References

1 Cortes Funes H, Martin C, Abratt R, Lund B. Safety profile of gemcitabine, a novel anticancer agent, in non small cell lung cancer. *Anticancer Drugs* 1997; **8**:582 587.

2 Heinemann V, Wilke H, Mergenthaler HG, Clemens M, Konig H, Illiger HJ, *et al.* Gemcitabine and cisplatin in the treatment of advanced or metastatic pancreatic cancer. *Ann Oncol* 2000; **11**:1399 1403.

3 Tanaka T, Ikeda M, Okusaka T, Ueno H, Morizane C, Hagihara A, *et al.* Prognostic factors in Japanese patients with advanced pancreatic cancer treated with single agent gemcitabine as first line therapy. *Jpn J Clin Oncol* 2008; **38**:755 761.

4 Lee JO, Kim DY, Lim JH, Seo MD, Yi HG, Oh DY, *et al.* Palliative chemotherapy for patients with recurrent hepatocellular carcinoma after liver transplantation. *J Gastroenterol Hepatol* 2009; **24**:800 805.

5 Mackey JR, Yao SY, Smith KM, Karpinski E, Baldwin SA, Cass CE, *et al.* Gemcitabine transport in *xenopus* oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. *J Natl Cancer Inst* 1999; **91**:1876 1881.

6 Ritzel MW, Ng AM, Yao SY, Graham K, Loewen SK, Smith KM, *et al.* Molecular identification and characterization of novel human and mouse concentrative Na^+ nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). *J Biol Chem* 2001; **276**:2914 2927.

7 Mackey JR, Mani RS, Selner M, Mowles D, Young JD, Belt JA, *et al.* Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. *Cancer Res* 1998; **58**:4349 4357.

8 Van Rompay AR, Johansson M, Karlsson A. Substrate specificity and phosphorylation of antiviral and anticancer nucleoside analogues by human deoxyribonucleoside kinases and ribonucleoside kinases. *Pharmacol Ther* 2003; **100**:119 139.

9 Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, *et al.* Inhibition of ribonucleotide reduction in CCRF CEM cells by 2′,2′ difluorodeoxycytidine. *Mol Pharmacol* 1990; **38**:567 572.

10 Plunkett W, Huang P, Gandhi V. Preclinical characteristics of gemcitabine. *Anticancer Drugs* 1995; **6 (Suppl 6)**:7 13.

11 Rha SY, Jeung HC, Choi YH, Yang WI, Yoo JH, Kim BS, *et al.* An association between *RRM1* haplotype and gemcitabine induced neutropenia in breast cancer patients. *Oncologist* 2007; **12**:622 630.

12 Sugiyama E, Kaniwa N, Kim SR, Kikura Hanajiri R, Hasegawa R, Maekawa K, *et al.* Pharmacokinetics of gemcitabine in Japanese cancer patients: the impact of a cytidine deaminase polymorphism. *J Clin Oncol* 2007; **25**:32 42.

13 Tanaka M, Javle M, Dong X, Eng C, Abbruzzese JL, Li D. Gemcitabine metabolic and transporter gene polymorphisms are associated with drug toxicity and efficacy in patients with locally advanced pancreatic cancer. *Cancer* 2010; **116**:5325 5335.

14 Chew HK, Doroshow JH, Frankel P, Margolin KA, Somlo G, Lenz HJ, *et al.* Phase II studies of gemcitabine and cisplatin in heavily and minimally pretreated metastatic breast cancer. *J Clin Oncol* 2009; **27**:2163 2169.

15 Nakamura Y. The BioBank Japan Project. *Clin Adv Hematol Oncol* 2007; **5**:696 697.

16 Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high throughput SNP typing system for genome wide association studies. *J Hum Genet* 2001; **46**:471 477.

17 International HapMap Consortium. The International HapMap Project. *Nature* 2003; **426**:789 796.

18 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B* 1995; **57**: 289 300.

19 Purcell S, Neale B, Todd Brown K, Thomas L, Ferreira MA, Bender D, *et al.* PLINK: a tool set for whole genome association and population based linkage analyses. *Am J Hum Genet* 2007; **81**:559 575.

20 Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**:263 265.

21 Levy Strumpf N, Kimchi A. Death associated proteins (DAPs): from gene identification to the analysis of their apoptotic and tumor suppressive functions. *Oncogene* 1998; **17**:3331 3340.

22 Kimchi A. DAP genes: novel apoptotic genes isolated by a functional approach to gene cloning. *Biochim Biophys Acta* 1998; **1377**:F13 F33.

23 Larramendy ML, Niini T, Elonen E, Nagy B, Ollila J, Vihinen M, *et al.* Overexpression of translocation associated fusion genes of *FGFR1*, *MYC*, *NPM1*, and *DEK*, but absence of the translocations in acute myeloid leukemia. A microarray analysis. *Haematologica* 2002; **87**:569 577.

24 Holleman A, den Boer ML, de Menezes RX, Cheok MH, Cheng C, Kazemier KM, *et al.* The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia. *Blood* 2006; **107**:769 776.

25 Zhang X, Yashiro M, Qiu H, Nishii T, Matsuzaki T, Hirakawa K. Establishment and characterization of multidrug resistant gastric cancer cell lines. *Anticancer Res* 2010; **30**:915 921.

26 Muller T, Engels P, Fozard JR. Subtypes of the type 4 cAMP phosphodiesterases: structure, regulation and selective inhibition. *Trends Pharmacol Sci* 1996; **17**:294 298.

27 Torphy TJ. Phosphodiesterase isozymes: molecular targets for novel antiasthma agents. *Am J Respir Crit Care Med* 1998; **157**:351 370.

28 Tooker P, Yen WC, Ng SC, Negro Vilar A, Hermann TW. Bexarotene (LGD1069, Targretin), a selective retinoid X receptor agonist, prevents and reverses gemcitabine resistance in NSCLC cells by modulating gene amplification. *Cancer Res* 2007; **67**:4425 4433.

29 Tibaldi C, Giovannetti E, Vasile E, Mey V, Laan AC, Nannizzi S, *et al.* Correlation of *CDA*, *ERCC1*, and *XPD* polymorphisms with response and survival in gemcitabine/cisplatin treated advanced non small cell lung cancer patients. *Clin Cancer Res* 2008; **14**:1797 1803.

30 Okazaki T, Javle M, Tanaka M, Abbruzzese JL, Li D. Single nucleotide polymorphisms of gemcitabine metabolic genes and pancreatic cancer survival and drug toxicity. *Clin Cancer Res* 2010; **16**:320 329.

31 Yonemori K, Ueno H, Okusaka T, Yamamoto N, Ikeda M, Saijo N, *et al.* Severe drug toxicity associated with a single nucleotide polymorphism of the cytidine deaminase gene in a Japanese cancer patient treated with gemcitabine plus cisplatin. *Clin Cancer Res* 2005; **11**:2620 2624.

32 Soo RA, Wang LZ, Ng SS, Chong PY, Yong WP, Lee SC, *et al.* Distribution of gemcitabine pathway genotypes in ethnic Asians and their association with outcome in non small cell lung cancer patients. *Lung Cancer* 2009; **63**:121 127.

33 Ueno H, Kaniwa N, Okusaka T, Ikeda M, Morizane C, Kondo S, *et al.* Homozygous *CDA*3* is a major cause of life threatening toxicities in gemcitabine treated Japanese cancer patients. *Br J Cancer* 2009; **100**:870 873.

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Genome-wide association study for intracranial aneurysm in the Japanese population identifies three candidate susceptible loci and a functional genetic variant at *EDNRA*

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Aneurysmal subarachnoid hemorrhage (aSAH) is the most serious subtype of stroke. Genetic factors have been known to play an important role in the development of intracranial aneurysm (IA), some of which further progress to subarachnoid hemorrhage (SAH). In this study, we conducted a genome-wide association study (GWAS) to identify common genetic variants that are associated with the risk of IA, using 1383 aSAH subjects and 5484 control individuals in the Japanese population. We selected 36 single-nucleotide polymorphisms (SNPs) that showed suggestive association ($P < 1 \times 10^{-4}$) in the GWAS as well as additional 7 SNPs that were previously reported to be associated with IA, and further genotyped an additional set of 1048 IA cases and 7212 controls. We identified an SNP, rs6842241, near *EDNRA* at chromosome 4q31.22 (combined P-value = 9.58×10^{-9}; odds ratio = 1.25), which was found to be significantly associated with IA. Additionally, we successfully replicated and validated rs10757272 on *CDKN2BAS* at chromosome 9p21.3 (combined P-value = 1.55×10^{-7}; odds ratio = 1.21) to be significantly associated with IA as previously reported. Furthermore, we performed functional analysis with the associated genetic variants on *EDNRA*, and identified two alleles of rs6841581 that have different binding affinities to a nuclear protein(s). The transcriptional activity of the susceptible allele of this variant was significantly lower than the other, suggesting that this functional variant might affect the expression of EDNRA and subsequently result in the IA susceptibility. Identification of genetic variants on *EDNRA* is of clinical significance probably due to its role in vessel hemodynamic stress. Our findings should contribute to a better understanding of physiopathology of IA.

INTRODUCTION

Intracranial aneurysms (IAs) are balloon-like dilations of the intracranial arterial wall in the brain. Rupture of IA causes subarachnoid hemorrhage (SAH), a serious subtype of stroke, which leads to fatality in ~50% of the cases and results in significant disability in 30% of the cases (1). The age- and sex-adjusted annual incidence and mortality rates of SAH were 23 and 9 per 100 000 for all ages, respectively, in Japan (1). The annual rupture risk of IA in Japan is relatively high at ~2.7% (2). The incidence of SAH is particularly higher in Finland and Japan than in the rest of the world (1,3–5).

Both environmental and genetic factors are known to be involved in the development of IA, and several studies have indicated that hypertension, hypercholesterolemia, cigarette smoking and female gender are risk factors for IA (6–10). Various Mendelian hereditary connective tissue disorders such as autosomal dominant polycystic kidney disease (11) and

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type-IV Ehlers Danlos syndrome (12) are the inherited conditions that increase the risk of IA. Additionally, a positive family history for IA is considered to be an important risk factor, as the incidence of harboring IA for individuals who have at least two affected first-degree relatives was reported to range from 6 to 10% (6,13,14). With the high incidence of familial IA, several susceptible loci which include chromosomes 1p34.3 p36.13 (15), 7q11 (16), 19q13.3 (17) and Xp22 (18) were successfully identified to be linked to familial IA through the linkage analysis. All this evidence has strongly implied the role of genetic factors contributing to the physiopathology of IAs.

Since there is no clear symptom of IA, identification of risk factors, particularly genetic risk factors, that lead to an increase in the risk of the formation and rupture of IA, which result in aneurysmal subarachnoid hemorrhage (aSAH), is critically essential. With the current advances in biotechnology, it is now feasible to identify common genetic variants that are associated with polygenic diseases by means of genome-wide high-density SNP array. Two genome-wide association studies (GWAS) of multiple European populations have successfully identified common variants located on chromosomes 8q12.1, 9p21.3, 10q24.32, 13q13.1 and 18q11.2 that are associated with IA. Notably, these associated loci were successfully replicated in the Japanese population (19,20). Because of the complex linkage disequilibrium (LD) structures across different populations and potential interaction between genetic variants and environment factors, it is well known that the effect size of common genetic variants associated with the disease varies among different populations. Although a GWAS of IA in the Japanese population was reported previously, the association was not conclusive due to the lack of validation analysis as well as the insufficient statistical power of the study (21). Hence, we conducted an independent GWAS of IA with a larger samples size for the identification of genetic variants associated with IA in the Japanese population.

RESULTS

To identify genetic variants associated with susceptibility to IA in the Japanese population, we performed a GWAS, using 1383 aSAH patients and 5484 control (Supplementary Material, Table S1) subjects, with Illumina OmniExpress BeadChip Kits that contained 733 202 SNPs. After quality check of the SNP genotyping data, a total of 565 149 autosomal SNPs were used for association analysis (Supplementary Material, Table S2).

Principal component analysis (PCA) revealed that all the subjects participating in this study were clustered in the Asian population (Supplementary Material, Fig. S1). The detailed PCA analysis on the basis of the genotype information from the cases and controls classified the sample populations mostly into two major clusters consisting of the Ryuukyu cluster (southern islands of Japan) and the Hondo cluster (mainland cluster) (Supplementary Material, Fig. S2). To avoid influences of population substructure in the sample populations as well as age and gender biases, the association study was performed by logistic regression analysis with associated eigenvectors, age and gender as covariates.

