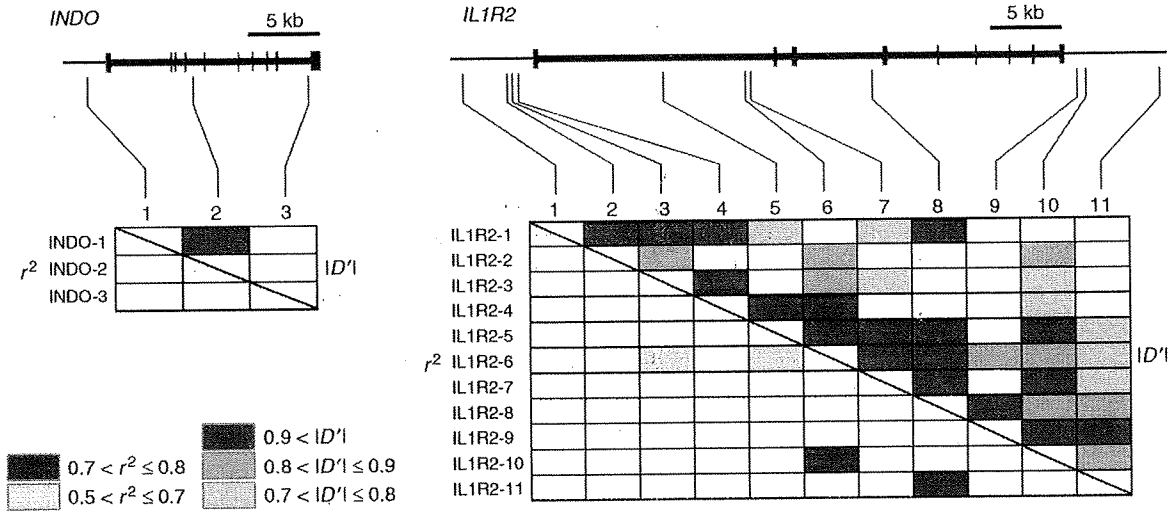

Fig. 3. Linkage disequilibrium pattern of *INDO* and *IL1R2*. The gene structures of *INDO* (left) and *IL1R2* (right), together with positions of the 14 single nucleotide polymorphisms (SNPs) examined, are shown. Pairwise LD coefficients, D' and r^2, were determined and expressed as a block structure. 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.

Acknowledgements

We thank tissue and DNA donors and supporting medical staff for making this study possible. This work was supported in part by a Grant-in-Aid for scientific research from the Japanese Ministry of Education, Science, Sports, and Culture. We are grateful to Yoshiko Sakamoto, Hiromi Kamura, and Kozue Otaka for their technical assistance. All of authors have no declared conflicts of interest.

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.

Original Paper

Cerebrovascular Diseases

Cerebrovasc Dis 2009;28:131–134
DOI: 10.1159/000223438

Received: December 31, 2008
Accepted: April 6, 2009
Published online: June 5, 2009

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.

Acknowledgments

Y.M.R. was supported by a grant from the Dr. E. Dekker program of the Netherlands Heart Foundation (2005T014). B.K. was supported by the Alexander-von-Humboldt Foundation and the Japanese Society for Promotion of Science. I.I. was supported by KAKENHI (Grant-in-Aid for Scientific Research) on priority areas 'Applied Genomics' from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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.

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Human Mutation

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The Phenotype and Genotype Experiment Object Model (PaGE-OM): A Robust Data Structure for Information Related to DNA Variation

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Communicated by Richard G. H. Cotton

Received 12 November 2008; accepted revised manuscript 19 December 2008.
Published online 18 March 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/humu.20973

ABSTRACT: Torrents of genotype–phenotype data are being generated, all of which must be captured, processed, integrated, and exploited. To do this optimally requires the use of standard and interoperable "object models," providing a description of how to partition the total spectrum of information being dealt with into elemental "objects" (such as "alleles," "genotypes," "phenotype values," "methods") with precisely stated logical interrelationships (such as "A objects are made up from one or more B objects"). We herein propose the Phenotype and Genotype Experiment Object Model (PaGE-OM; www.pageom.org), which has been tested and implemented in conjunction with several major databases, and approved as a standard by the Object Management Group (OMG). PaGE-OM is open-source, ready for use by the wider community, and can be further developed as needs arise. It will help to improve information management, assist data integration, and simplify the task of informatics resource design and construction for genotype and phenotype data projects.
Hum Mutat 30, 968–977, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: bioinformatics; data model; genotype–phenotype; database

Introduction

Individual genomes vary extensively, and much of this variation can impact disease and other phenotypes. Technological progress has made it possible to study such genotype to phenotype (G2P) relationships in a genome-wide manner, and deep whole-genome resequencing may soon be economically available as the ultimate experimental strategy [Mardis, 2008]. To complement this, clinical sample biobanks have been steadily growing in size and proficiency, providing large-scale resources to support the G2P field [Smith et al., 2005]. Consequently, new G2P correlations are being identified with increasing frequency, and the pressure is on to use this elemental information in the most optimal fashion—both for improved biomedical understanding and in the context of drug development and clinical practice. To enable this, databases and informatics resources must be developed to support the data-handling challenges posed by vast numbers of dispersed and multifarious G2P datasets. Those systems must be able to interoperate on many levels of data processing—such as security, validation, integration, exchange, interrogation, presentation, and analysis.

To achieve the desired widespread interoperability, G2P data systems must be based upon well-designed and robust standards. The role of standards and unified effort in modern biomedicine is

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