A quantile quantile (Q Q) plot for this GWAS based on 565 149 SNPs is shown in Supplementary Material, Figure S3. The genomic inflation factor (λ_{GC}) of the test statistic in this study was 1.055. Since it is known that the λ_{GC} value increases with an increase of the sample size, we calculated the λ_{GC} value adjusted to a sample size of 1000 which was 1.031, indicating a low possibility of false-positive association by population stratification. We subsequently applied the genomic control method to adjust the P values and used the adjusted P-values (P_{GC}) for further analysis. The Manhattan plot shown in Figure 1 indicated no SNP to have achieved genome-wide significance level with the threshold at a P_{GC} value of $<5 \times 10^{-8}$.

Six SNPs (rs10958409, rs9298506, rs1333040, rs11191514, rs1980781 and rs11661542) on chromosomes 8q11.23, 9p21.3, 10q24.32, 13q13.1 and 18q11.2, which were previously reported to be associated with IA in the European population, showed nominal association with P-values from 4.50×10^{-2} to 9.52×10^{-5} (Supplementary Material, Table S3). On chromosome 9p21.3, although the previously reported SNP, rs1333040, revealed a P value of 3.79×10^{-2}, we identified another SNP, rs10757272, on this locus that showed stronger association with IA with a P value of 7.75×10^{-4}. However, we observed no association with P-values of >0.05 for the five SNPs (rs7542311, rs358345, rs4628172, rs6461176, rs10217224) that were previously indicated in the GWAS in the Japanese population (Supplementary Material, Table S3).

To further validate a possible genetic variant(s) associated with IA, we selected a total of 64 SNPs showing suggestive association ($P_{GC} < 1 \times 10^{-4}$) with IA. After excluding SNPs that possess LD coefficient (r^2) of >0.8 within each LD block, we performed a replication study of 36 SNPs, using an independent set of samples consisting of 1048 IA patients and 7212 controls. In addition, we further analyzed seven previously reported SNPs, rs10958409, rs9298506, rs1333040, rs11191514, rs1980781 rs11661542 and rs10757272, that showed nominal association (P-value of <0.05) with IA in our first stage.

Among 43 SNPs in the replication study, two SNPs (rs6842241 on 4q31.22, rs10757272 on 9p21.3) were successfully replicated with Bonferroni-corrected P-value of $<1.16 \times 10^{-3}$ (0.05/43 independent tests) at the replication phase as shown in Table 1 and Supplementary Material, Table S4. The association of these two SNPs with IA was statistically significant, considering strict multiple testing with the Bonferroni correction. After evaluating the combined association of the discovery GWAS and replication stage using weighted inverse-variance meta-analysis, we identified an SNP, rs6842241, to have achieved the genome-wide significant level of association with IA in the Japanese population, yielding a combined P-value of 9.58×10^{-9} (OR = 1.25; 95% CI = 1.16 1.34) without any significant heterogeneity (P for heterogeneity = 0.606 with $I^2 = 0.0\%$). This SNP is located within the regulatory region of the *EDNRA* gene on the chromosome locus 4q31.22. We also identified another *EDNRA* intronic SNP, rs17612742 ($r^2 = 0.99$ with rs6842241), from the GWAS to be significantly associated with IA. Imputation analysis of this locus identified two additional SNPs, rs6841581 and rs1878406, with an r^2-value of >0.8 showing similar levels of association with rs6842241 (Fig. 2A).

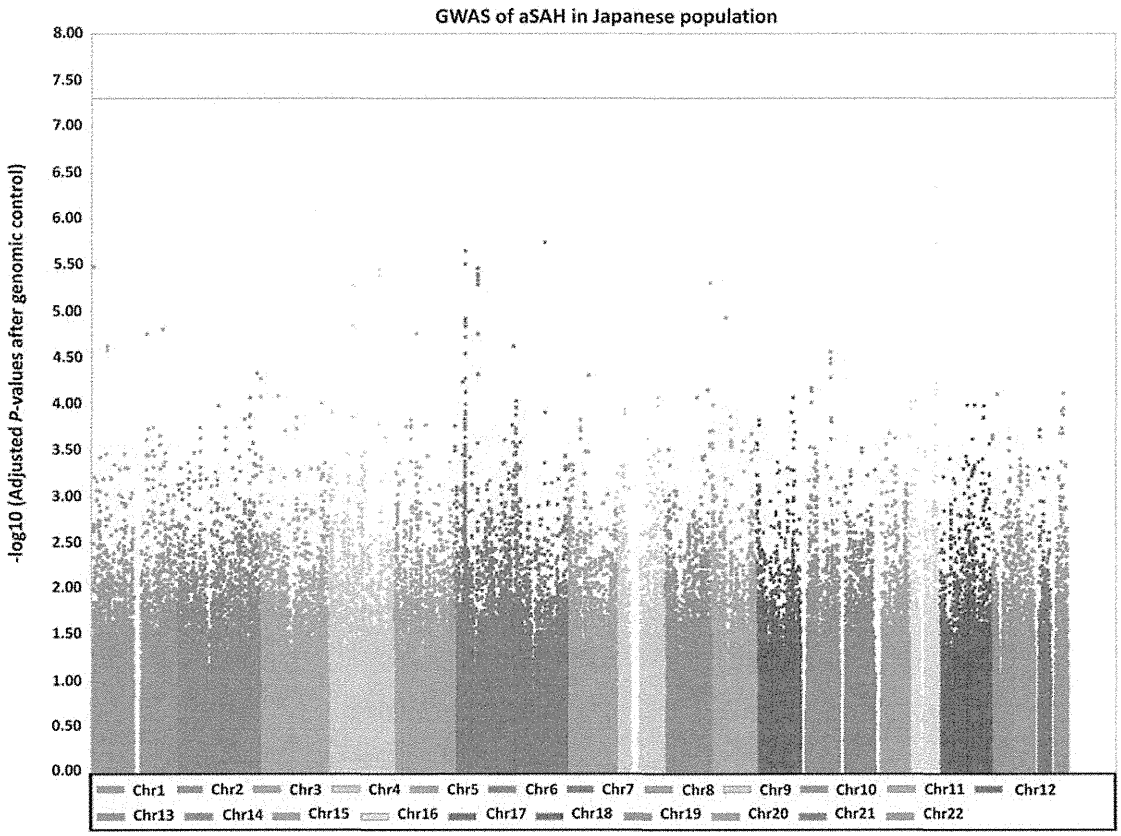

Figure 1. Manhattan plot of the GWAS of aSAH. A plot that utilized genomic-control adjustment *P*-values after eigenvectors, age and gender correction for 565 149 autosomal SNPs against their respective position on each chromosome.

Among the previously reported candidate loci (8q11.23, 9p21.3, 10q24.32, 13q13.1 and 18q11.2) associated with IA, the SNPs, rs1333040 and rs10757272 ($r^2 = 0.57$ with rs1333040), within the *CDKN2BAS* gene on a 9p21.3 region were replicated their significant association with IA in the Japanese population. In this study, rs10757272 revealed a combined *P* value of 1.55×10^{-7} (OR = 1.21; 95% CI = 1.13 1.30) (Table 1). The other SNP, rs1333040, on the same locus was also successfully replicated, but the effect of this variant was less significant with a *P* value of 5.56×10^{-5} (OR = 1.16, 95% CI = 1.09 1.25) than rs10757272 (Supplementary Material, Table S4). Imputation analysis on the 9p21.3 region indicated that most of the strongly associated SNPs were located at the 3′ end of *CDKN2BAS* (Fig. 2B).

After adjustment for known IA risk factors, which included smoking and hypertension, the association of rs6842241 and rs10757272 with IA remained strong (2.40×10^{-9}, OR = 1.26, 95% CI = 1.17 1.36; 1.32×10^{-8}, OR = 1.23, 95% CI = 1.24 1.33) (Supplementary Material, Table S5).

Among the SNPs that were previously reported, rs11191514 on 10q24.32 and rs1980781 on 13q13.1 revealed less significant association with IA in the Japanese population; the combined analysis of the two stages showed a *P*-value of 9.68×10^{-5} (OR = 1.17, 95% CI = 1.09 1.26) for rs11191514 on 10q24.32 and 9.20×10^{-5} (OR = 1.17, 95% CI = 1.09 1.26) for rs1980781 on 13q13.1.

Although the SNP rs671 located in *ALDH2* on chromosome 12q24.12 was marginally replicated with a *P*-value of 5.56×10^{-3}, the meta-analysis combining the two stages revealed stronger association with a *P*-value of 2.63×10^{-6} (OR = 1.24, 95% CI = 1.15 1.34). Owing to the functional relevance of this locus to IA, this SNP was considered to be significant in the susceptibility to IA in the Japanese population (Table 1).

Identification of novel genetic variants on the *EDNRA* gene revealed important insights into IA pathogenesis because of the biological function of this gene. Hence, we further investigated the role of genetic variants in the transcriptional level or the protein function. We identified four SNPs in an intron (rs17612742), or an upstream (rs6841581, 1878406 and rs6842241) of *EDNRA* to be significantly associated with IA through GWAS and imputation analysis. To examine the effect of these SNPs on the transcription, we performed electrophoretic mobility shift assays (EMSAs) and identified allelic differences in the binding affinity of a nuclear protein(s) from HEK293 cells, using the oligonucleotides corresponding to each allele of rs6841581 and rs1878406. The bands corresponding to the susceptible alleles G for rs6841581 and C for rs1878406 appeared to be strong, but those for protective alleles A and T appeared to be weak or undetectable (Fig. 3A). The different binding affinity between the two alleles for rs6841581 and those for rs1878406 were confirmed

Table 1. Association analysis of SNPs on chromosomes 4, 9 and 12 with IA

Chr	SNP	Chrloc	Gene	Stage	Allele		Case			Control			Risk allele frequency		*P*-value[a]	OR	95% confidence interval		P^{b}_{hetero}	I^2 (%)
					1	2	11	12	22	11	12	22	Case	Control			L95	U95		
4	rs6842241	148400819	*EDNRA*	GWAS	A	C	105	512	766	503	2356	2624	0 739	0 693	4 04E − 06	1 262	1 146	1 391		
				Replication	A	C	83	394	570	650	3110	3451	0 733	0 694	3 67E − 04	1 226	1 096	1 372		
				Combined	A	C	188	906	1336	1153	5466	6075	0 736	0 694	9 58E − 09	1 249	1 161	1 343	6 06E − 01	0 0
9	rs10757272	22088260	*CDKN2BAS*	GWAS	C	T	137	571	674	663	2474	2346	0 694	0 654	7 75E − 04	1 175	1 072	1 289		
				Replication	C	T	97	463	481	931	3253	3024	0 684	0 645	5 45E − 05	1 245	1 119	1 384		
				Combined	C	T	234	1034	1155	1594	5727	5370	0 690	0 649	1 55E − 07	1 213	1 133	1 300	8 88E − 01	0 0
Suggestive association																				
				GWAS	T	C	64	465	854	372	2083	3029	0 786	0 742	8 43E − 05	1 238	1 116	1 372		
12	rs671	112241766	*ALDH2*	Replication	T	C	67	343	638	491	2629	4091	0 772	0 750	5 56E − 03	1 180	1 050	1 326		
				Combined	T	C	131	808	1492	863	4712	7120	0 780	0 746	2 63E − 06	1 240	1 148	1 338	1 26E − 01	57 2

Chr , chromosome; Chrloc , chromosome location (bp); 11, homozygous genotype for allele 1; 12, heterozygous genotype; 22, homozygous genotype for allele 2; RAF, risk allele frequency; OR, odds ratio (calculated referred to risk allele)

[a]*P*-values of the GWAS were obtained from logistic regression analysis with eigenvectors, age and gender as covariates after genomic controls; *P*-values of the replication stage were obtained from logistic regression analysis with age and gender as covariates; *P*-values for combined were based on the weighted inverse-variance meta-analysis

[b]P_{hetero} values were obtained from heterogeneity analysis based on Cochran's *Q* statistic and I^2 statistic (46)

by the competition assay in which non-labeled oligonucleotides were added at different concentrations; the increase of the non-labeled oligonucleotides inhibited the binding of the labeled oligonucleotides in a dose-dependent manner (Fig. 3B).

To identify whether these SNPs could affect the transcriptional activity of *EDNRA*, we performed reporter assays by inserting either of the oligonucleotides corresponding to the two alleles at the two SNP loci into luciferase-expressing vectors. Plasmids containing the susceptible allele G for the SNP rs6841581, which showed higher binding affinity to the nuclear protein(s), revealed significantly lower luciferase activity than the non-susceptible allele A (Fig. 3C), although no difference between the alleles was observed for the SNP rs1878406. It is notable that the non-susceptible allele A for rs6841581 revealed no enhancer activity in comparison with the mock (empty) vector, but plasmids containing the susceptible allele G revealed the suppressive effect on the activity (Fig. 3C). Taking together, our findings from the EMSA and reporter assays suggest that the 5′ flanking region including the SNP rs6841581 on *EDNRA* might function as a transcriptional repressor and that this SNP is likely to be a functional variant conferring IA susceptibility.

DISCUSSION

To identify genetic variants associated with IA in the Japanese population, we performed a GWAS and a replication study with a total of 2431 aSAH/IA subjects and 12 696 control individuals. Among the identified SNPs, we did not observe significant differences in odds ratio (risk) between the aSAH patients in the discovery GWAS phase and the IA patients in the replication stage, indicating that the identified SNPs are likely to be associated with the risk of IA development.

The SNP rs6842241, which is located 1.25 kb upstream from the *EDNRA* gene encoding endothelin receptor A, revealed the most significant association with the combined *P*-value of 9.58×10^{-9}. Identification of this genetic variant in the regulatory region of *EDNRA* is clinically interesting since endothelin-1 (EDN-1) and its receptors, EDNRA and EDNRB, have been known to play a significant role in IA pathophysiology. EDN-1 is a potent vasoconstrictor produced by the endothelial cells in the vasculature system. The effect of EDN-1 is mediated by two major receptor subtypes, EDNRA and EDNRB, which activate a G-protein(s) and their second messenger system. EDNRA is located predominantly on vascular smooth muscle cells of the cerebrovascular system (22) and mediates vasoconstriction and proliferation (23). Accumulated evidence strongly implies that the EDN-1/EDNRA and EDNRB pathways are critically important to maintain the balance of vasoconstriction and vasodilatation in response to the hemodynamic stress. Previous reports suggested correlation of the elevated level of EDN-1 in the cerebrospinal fluid and plasma in patients with aSAH having persistent cerebral vasospasm (24,25). Interestingly, *EDNRA* variants were previously shown to be associated with a few cerebrovascular diseases including migraine (26), myocardiac infarction (27,28) and cystic fibrosis pulmonary disease (29). Furthermore, *EDNRA* variants were also associated with essential hypertension (30) and it is overexpressed in the arteries of

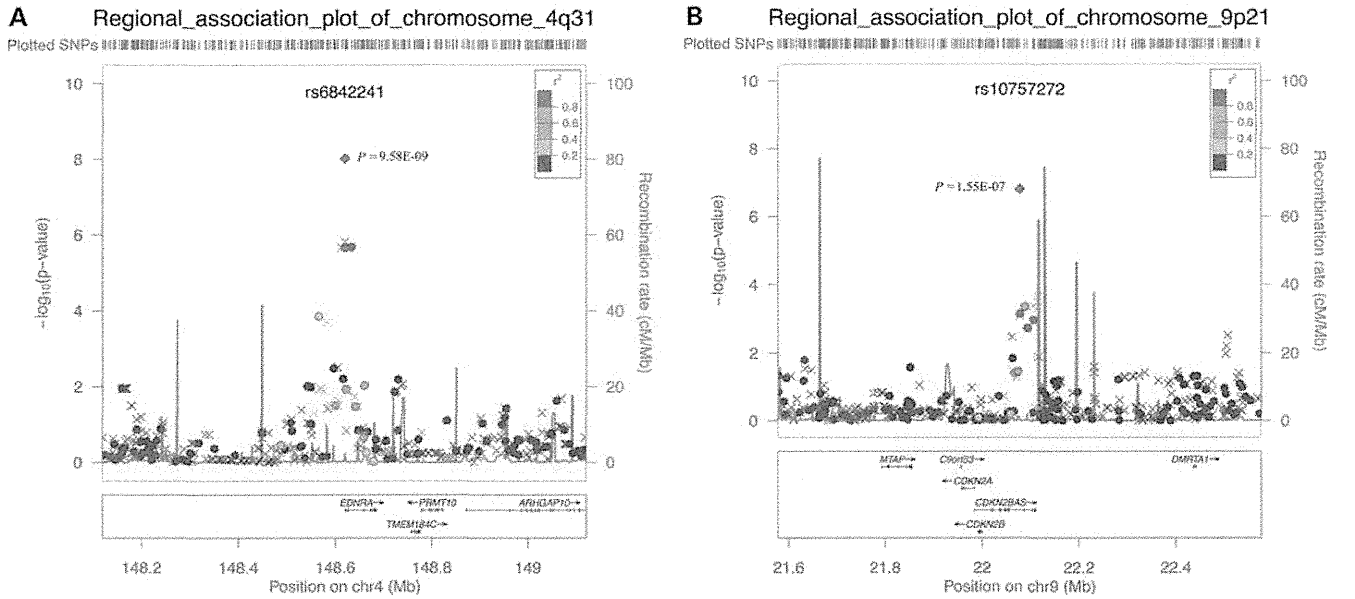

Figure 2. Regional association plots of two associated chromosome loci with IA after imputation analysis at 4q31 (**A**) and 9p21 (**B**) regions. The association of marker SNP is marked by a purple diamond before and after additional replication samples. SNPs from the GWAS are plotted as circles; imputed SNPs are plotted as crosses. The color intensity reflects the extent of LD with the marker SNP: red ($r^2 \geq 0.8$), orange ($0.6 \leq r^2 < 0.8$), green ($0.4 \leq r^2 < 0.6$), light blue ($0.2 \leq r^2 < 0.4$) and dark blue ($r^2 < 0.2$). Purplish blue lines represent local recombination rate. The SNP position is based on NCBI build 37.

hypertensive patients (31). The other three genes that reside at the same locus on chromosome 4q31.22 are *PRMT10* with an unknown function; *TMEM184C*, which is a possible tumor suppressor that may play some role in cell growth (32); and *ARHGAP10*, which is a Rho GTPase-activating protein 10 which stabilizes PAK and stimulates cell death (33).

With strong biological evidence for the role of *EDNRA* in IA pathogenesis, we carried out functional analysis of the identified SNPs from the GWAS and imputation analyses. It is well known that SNPs located in the transcriptional regulatory regions such as promoter and enhancer regions could affect the expression levels of the gene product through alteration of the binding affinity to a specific transcription factor(s) (34). The results of the EMSA and reporter assays indicated that the susceptible allele G of rs6841581 has higher affinity to the specific transcription factor(s) that might repress the transcriptional activity of *EDNRA*, compared with the non-susceptible allele A. We observed a different binding affinity to a nuclear protein(s) between the two alleles of rs1878406, and the reporter assay revealed no effect on the transcriptional activity between them. We suspect it might reflect that the 31 bp oligonucleotides may not be long enough to cover the enhancer- or repressor-binding regions. The other possibility is that a nuclear protein bound to this region has no stronger effect on the transcriptional regulation.

For SNPs on chromosome 9p21.3, we successfully validated the association of this locus with IA as reported previously. We identified a stronger association of an SNP, rs10757272 (combined $P = 1.55 \times 10^{-7}$), with IA than the SNP, rs1333040 (combined $P = 5.56 \times 10^{-5}$), that was previously identified in the study of the European population. Interestingly, the SNP rs10757272 was also shown to be associated with coronary artery disease (CAD) and platelet reactivity (a potential mechanism for increased vascular disease) in the European population (35,36). This finding suggests that rs10757272 or SNPs that are in high LD with it might be a common genetic risk factor(s) for multiple cardiovascular disorders. SNPs on a chromosome 9p21.3 region, which consist of $p15^{INK4b}$, $p16^{INK4a}$ and *CDKN2BAS*, have been indicated to be associated with several atherosclerotic vascular diseases such as CADs (37–39), stroke (40), myocardial infarction (38), abdominal aortic aneurysm (41) and IA (20). A recent study revealed that targeted deletion of the 9p21 CAD risk interval in a mouse model resulted in severely attenuated expression of two *CDKN2BAS*s' neighboring tumor-suppressor genes, $p15^{INK4b}$ and $p16^{INK4a}$, which subsequently affects the CAD progression by altering the dynamics of vascular cell proliferation (42). The findings of multiple vascular diseases associated with this locus have provided a new direction for the pathogenesis of these diseases.

Finally, although the association of rs671 (combined $P = 2.63 \times 10^{-6}$) on the *ALDH2* gene was marginally replicated in this study, the association of this locus remained to be of interest in the Japanese population. *ALDH2* belongs to the aldehyde dehydrogenase 2 family (mitochondrial), which is the second enzyme of the major oxidative pathway of alcohol metabolism. rs671 in this gene is the functional variant (Glu504Lys), and an A allele results in the inactivation of ALDH2, inducing 'alcohol flush'. The allelic frequency of this variant is uniquely high in the Asian population. In this study, the variant rs671A allele seemed to act as a protective allele, suggesting that individuals with the A allele might drink less amount of alcohol, which would result in the reduction of IA risk. Since alcohol drinking has been consistently indicated as one of the risk factors for IA, further validation of the association on this locus would be of medical importance.

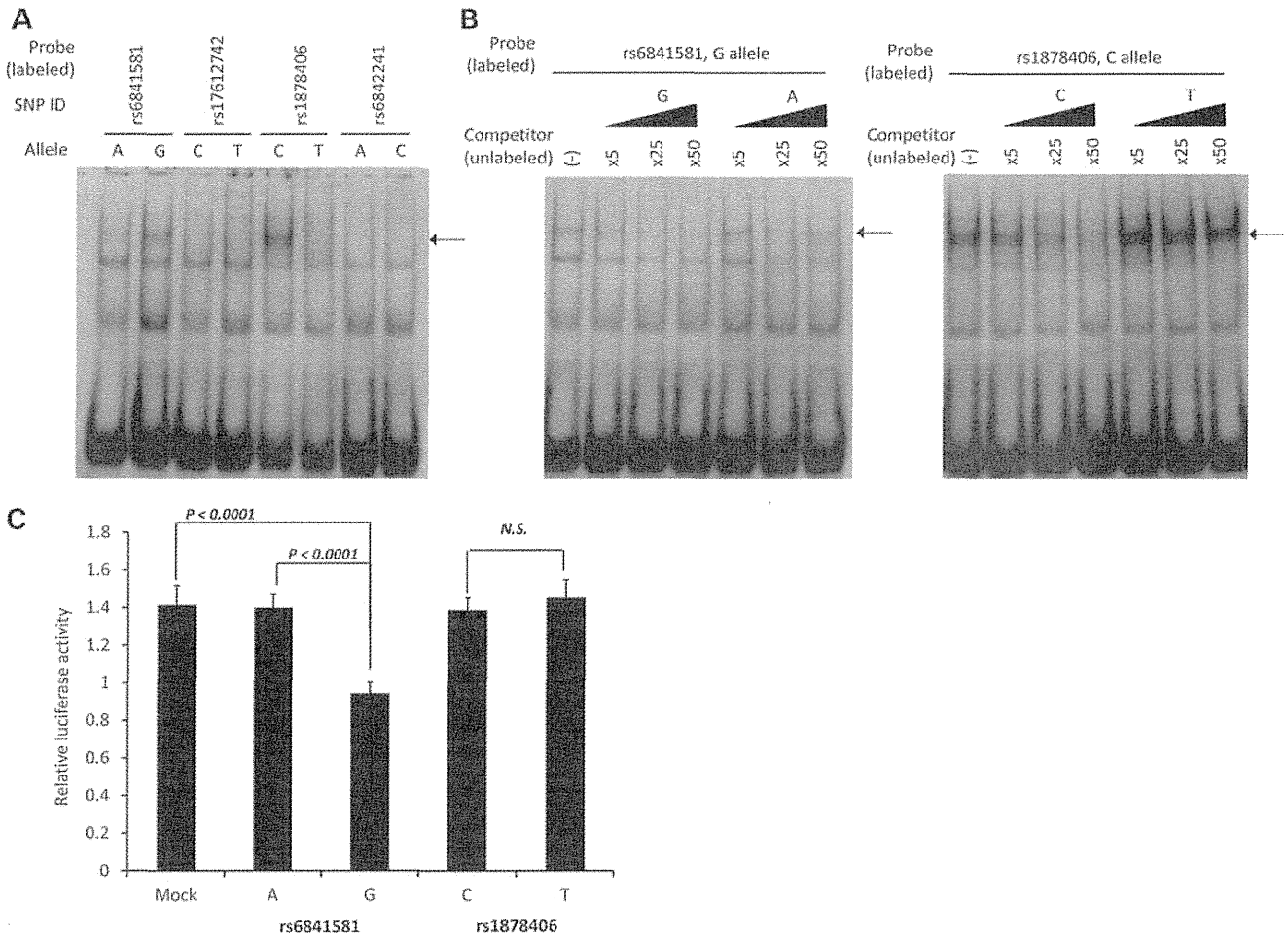

Figure 3. Functional analysis of SNPs at the *EDNRA* locus. (**A**) EMSA screening for variants associated with the binding of a nuclear protein(s) from nuclear extracts of HEK293 cells. The susceptible alleles G and C for rs6841581 and rs1878406, respectively, formed DNA protein complexes, as pointed with arrows. (**B**) The specific interaction of labeled oligonucleotides corresponding to alleles G and C was completed in a dose-dependent manner using an unlabeled oligonucleotide with the G and C alleles, but not oligonucleotides corresponding to the A and T alleles of rs6841581 and rs1878406, respectively. (**C**) Differences in the transcriptional activity between the susceptible and non-susceptible alleles of rs6841581 and rs1878406 measured by dual-luciferase reporter assay. The values of the relative luciferase activity are shown with standard deviation after normalization with internal control *renilla* luciferase activity. The relative luciferase activity of the susceptible genotype G of rs6841581 was significantly lower than that of the non-susceptible allele A and mock (empty) vector ($P < 0.0001$, Student's *t*-test).

In conclusion, by a large-scale GWAS, we successfully identified two genomic loci, *EDNRA* (4q31.22) and *CDKN2BAS* (9p21.3), to be significantly associated with IA in the Japanese population. Owing to the complexity of detecting IA in the general population, the associations of genetic variants have not just served as a promising prediction tool to identify individuals who have a higher risk of IA, but have also provided a better understanding of the disease pathogenesis which subsequently leads to the development of clinical intervention for IA.

MATERIALS AND METHODS

Study population

All DNA samples for this study were recruited from the Biobank Japan Project that began in 2003 (http://biobankjp.org). The Biobank Japan Project has a collaborative network of 66 hospitals throughout Japan to collect DNAs and serum samples from nearly 300 000 cases with any of the 47 diseases we assigned. One of the major aims of this project was to identify common genetic variants that confer risk to common diseases, including metabolic diseases and cancers. As a discovery step of this study, we selected 1383 patients registered as aneurysmal SAH (aSAH) cases, which is caused by the rupture of IA. We selected aSAH patients for the discovery screening because aSAH is a severe form of IA in which the disease-associated variants are likely to be enriched. We verified the association results of the first set by utilizing an independent set of samples consisting of 1048 IA patients. Identification of IA in the case samples was done using computerized tomography angiogram, magnetic resonance angiogram or cerebral digital subtraction angiogram. We included 5484 and 7212 controls consisting of healthy volunteers from Midosuji Rotary Club, Osaka, Japan, Health Science Research Resource Bank and individuals in the Biobank

Japan who were registered not to have SAH/IA in the discovery and replication stages, respectively. The detailed demographic and clinical parameters of cases and controls are summarized in Supplementary Material, Table S1. Participants of this study provided written inform consent and this project was approved by the ethical committee from the Institute of Medical Sciences, the University of Tokyo and RIKEN Center of Genomic Medicine.

Genotyping and quality control

For the GWAS discovery stage, we genotyped both case and control samples using Illumina OmniExpress BeadChip that contained a total of 733 202 SNPs. We performed standard SNP quality control by excluding SNPs with a call rate of <0.99, those deviated from the Hardy–Weinberg equilibrium ($P \leq 1.0 \times 10^{-6}$), non-polymorphic ones and those on the X chromosome. The cluster plots of top 100 SNPs that revealed the strongest association with aSAH were checked by visual observation to exclude SNPs with ambiguous patterns. The number of excluded SNPs in each quality control process is summarized in Supplementary Material, Table S2. We utilized the identity-by-state method to evaluate cryptic relatedness for each sample; samples that possess an average estimate value of ≥ 1.7 were eliminated from subsequent analyses. Additionally, we examined population stratification by principal component analysis (PCA) using the EIGENSTRAT software v2.0 (http://genepath.med.harvard.edu/~reich/Software.htm). We first performed PCA, utilizing four populations in the HapMap database, which included Europeans (represented by Caucasian from UTAH, CEU), Africans (represented by Yoruba from Ibadan, YRI) and East Asians (represented by Japanese from Tokyo, JPT, and Han Chinese from Beijing, CHB) as reference populations for PCA. The top two principal components were utilized to produce a scatter plot for the identification of outliers who did not belong to the Asian cluster. To further investigate the population substructure in the sample population, we performed PCA using the genotype information of the case and control subjects in this study. The Q–Q plot that was generated between observed *P*-values against expected *P*-values and inflation factor (λ) values were used to evaluate the potential population substructure. Manhattan plot of the study was plotted using Haploview 4.1 (43).

For a replication study, a total of 36 SNPs that showed suggestive association with IA in the Japanese population ($P_{GC} < 1.0 \times 10^{-4}$) as well as 7 SNPs that were previously reported to be associated with IA (19,20) were selected for further evaluation with an independent set of 1048 IA cases and 7212 controls. We genotyped the cases with the multiplex-PCR Invader assay (44) and the control samples with Illumina OmniExpress BeadChip Kits. SNPs with a call rate of <99% and those that were deviated from the Hardy–Weinberg equilibrium ($P \leq 0.05$) were excluded for further analysis.

Statistical analysis

The case–control association was evaluated using logistic regression analysis with associated eigenvectors, age and gender as covariates in the discovery (GWAS) and replication phases of this study. The *P*-values obtained from the discovery phase were subsequently corrected using the genomic control method (45), and the corrected *P*-values were used for further analysis. Meta-analysis for the combined analysis of discovery and replication phases was performed using the weighted inverse-variance method implemented in the METAL software (http://www.sph.umich.edu/csg/abecasis/Metal/index.html). *P*-values for the heterogeneity test are evaluated with Cochran's Q statistic and I^2 statistic (46).

After the identification of candidate loci to be possibly associated with IA, imputation of the missing genotypes was performed with MACH 1.0 (http://www.sph.umich.edu/csg/abecasis/MACH/index.html). For imputation analysis, we included SNPs that were located <500 kb upstream or downstream of the marker SNP except SNPs that had a low genotyping rate (<99%), showed deviations from Hardy–Weinberg equilibrium ($<1.0 \times 10^{-6}$) or had an MAF of <0.01. Genotype information from the Phase III HapMap database was used as reference. Using the MACH version 1.0 program, we estimated haplotypes, map crossover and error rates using 50 iterations of the Markov chain Monte Carlo algorithm. For imputation quality control, we excluded SNPs with r^2 values of <0.3. Regional association plots were generated using Locus Zoom (https://statgen.sph.umich.edu/locuszoom/genform.php?type=yourdata).

Cell line

A human embryonic kidney cell line, HEK293, was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA; ATCC) supplemented with 10% bovine serum (GIBCO) and 1% antibiotic/antimycotic solution (Sigma-Aldrich, St Louis, MO, USA). The cells were maintained at 37°C in atmospheres of humidified air with 5% CO_2.

Electrophoretic mobility shift assay

A nuclear fraction of HEK293 cells was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Thirty-one base pairs of sense and antisense oligonucleotides corresponding to the genomic sequence which contained the SNPs of interest (rs6841581, rs17612742, rs1878406 and rs6842241) were synthesized (Supplementary Material, Table S6) and labeled with the DIG Gel Shift kit, second generation (Roche), according to the manufacturer's protocol. The reaction was started with pre-incubation of labeled oligonucleotide with poly[d(I-C)] and poly-L-lysine, and 10 μg of nuclear extract from HEK293, for 20 min at 25°C. For a competition assay, 5-fold, 25-fold or 50-fold excess of unlabeled oligonucleotide was added to nuclear extracts before adding the either of DIG-labeled probes. The protein–DNA complex was separated by electrophoresis on a 6% non-denaturing polyacrylamide gel with 0.5× Tris–borate EDTA buffer and transferred onto a nylon membrane. The protein complexes were visualized by autoradiography. All EMSAs were repeated twice to check for reproducibility.

Dual-luciferase reporter assays

To construct luciferase reporter plasmids containing the SNPs of interest (rs6841581 and rs1878406), *Sma*I and *Bgl*II restriction enzyme sites were added to the 31 bp oligonucleotides that were used as a probe in the EMSA assay and either of the annealed double-stranded oligonucleotides was inserted into the upstream of the luciferase reporter gene in the pGL3 promoter (Promega). The sequences of the constructs were verified using the ABI3730 Genetic Analyzer (Applied Biosystems). After 24 h incubation of HEK293 cells (2×10^4) on a 12-well plate, the cells were co-transfected with 400 ng of each reporter construct and 8 ng of the internal control pRL-TK (*renilla* luciferase), using the FuGene 6 transfection reagent (Roche). After 48 h incubation, the cells were lysed in passive lysis buffer and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The results were normalized by *renilla* luciferase activity.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. The authors declare no competing financial interests.

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REFERENCES

1. Inagawa, T. (2005) Trends in surgical and management outcomes in patients with aneurysmal subarachnoid hemorrhage in Izumo city, Japan, between 1980–1989 and 1990–1998. *Cerebrovasc. Dis.*, **19**, 39–48.
2. Morita, A., Fujiwara, S., Hashi, K., Ohtsu, H. and Kirino, T. (2005) Risk of rupture associated with intact cerebral aneurysms in the Japanese population: a systematic review of the literature from Japan. *J. Neurosurg.*, **102**, 601–606.
3. Sarti, C., Tuomilehto, J., Salomaa, V., Sivenius, J., Kaarsalo, E., Narva, E.V., Salmi, K. and Torppa, J. (1991) Epidemiology of subarachnoid hemorrhage in Finland from 1983 to 1985. *Stroke*, **22**, 848–853.
4. Ingall, T., Asplund, K., Mähönen, M. and Bonita, R. (2000) A multinational comparison of subarachnoid hemorrhage epidemiology in the WHO MONICA stroke study. *Stroke*, **31**, 1054–1061.
5. Ohkuma, H., Fujita, S. and Suzuki, S. (2002) Incidence of aneurysmal subarachnoid hemorrhage in Shimokita, Japan, from 1989 to 1998. *Stroke*, **33**, 195–199.
6. Schievink, W.I., Schaid, D.J., Michels, V.V. and Piepgras, D.G. (1995) Familial aneurysmal subarachnoid hemorrhage: a community-based study. *J. Neurosurg.*, **83**, 426–429.
7. Teunissen, L.L., Rinkel, G.J., Algra, A. and van Gijn, J. (1996) Risk factors for subarachnoid hemorrhage: a systematic review. *Stroke*, **27**, 544–549.
8. Connolly, E.S. Jr., Choudhri, T.F., Mack, W.J., Mocco, J., Spinks, T.J., Slosberg, J., Lin, T., Huang, J. and Solomon, R.A. (2001) Influence of smoking, hypertension, and sex on the phenotypic expression of familial intracranial aneurysms in siblings. *Neurosurgery*, **48**, 64–68; Discussion, 68–69.
9. Ronkainen, A., Miettinen, H., Karkola, K., Papinaho, S., Vanninen, R., Puranen, M. and Hernesniemi, J. (1998) Risk of harboring an unruptured intracranial aneurysm. *Stroke*, **29**, 359–362.
10. Inagawa, T. (2010) Risk factors for the formation and rupture of intracranial saccular aneurysms in Shimane, Japan. *World Neurosurg.*, **73**, 155–164; Discussion, e123.
11. Chapman, A.B., Rubinstein, D., Hughes, R., Stears, J.C., Earnest, M.P., Johnson, A.M., Gabow, P.A. and Kaehny, W.D. (1992) Intracranial aneurysms in autosomal dominant polycystic kidney disease. *N. Engl. J. Med.*, **327**, 916–920.
12. Schievink, W.I. (1997) Genetics of intracranial aneurysms. *Neurosurgery*, **40**, 651–662; Discussion, 662–653.
13. Ronkainen, A., Hernesniemi, J. and Ryynänen, M. (1993) Familial subarachnoid hemorrhage in east Finland, 1977–1990. *Neurosurgery*, **33**, 787–796; Discussion, 796–797.
14. Wang, P.S., Longstreth, W.T. and Koepsell, T.D. (1995) Subarachnoid hemorrhage and family history. A population-based case–control study. *Arch. Neurol.*, **52**, 202–204.
15. Nahed, B.V., Seker, A., Guclu, B., Ozturk, A.K., Finberg, K., Hawkins, A.A., DiLuna, M.L., State, M., Lifton, R.P. and Gunel, M. (2005) Mapping a Mendelian form of intracranial aneurysm to 1p34.3-p36.13. *Am. J. Hum. Genet.*, **76**, 172–179.
16. Onda, H., Kasuya, H., Yoneyama, T., Takakura, K., Hori, T., Takeda, J., Nakajima, T. and Inoue, I. (2001) Genomewide-linkage and haplotype-association studies map intracranial aneurysm to chromosome 7q11. *Am. J. Hum. Genet.*, **69**, 804–819.
17. Olson, J.M., Vongpunsawad, S., Kuivaniemi, H., Ronkainen, A., Hernesniemi, J., Ryynänen, M., Kim, L.L. and Tromp, G. (2002) Search for intracranial aneurysm susceptibility gene(s) using Finnish families. *BMC Med. Genet.*, **3**, 7.
18. Yamada, S., Utsunomiya, M., Inoue, K., Nozaki, K., Inoue, S., Takenaka, K., Hashimoto, N. and Koizumi, A. (2004) Genome-wide scan for Japanese familial intracranial aneurysms: linkage to several chromosomal regions. *Circulation*, **110**, 3727–3733.
19. Bilguvar, K., Yasuno, K., Niemela, M., Ruigrok, Y.M., von Und Zu Fraunberg, M., van Duijn, C.M., van den Berg, L.H., Mane, S., Mason, C.E., Choi, M. *et al.* (2008) Susceptibility loci for intracranial aneurysm in European and Japanese populations. *Nat. Genet.*, **40**, 1472–1477.
20. Yasuno, K., Bilguvar, K., Bijlenga, P., Low, S.K., Krischek, B., Auburger, G., Simon, M., Krex, D., Arlier, Z., Nayak, N. *et al.* (2010) Genome-wide association study of intracranial aneurysm identifies three new risk loci. *Nat. Genet.*, **42**, 420–425.
21. Akiyama, K., Narita, A., Nakaoka, H., Cui, T., Takahashi, T., Yasuno, K., Tajima, A., Krischek, B., Yamamoto, K., Kasuya, H. *et al.* (2010) Genome-wide association study to identify genetic variants present in Japanese patients harboring intracranial aneurysms. *J. Hum. Genet.*, **55**, 656–661.
22. Yu, J.C., Pickard, J.D. and Davenport, A.P. (1995) Endothelin ETA receptor expression in human cerebrovascular smooth muscle cells. *Br. J. Pharmacol.*, **116**, 2441–2446.
23. Janakidevi, K., Fisher, M.A., Del Vecchio, P.J., Tiruppathi, C., Figge, J. and Malik, A.B. (1992) Endothelin-1 stimulates DNA synthesis and proliferation of pulmonary artery smooth muscle cells. *Am. J. Physiol.*, **263**, C1295–C1301.
24. Fassbender, K., Hodapp, B., Rossol, S., Bertsch, T., Schmeck, J., Schütt, S., Fritzinger, M., Horn, P., Vajkoczy, P., Wendel-Wellner, M. *et al.*

(2000) Endothelin-1 in subarachnoid hemorrhage: an acute-phase reactant produced by cerebrospinal fluid leukocytes. *Stroke*, **31**, 2971–2975.
25. Juvela, S. (2000) Plasma endothelin concentrations after aneurysmal subarachnoid hemorrhage. *J. Neurosurg.*, **92**, 390–400.
26. Tzourio, C., El Amrani, M., Poirier, O., Nicaud, V., Bousser, M.G. and Alpérovitch, A. (2001) Association between migraine and endothelin type A receptor (ETA -231 A/G) gene polymorphism. *Neurology*, **56**, 1273–1277.
27. Nicaud, V., Poirier, O., Behague, I., Herrmann, S.M., Mallet, C., Troesch, A., Bouyer, J., Evans, A., Luc, G., Ruidavets, J.B. *et al.* (1999) Polymorphisms of the endothelin-A and -B receptor genes in relation to blood pressure and myocardial infarction: the Etude Cas-Témoins sur l'Infarctus du Myocarde (ECTIM) Study. *Am. J. Hypertens.*, **12**, 304–310.
28. Oguri, M., Kato, K., Yokoi, K., Itoh, T., Yoshida, T., Watanabe, S., Metoki, N., Yoshida, H., Satoh, K., Aoyagi, Y. *et al.* (2009) Association of genetic variants with myocardial infarction in Japanese individuals with metabolic syndrome. *Atherosclerosis*, **206**, 486–493.
29. Darrah, R., McKone, E., O'Connor, C., Rodgers, C., Genatossio, A., McNamara, S., Gibson, R., Stuart Elborn, J., Ennis, M., Gallagher, C.G. *et al.* (2010) EDNRA variants associate with smooth muscle mRNA levels, cell proliferation rates, and cystic fibrosis pulmonary disease severity. *Physiol. Genomics*, **41**, 71–77.
30. Benjafield, A.V., Katyk, K. and Morris, B.J. (2003) Association of EDNRA, but not WNK4 or FKBP1B, polymorphisms with essential hypertension. *Clin. Genet.*, **64**, 433–438.
31. Hasegawa, K., Fujiwara, H., Doyama, K., Inada, T., Ohtani, S., Fujiwara, T., Hosoda, K., Nakao, K. and Sasayama, S. (1994) Endothelin-1-selective receptor in the arterial intima of patients with hypertension. *Hypertension*, **23**, 288–293.
32. Akaishi, J., Onda, M., Okamoto, J., Miyamoto, S., Nagahama, M., Ito, K., Yoshida, A. and Shimizu, K. (2007) Down-regulation of an inhibitor of cell growth, transmembrane protein 34 (TMEM34), in anaplastic thyroid cancer. *J. Cancer Res. Clin. Oncol.*, **133**, 213–218.
33. Koeppel, M.A., McCarthy, C.C., Moertl, E. and Jakobi, R. (2004) Identification and characterization of PS-GAP as a novel regulator of caspase-activated PAK-2. *J. Biol. Chem.*, **279**, 53653–53664.
34. Chorley, B.N., Wang, X., Campbell, M.R., Pittman, G.S., Noureddine, M.A. and Bell, D.A. (2008) Discovery and verification of functional single nucleotide polymorphisms in regulatory genomic regions: current and developing technologies. *Mutat. Res.*, **659**, 147–157.
35. Erdmann, J., Grosshennig, A., Braund, P.S., König, I.R., Hengstenberg, C., Hall, A.S., Linsel-Nitschke, P., Kathiresan, S., Wright, B., Trégouët, D.A. *et al.* (2009) New susceptibility locus for coronary artery disease on chromosome 3q22.3. *Nat. Genet.*, **41**, 280–282.
36. Musunuru, K., Post, W.S., Herzog, W., Shen, H., O'Connell, J.R., McArdle, P.F., Ryan, K.A., Gibson, Q., Cheng, Y.C., Clearfield, E. *et al.* (2010) Association of single nucleotide polymorphisms on chromosome 9p21.3 with platelet reactivity: a potential mechanism for increased vascular disease. *Circ. Cardiovasc. Genet.*, **3**, 445–453.
37. W.T.C.C. Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, **447**, 661–678.
38. Helgadottir, A., Thorleifsson, G., Manolescu, A., Gretarsdottir, S., Blondal, T., Jonasdottir, A., Sigurdsson, A., Baker, A., Palsson, A., Masson, G. *et al.* (2007) A common variant on chromosome 9p21 affects the risk of myocardial infarction. *Science*, **316**, 1491–1493.
39. McPherson, R. (2010) Chromosome 9p21 and coronary artery disease. *N. Engl. J. Med.*, **362**, 1736–1737.
40. Anderson, C.D., Biffi, A., Rost, N.S., Cortellini, L., Furie, K.L. and Rosand, J. (2010) Chromosome 9p21 in ischemic stroke: population structure and meta-analysis. *Stroke*, **41**, 1123–1131.
41. Helgadottir, A., Thorleifsson, G., Magnusson, K.P., Gretarsdottir, S., Steinthorsdottir, V., Manolescu, A., Jones, G.T., Rinkel, G.J., Blankensteijn, J.D., Ronkainen, A. *et al.* (2008) The same sequence variant on 9p21 associates with myocardial infarction, abdominal aortic aneurysm and intracranial aneurysm. *Nat. Genet.*, **40**, 217–224.
42. Visel, A., Zhu, Y., May, D., Afzal, V., Gong, E., Attanasio, C., Blow, M.J., Cohen, J.C., Rubin, E.M. and Pennacchio, L.A. (2010) Targeted deletion of the 9p21 non-coding coronary artery disease risk interval in mice. *Nature*, **464**, 409–412.
43. Barrett, J.C., Fry, B., Maller, J. and Daly, M.J. (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*, **21**, 263–265.
44. Ohnishi, Y., Tanaka, T., Ozaki, K., Yamada, R., Suzuki, H. and Nakamura, Y. (2001) A high-throughput SNP typing system for genome-wide association studies. *J. Hum. Genet.*, **46**, 471–477.
45. Devlin, B. and Roeder, K. (1999) Genomic control for association studies. *Biometrics*, **55**, 997–1004.
46. Higgins, J.P., Thompson, S.G., Deeks, J.J. and Altman, D.G. (2003) Measuring inconsistency in meta-analyses. *BMJ*, **327**, 557–560.

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ORIGINAL ARTICLE

Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-Akt pathway in gastric cancer

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Abstract

AIM: To investigate human epidermal growth factor receptor 2 (HER2)-phosphatidylinositol 3-kinase (PI3K)-v-Akt murine thymoma viral oncogene homolog signaling pathway.

METHODS: We analyzed 231 formalin-fixed, paraffin-embedded gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of the medical records. Expression of HER2 was analyzed by immunohistochemistry (IHC) using the HercepTest™ kit. Standard criteria for HER2 positivity (0, 1+, 2+, and 3+) were used. Tumors that scored 3+ were considered HER2-positive. Expression of phospho Akt (pAkt) was also analyzed by IHC. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more. PI3K, catalytic, alpha polypeptide (PIK3CA) mutations in exons 1, 9 and 20 were analyzed by pyrosequencing. Epstein-Barr virus (EBV) infection was analyzed by *in situ* hybridization targeting EBV-encoded small RNA (EBER) with an EBER-RNA probe. Microsatellite instability (MSI) was analyzed by polymerase chain reaction using the mononucleotide markers BAT25 and BAT26.

RESULTS: HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively. HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type (15/20 *vs* 98 /205, $P = 0.05$). PIK3CA mutations were present in 20 cases (8.7%) and significantly correlated with MSI (10/20 *vs* 9/211, $P < 0.01$).

The mutation frequency was high (21%) in T4 cancers and very low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively. Two new types of PIK3CA mutation, R88Q and R108H, were found in exon1. All PIK3CA mutations were heterozygous missense single-base substitutions, the most common being H1047R (6/20, 30%) in exon20. Eighteen cancers (8%) were EBV-positive and this positivity significantly correlated with a diffuse histological type (13/18 *vs* 93/198, P = 0.04). There were 7 cases of lymphoepithelioma-like carcinomas (LELC) and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). pAkt expression was positive in 119 (53%) cases but showed no correlation with clinicopathological characteristics. pAkt expression was significantly correlated with HER2 overexpression (16/20 *vs* 103/211, $P < 0.01$) but not with PIK3CA mutations (12/20 *vs* 107/211, P = 0.37) or EBV infection (8/18 *vs* 103/211, P = 0.69). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations. One case showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However, no cases showed both PIK3CA mutations and HER2 overexpression. One EBV-positive cancer with PIK3CA mutation (H1047R) was MSI-positive. Three of these 4 cases were positive for pAkt expression. In survival analysis, pAkt expression significantly correlated with a poor prognosis (hazard ratio 1.75; 95%CI: 1.12-2.80, P = 0.02).

CONCLUSION: HER2 expression, PIK3CA mutations and EBV infection in gastric cancer were characterized. pAkt expression significantly correlates with HER2 expression and with a poor prognosis.

Key words: Human epidermal growth factor receptor 2; Phosphatidylinositol 3-kinase; Catalytic; Alpha polypeptide; Epstein-Barr virus; Akt; Gastric cancer

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INTRODUCTION

Gastric cancer is one of the most common cancer types and the second leading cause of cancer-related deaths worldwide[1]. Genetic and epigenetic alterations play important roles in the development and progression of these tumors[1,2]. Considerable attention has been given to the potential role of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway in gastric cancer[3,4]. Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and inactivation of phosphatase and tensin homolog (PTEN) lead to activation of the PI3K-Akt signaling pathway. With regards to growth factor receptors, there is growing evidence that human epidermal growth factor receptor 2 (HER2) is a key driver of tumorigenesis and an important biomarker in gastric cancer. The amplification or overexpression of HER2 is observed in 7%-34% of these cases[5-9].

PIK3CA is mutated in a wide variety of human tumor types[10,11], including gastric cancers[12-15]. Activating mutations in this gene up-regulate the PI3K-Akt signaling pathway, making it a potentially useful therapeutic target. For example, oncogenic mutations of PIK3CA reportedly render breast cancers more resistant to treatment with the anti-HER2 receptor antibody trastuzumab[16]. Thus, this signaling pathway is thought to be one of the mechanisms underlying resistance to trastuzumab. Trastuzumab has recently been approved for treatment of advanced gastric cancers[5,6].

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden[17]. PIK3CA mutations have been detected in 4%-25% of gastric cancers[12-15]. However, in most previous studies, only exons 9 and 20 hot spot mutations in PIK3CA were analyzed by DNA sequencing. Moreover, the association between HER2 expression and PIK3CA mutations in gastric cancer has not been reported.

A significant correlation has been found between Epstein-Barr virus (EBV) and the methylation of multiple genes in gastric cancers[18-20]. EBV infection reportedly induces PTEN expression loss through CpG island methylation of its promoter, leading to activation of the PI3K-Akt signaling pathway, in EBV-associated gastric cancer[21].

The aim of our present study was to systematically characterize HER2 expression, PIK3CA mutations, and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of gastric cancers (n = 231). We wished to determine the prevalence of each of these factors with a high precision and thereby correlate them with clinicopathological and molecular features of gastric lesions, including microsatellite instability (MSI) and phospho Akt (pAkt) expression.

MATERIALS AND METHODS

Tissue samples

A total of 231 formalin-fixed, paraffin-embedded (FFPE) gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment was analyzed in

Table 1 Clinicopathological characteristics of patients with gastric cancer

Variables (n = 231)		n (%)
Sex	Male	157 (68)
	Female	74 (32)
Age (yr)	Median (range)	71 (25-91)
Location	Cardias	82 (35)
	Body	62 (27)
	Antrum	83 (36)
	Unknown	4 (2)
Depth of invasion	T2	125 (54)
	T3	92 (40)
	T4	14 (6)
Lymph node metastasis	N0	65 (28)
	N+	158 (68)
	N1	73 (32)
	N2	56 (24)
	N3	29 (13)
	Unknown	8 (3)
Stage	I B	49 (21)
	II	45 (19)
	IIIA + IIIB	82 (35)
	IV	51 (22)
	Unknown	4 (2)
Lauren histotype	Intestinal	113 (49)
	Diffuse	112 (48)
	Others	6 (3)

this study. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of their medical records. Clinicopathological findings were determined according to the criteria of the Japanese Research Society for Gastric Cancer (Table 1). Our institutional review committee approved the study.

Immunohistochemistry

HER2 expression was analyzed using the HercepTest™ kit (DAKO, Carpinteria, CA) by manual sample processing in accordance with the manufacturer's instructions. Standard criteria for HER2 positivity (0, 1+, 2+ and 3+) were used. Tumors that scored 3+ were considered HER2-positive. For the immunohistochemical analysis of pAkt, FFPE specimens were processed using SignalStain Boost Detection Reagent (Cell Signaling Technology, Beverly, MA). Briefly, 5-μm-thick sections were dewaxed in xylene, rehydrated in ethanol, and heated with target retrieval solution (DAKO) in an autoclave for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in methanol for 10 min. The tissue sections were then washed twice with tris-buffered saline (TBS) and preblocked with 10% goat serum in TBS for 60 min. After washing with TBS, the sections were incubated with an anti-phospho-Akt (Ser473) polyclonal antibody (D9E, Cell Signaling Technology) at a dilution of 1:100 for 30 h at 4 ℃. The sections were washed three times in TBS and incubated with SignalStain Boost Detection Reagent for 45 min. After three further washes in TBS, a diamino-benzidine tetrahydrochloride working solution was applied. Finally, the sections were counterstained with hematoxylin. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more[22]. Only clear staining of the tumor cell nucleus and/or cytoplasm was considered positive.

Mutation analysis of the PIK3CA gene by pyrosequencing

Genomic DNA was extracted from tumor specimens and mutations in exon9 and exon20 of the *PIK3CA* gene were analyzed by pyrosequencing as described previously[23,24]. We also developed a pyrosequencing assay to detect PIK-3CA exon1 mutations using the primer sets exon1-RS1 (5'-GGGAAGAATTTTTTGATGAAACA-3' for the biotinylated forward primer and 5'-GGTTGCCTACT-GGTTCAATTACTT-3' for the reverse primer) and exon1-RS2 (5'-CGGCTTTTTCAACCCTTTTT-3' for the forward primer and 5'-ATTTCTCGATTGAG-GATCTTTTCT-3' for the biotinylated reverse primer). Each polymerase chain reaction (PCR) mix contained the forward and reverse primers (each 10 μmol/L), a 25 mmol/L dNTP mix with dUTP, 75 mmol/L $MgCl_2$, 1 × PCR buffer, 1.0 U of exTaq, and 2 μL of template DNA in a total volume of 25 μL. PCR conditions were as follows: initial denaturing at 95 ℃ for 5 min; 50 cycles of 94 ℃ for 20 s, 50 ℃ for 20 s and 74 ℃ for 40 s; and a final extension at 72 ℃ for 1 min. The PCR products (each 25 μL) were sequenced using the PyroMark kit and Pyrosequencing PSQ96 HS System (Qiagen, Valencia, CA).

In situ hybridization for EBER1

The presence of EBV in the carcinoma tissues was evaluated by *in situ* hybridization (ISH) targeting of EBV-encoded small RNA (EBER-ISH) with an EBER-RNA probe (Dako Cytomation).

Microsatellite instability analysis

MSI was analyzed by PCR using the mononucleotide markers (BAT25 and BAT26). Based on the number of markers showing instability per tumor sample, cancers were divided into two groups; those with one or more of the two markers displaying MSI and those with no instability (microsatellite stable).

Statistical analysis

For all statistical analysis, the JMP program was used. All P values were two-sided and statistical significance was set at $P \leq 0.05$. For categorical data, the χ^2 test was used. For survival analysis, Kaplan-Meier method and log-rank test were used. For analysis of cancer-specific mortality, we excluded surgery-related deaths (deaths within one month of surgery).

RESULTS

HER2 expression in gastric cancer tissues

HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively (Figure 1). HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type

Table 2 Clinicopathological characteristics of patients with gastric cancer based on human epidermal growth factor receptor 2 expression, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations and Epstein-Barr virus infection *n* (%)

		HER2			PIK3CA			EBV		
		Positive (*n* = 20)	Negative (*n* = 211)	*P* value	Mutation (*n* = 20)	Wild type (*n* = 211)	*P* value	Positive (*n* = 18)	Negative (*n* = 204)	*P* value
Sex	Male	15 (75)	142 (67)	0.48	13 (65)	144 (68)	0.77	14 (78)	138 (68)	0.38
	Female	5 (25)	69 (33)		7 (35)	70 (32)		4 (22)	66 (32)	
Age	Median	69 (50-84)	71 (25-91)	0.26	71 (25-85)	70 (38-91)	0.40	72 (48-90)	70 (38-91)	0.41
Location	Cardias	10 (50)	72 (34)	0.49	5 (25)	77 (36)	0.31	8 (44)	73 (36)	0.70
	Body	5 (25)	57 (27)		4 (20)	58 (27)		5 (28)	55 (27)	
	Antrum	5 (25)	78 (37)		10 (50)	73 (35)		5 (28)	75 (37)	
	Unknown	0	4 (2)		1 (5)	2 (1)		0	1 (0)	
Depth	T2	12 (60)	113 (54)	0.48	8 (40)	117 (55)	0.15	12 (67)	106 (52)	0.35
	T3	8 (40)	84 (40)		9 (45)	83 (39)		6 (33)	85 (42)	
	T4	0	14 (6)		3 (15)	11 (5)		0	13 (6)	
L/N meta	N0	5 (25)	60 (28)	0.71	4 (20)	61 (29)	0.37	3 (17)	57 (28)	0.28
	N+	14 (70)	144 (68)		16 (80)	142 (67)		14 (77)	140 (69)	
	N1	5 (25)	68 (32)		8 (40)	65 (31)		8 (44)	63 (31)	
	N2	6 (30)	50 (24)		6 (30)	50 (24)		2 (11)	53 (26)	
	N3	3 (15)	26 (12)		2 (10)	27 (13)		4 (22)	24 (12)	
	Unknown	1 (5)	7 (3)		0	8 (4)		1 (6)	7 (3)	
Stage	I	5 (25)	44 (21)	0.89	1 (5)	48 (23)	0.14	3 (17)	41 (20)	0.98
	II	3 (15)	42 (20)		7 (35)	38 (18)		4 (22)	39 (19)	
	III	6 (30)	76 (36)		8 (40)	74 (35)		6 (33)	75 (37)	
	IV	5 (25)	46 (22)		4 (20)	47 (22)		4 (22)	46 (23)	
	Unknown	1 (5)	3 (1)		0	4 (2)		1 (6)	3 (1)	
Lauren	Intestinal	15 (75)	98 (46)	0.05	14 (70)	99 (47)	0.13	5 (28)	105 (51)	0.04
histotype	Diffuse	5 (25)	107 (51)		6 (30)	106 (50)		13 (72)	93 (46)	
	LELC	0	6 (3)		2 (10)	4 (2)		5 (28)	0	
	Others	0	6 (3)		0	6 (3)		0	6 (3)	
MSI		2 (10)	28 (13)	0.72	10 (50)	20 (9)	< 0.01	1 (6)	26 (13)	0.36
pAkt		16 (84)	103 (51)	< 0.01	12 (63)	107 (53)	0.37	8 (47)	103 (52)	0.69
3 yr OS (%)		29.4	59.2	0.24	57.3	56.8	0.59	57.4	57.3	0.98

MSI: Microsatellite instability; LELC: Lymphoepithelioma-like carcinoma; HER2: Human epidermal growth factor receptor 2; PIK3CA: Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations; EBV: Epstein-Barr virus; pAkt: Phospho Akt; OS: Overall survival.

Table 3 Frequencies of phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations detected in gastric cancer tissues

	Mutation	Overall frequency	Percent/ total cases	Percent/ mutated cases	Microsatellite instability
Exon1	R88Q	1	0.4	5	1
	R108H	4	1.7	20	1
	Total	5	2.2		2
Exon9	E542K	5	2.2	25	1
	E545K	2	0.9	10	1
	E545G	2	0.9	10	1
	Total	9	4.0		3
Exon20	H1047Y	1	0.4	5	1
	H1047R	6	2.6	30	4
	Total	7	3.0		5

(15/20 *vs* 98/205, $P = 0.05$, Table 2). Three-year survival rates were 29% in patients with HER2 overexpression and 59% in cases without HER2 overexpression, respectively [hazard ratio (HR) 1.73; 95%CI: 0.87-3.14, $P = 0.24$].

Mutations of the PIK3CA gene in gastric cancer tissues

PIK3CA mutations were present in 20 cases (8.7%) (Table 2 and Figure 2). The mutation frequency was high (21%) in T4 cancers and low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 of PIK3CA were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively (Table 3). One case had multiple PIK3CA mutations (R108H and E542K). The exon20/exon9 prevalence ratio was 0.78 (7/9). Two new types of PIK3CA mutations, R88Q and R108H, were detected in exon1. All mutations were heterozygous missense single-base substitutions and the most common mutation was H1047R (6/20; 30%) in exon20. PIK3CA mutations were also found to significantly correlate with MSI (10/20 *vs* 9/211, $P < 0.01$) but not with other clinicopathological characteristics. The three-year survival rates were 57% in patients with PIK3CA mutations and 57% in cases without PIK3CA mutations, respectively (HR 1.37; 95%CI: 0.68-3.26, $P = 0.59$).

EBV infection

Eighteen samples in our cohort (8%) were EBV-positive and this positivity significantly correlated with diffuse histological type (13/18 *vs* 93/198, $P = 0.04$) (Table 2 and Figure 3). There were 7 cases of LELC and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). The three-year survival rates were 57% in patients with EBV infection and 57% in those without EBV infection (HR 0.81; 95%CI:

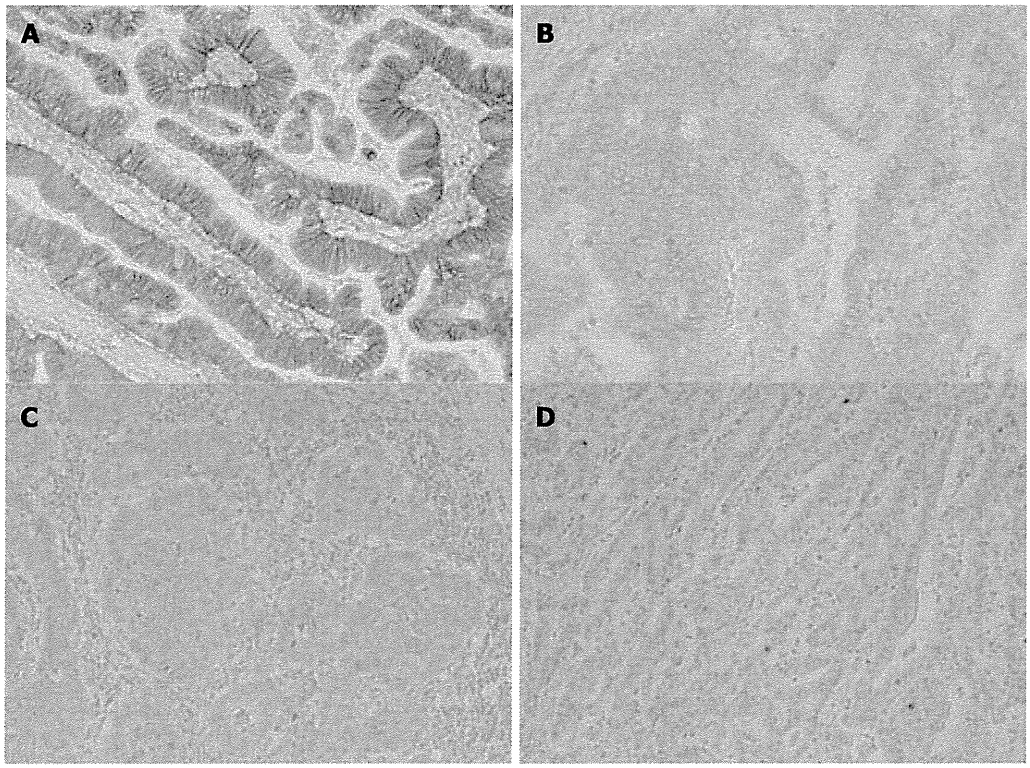

Figure 1 Immunohistochemical analysis of human epidermal growth factor receptor 2 in gastric cancer tissues. A: Human epidermal growth factor receptor 2 (HER2) 3+; B: HER2 2+; C: HER2 1+; D: HER2 0. Original magnification, ×200.

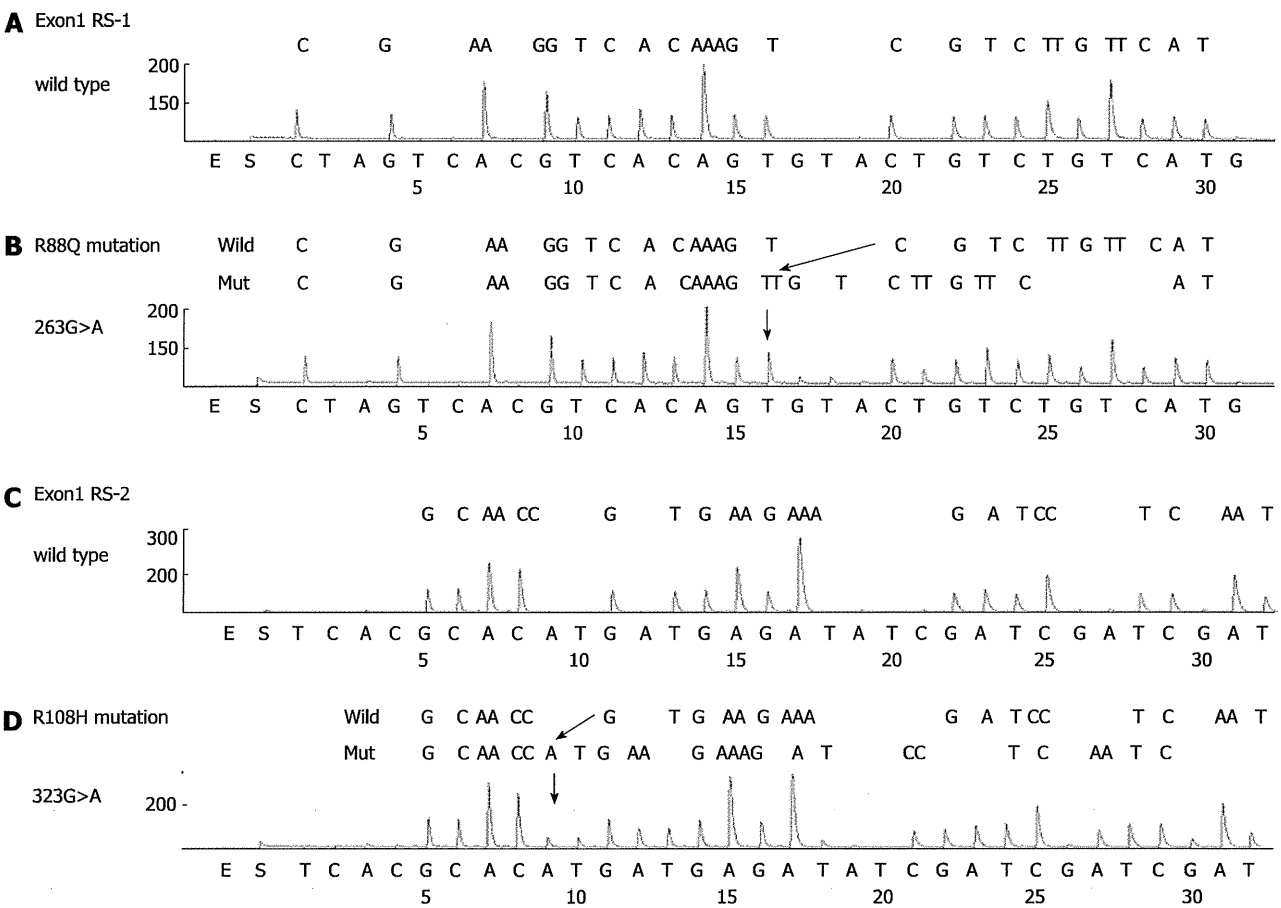

Figure 2 Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations detected by pyrosequencing in gastric cancer tissues. A: Exon1 RS1 wild type; B: 263G>A (R88Q) mutation; C: Exon1 RS2 wild type; D: 323G>A (R108H) mutation.

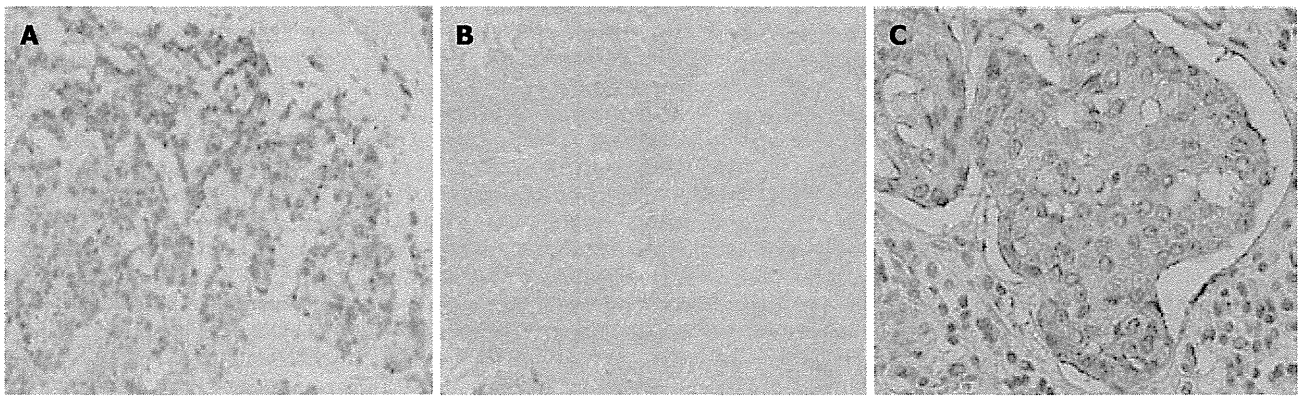

Figure 3 *In situ* hybridization analysis of Epstein-Barr virus-encoded small RNA-1 and human epidermal growth factor receptor 2 immunohistochemical expression in gastric cancer tissues. A: Gastric adenocarcinoma positive for Epstein-Barr virus-encoded small RNA-1 (EBER-1); B: Gastric adenocarcinoma negative for EBER-1; C: Immunohistochemical analysis of human epidermal growth factor receptor 2 (HER2) in an Epstein-Barr virus-positive and HER2-positive case. Original magnification, ×200.

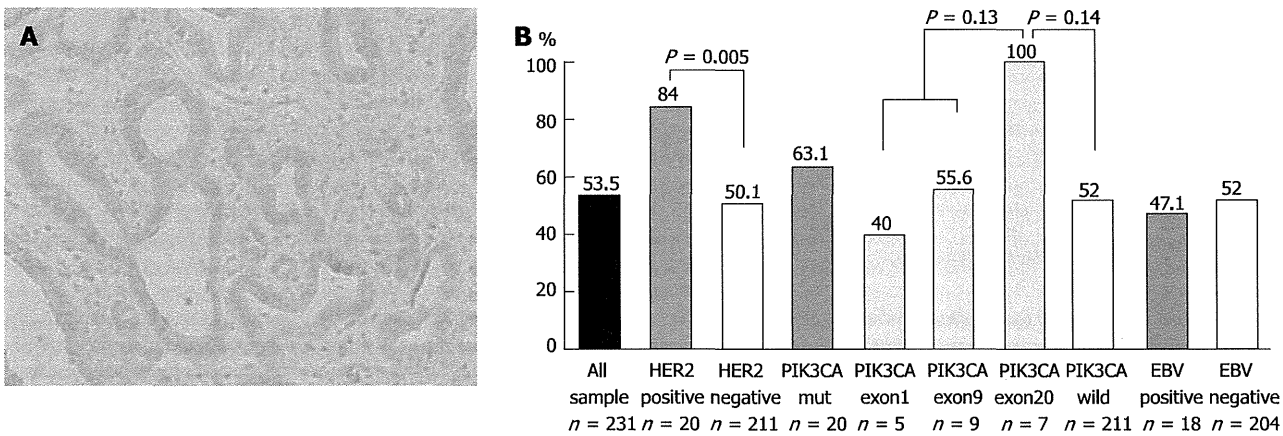

Figure 4 Immunohistochemical analysis and assessment of phospho Akt positivity based on molecular alterations in gastric cancer tissues. A: Gastric adenocarcinoma showing phospho Akt (pAkt) positivity. Original magnification, ×200; B: pAkt expression significantly correlates with human epidermal growth factor receptor 2 (HER2) overexpression ($P < 0.01$) but not with phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutations ($P = 0.37$) or Epstein-Barr virus (EBV) infection ($P = 0.69$).

0.36-2.31, $P = 0.98$).

Association of HER2 overexpression, PIK3CA mutations and EBV infection

One of our cases showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However there were no cases showing both PIK3CA mutations and HER2 overexpression. Three of the 4 cases were positive also for pAkt expression. PIK3CA mutations were present in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). One EBV-positive cancer with a PIK3CA mutation (H1047R) was MSI-positive.

pAkt expression

pAkt expression was positive in 119 (53%) of our cases but this showed no correlation with clinicopathological characteristics (Figure 4A). On the other hand, pAkt expression was found to be significantly correlated with HER2 overexpression (16/19 *vs* 103/204, $P < 0.01$) but not with PIK3CA mutations (12/19 *vs* 107/204, $P = 0.37$) or EBV infection (8/17 *vs* 103/198, $P = 0.69$) (Table 2). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations of PIK3CA, although this difference did not reach statistical significance (Figure 4B). The five-year survival rates were 37% in patients with pAkt expression and 59% in those without pAkt expression (HR 1.75; 95%CI: 1.12-2.80, $P = 0.02$) (Figure 5). Hence, pAkt expression significantly correlates with a poor prognosis in gastric cancer.

DISCUSSION

In our present study, we systematically characterized HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer ($n = 231$). We aimed to determine the prevalence of these characteristics with a high level of precision and to correlate them with clinicopathological and molecular features, such as MSI and pAkt expression.

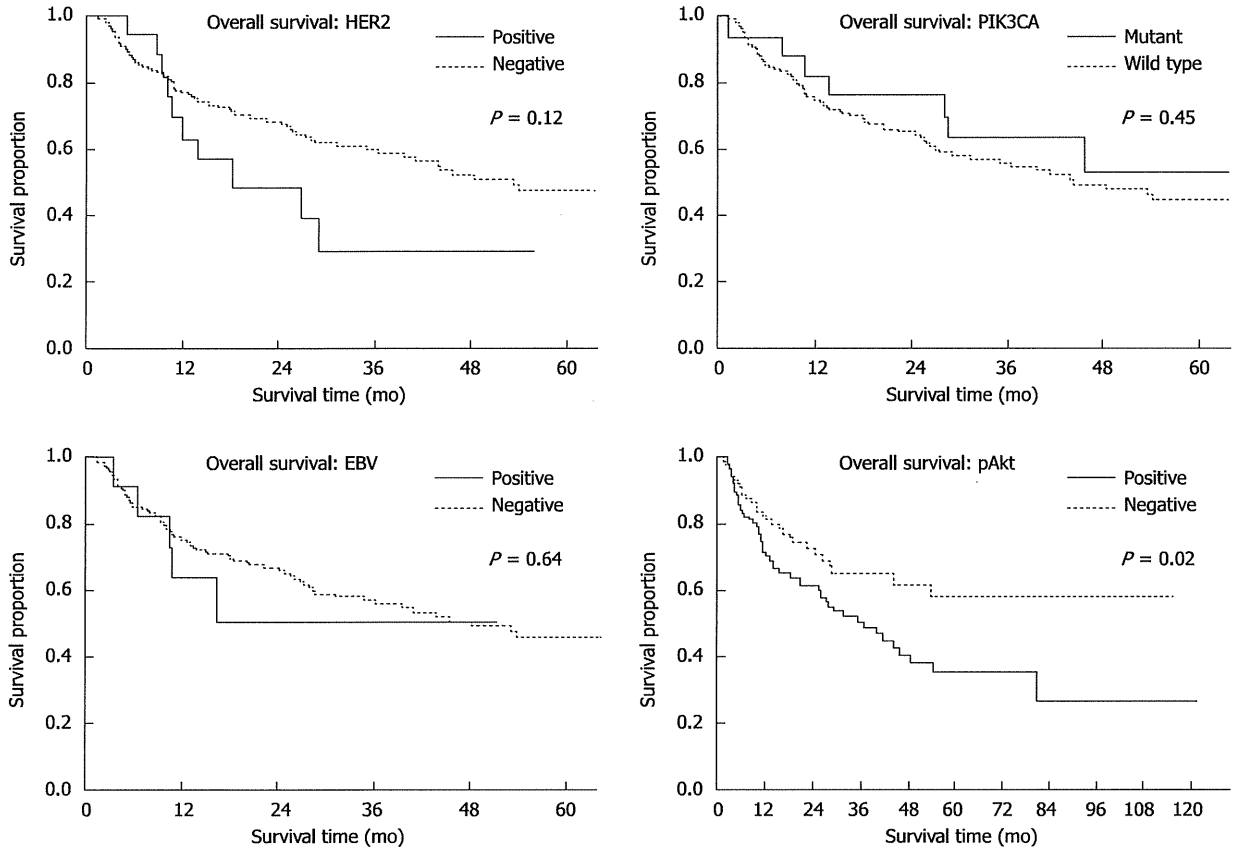

Figure 5 Survival analysis of gastric cancer patients. Three year survival of human epidermal growth factor receptor 2 (HER2)-positive *vs* HER2-negative, 29.1 mo *vs* 59.4 mo; Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutation *vs* wild type, 63.7 mo *vs* 56.3 mo; Epstein-Barr virus (EBV)-positive *vs* EBV-negative, 51.3 mo *vs* 57.6 mo; And phospho Akt (pAkt)-positive *vs* pAkt-negative, 50.7 mo *vs* 64.8 mo. Five year survival of pAkt-positive *vs* pAkt-negative cases, 35.5 mo *vs* 58.1 mo.

HER2 overexpression (IHC 3+) was present in 20 samples (8.4%), a value that is within the range (7%-34%) reported in the current literature[5-9]. HER2 overexpression was found to significantly correlate with the intestinal histological type. Hence, the frequency of HER2 expression may depend on, at least in part, the distribution of histology in a cohort of gastric cancer samples. Some studies have suggested that HER2 positivity in gastric cancer is associated with poor outcomes and aggressive disease, but the results are conflicting. We found for the first time in our present analyses that HER2 overexpression significantly correlates with pAkt expression in gastric cancer tissues. Moreover, pAkt expression correlated with a poor prognosis in these patients. Thus, the HER2-Akt axis may play an important role in gastric cancer.

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden[17,23,24]. We characterized PIK3CA mutations in gastric cancer tissues using pyrosequencing for the first time. The overall prevalence of PIK3CA mutations was found in our analysis to be 8.7%, a value that is within the previously reported range (4% to 25%)[10,12-15]. The mutation frequency was found to be high (21.4%) in T4 cancers and low (6.4%) in T2 cancers in our sample cohort. Thus, PIK3CA mutations appear to be late events in gastric carcinogenesis, leading to tumor progression. These patients might therefore be appropriate for targeted therapies directed against the PI3K pathway.

The most common PIK3CA mutation found in our analysis was H1047R, which was also found previously[15]. Importantly, two new types of mutations were found in exon1. To our knowledge, PIK3CA mutations involving residues 88 and 108 (R88Q and R108H) have been never reported previously in gastric cancer, nor described in the COSMIC database, despite the large number of previous studies in which this region was investigated. These mutations have been detected in several other types of cancer tissues[25]. Importantly also, these mutations have been reported to be gain-of-function[26-28]. Our present results thus have potential clinical implications since the mutational status of PIK3CA could stratify patients for genotype-based molecular therapies targeting the PI3K pathway. Hence, exon1 of PIK3CA should be analyzed in gastric cancer patients in these clinical settings.

PIK3CA mutations were found to be significantly associated with the MSI phenotype in our experiments. An association between PIK3CA mutations and MSI has been reported, or at least suggested, for both gastric and colon cancers[12,13,29]. We found in our present study that PIK3CA mutations in cancers with MSI are distributed in exon1, exon9 and exon20. These results further sup-

port the notion that PIK3CA is one of the most important oncogenes activated by missense mutations in MSI-positive gastric cancers.

The frequency of pAkt expression was found to be higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations in PIK3CA. These results further support the notion that the functional significance of PIK3CA mutations depends on the mutation type and that the H1047R hotspot mutation has high oncogenic activity.

The previous ToGA study has shown that the addition of trastuzumab to the chemotherapeutic regimen improves survival in patients with advanced gastric or gastroesophageal junction cancer[5,6]. PIK3CA mutation is one of the mechanisms underlying the resistance to trastuzumab in breast cancer[30]. Trastuzumab is likely to be effective for HER2-overexpressing breast cancers with no PIK3CA mutations, with possible rescue using HER2-TKIs in cases of relapse[31]. For HER2-overexpressing breast cancer with PIK3CA mutations, inhibitors against molecules of the PI3K pathway are possibly more effective than anti-HER2 agents, which are unlikely to be beneficial[32]. In our present study, PIK3CA mutations were not found in gastric cancers with HER2 overexpression. Thus, it is unlikely that PIK3CA mutation is a major mechanism underlying the resistance to trastuzumab in gastric cancer.

HER2 overexpression was found in only one of the 18 EBV-positive gastric cancers in our sample cohort. This result can be explained, at least in part, by the fact that HER2 overexpression and EBV infection significantly correlate with intestinal and diffuse histological types, respectively. On the other hand, PIK3CA mutations were identified in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). Although not analyzed in our current study, EBV infection reportedly inactivates PTEN through the CpG island methylation of its promoter in EBV-associated gastric cancer[21]. Thus, alterations in the PI3K-Akt signaling pathway in EBV-positive gastric cancers may differ from those in EBV-negative cancers.

Finally, pAkt expression was found to correlate with a poor prognosis in gastric cancer. A significant association between increased pAkt expression and poor prognosis has been reported previously in patients with T3/T4 gastric cancer but not in those with T1/T2 cancer[33]. It has been reported also that pAkt expression is associated with increased resistance to multiple chemotherapeutic agents in gastric cancer patients, when chemotherapeutic sensitivities were tested using MTT assays[34]. Thus, Akt activation appears to lead to a poor prognosis and resistance to chemotherapeutic agents in gastric cancer. A positive correlation between a decrease in the pAkt levels after gefitinib administration and tumor apoptotic index in gastric cancer has also been reported[35]. Further analyses regarding the pAkt status in cancer tissues before and after chemotherapy and molecular targeted therapy will be necessary. Not all Akt activation events can be explained by HER2 expression, PIK3CA mutations, and EBV infection in gastric cancer. We have reported previously that a dominant negative insulin-like growth factor (IGF)-1 receptor blocks the Akt-1 activation induced by IGF-1 and IGF-2 in gastric cancer cell lines[36]. Thus, molecular alterations, such as the overexpression of IGF-1 receptor, might be involved in the activation of Akt in gastric cancer and this issue needs to be clarified in the near future.

COMMENTS

Background

Personalized therapy has begun also in advanced gastric cancer through the use of trastuzumab, an anti-human epidermal growth factor receptor 2 (HER2) antibody. Many drugs targeting the phosphatidylinositol 3-kinase (PI3K)-Akt pathway have now been developed and clinical trials are ongoing. An appropriate biomarker is necessary for successful molecular targeted therapy. The alterations of molecules in the PI3K-Akt pathway could be a good biomarker for such drugs.

Research frontiers

Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and Epstein-Barr virus (EBV) infection lead to activation of the PI3K-Akt signaling pathway. However, clinicopathological and molecular correlates among such alterations have not been clearly addressed. In the present study, the authors identify new clinicopathological and molecular correlations between HER2 expression, PIK3CA mutations, EBV infection and phospho Akt (pAkt) expression in gastric cancer.

Innovations and breakthroughs

This is the first study to systematically characterize HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer. The prevalence of these characteristics was thereby determined with a high level of precision and correlations with the clinicopathological and molecular features of gastric cancers, such as microsatellite instability and pAkt expression, could be assessed accurately for the first time.

Applications

The results have potentially important clinical implications since the mutational status of PIK3CA can be used to stratify cancer patients for genotype-based molecular therapies that target the HERs-PI3K pathway.

Terminology

PI3K-Akt pathway: Akt is believed to transduce the major downstream PI3K signals in cancer. Akt regulates cell growth and survival pathways by phosphorylating substrates such as GSK3, forkhead transcription factors, and the TSC2 tumor suppressor protein; PIK3CA: PIK3CA encodes a key enzymatic subunit of PI3K. Gain of function mutations in PIK3CA occur frequently in several cancer types. Hotspots of PIK3CA mutations are located in exons 9 and 20.

Peer review

The authors investigated HER2 expression, PIK3CA mutations and EBV infection in patients with gastric cancer. The results demonstrated that pAkt expression significantly correlates with the prognosis and the HER2 expression status in gastric cancer. This article is important for the further development of molecular targeted therapy in patients with advanced gastric cancer.

REFERENCES

1 **Hamilton JP**, Sato F, Greenwald BD, Suntharalingam M, Krasna MJ, Edelman MJ, Doyle A, Berki AT, Abraham JM, Mori Y, Kan T, Mantzur C, Paun B, Wang S, Ito T, Jin Z, Meltzer SJ. Promoter methylation and response to chemotherapy and radiation in esophageal cancer. *Clin Gastroenterol Hepatol* 2006; **4**: 701-708

2 **Tamura G**. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric

cancer. *World J Gastroenterol* 2006; **12**: 192-198
3 **Engelman JA**. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* 2009; **9**: 550-562
4 **Mueller A**, Bachmann E, Linnig M, Khillimberger K, Schimanski CC, Galle PR, Moehler M. Selective PI3K inhibition by BKM120 and BEZ235 alone or in combination with chemotherapy in wild-type and mutated human gastrointestinal cancer cell lines. *Cancer Chemother Pharmacol* 2012; **69**: 1601-1615
5 **Bang YJ**, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**: 687-697
6 **Sawaki A**, Ohashi Y, Omuro Y, Satoh T, Hamamoto Y, Boku N, Miyata Y, Takiuchi H, Yamaguchi K, Sasaki Y, Nishina T, Satoh A, Baba E, Tamura T, Abe T, Hatake K, Ohtsu A. Efficacy of trastuzumab in Japanese patients with HER2-positive advanced gastric or gastroesophageal junction cancer: a subgroup analysis of the Trastuzumab for Gastric Cancer (ToGA) study. *Gastric Cancer* 2012; **15**: 313-322
7 **Gravalos C**, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. *Ann Oncol* 2008; **19**: 1523-1529
8 **Hofmann M**, Stoss O, Shi D, Büttner R, van de Vijver M, Kim W, Ochiai A, Rüschoff J, Henkel T. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. *Histopathology* 2008; **52**: 797-805
9 **Tanner M**, Hollmén M, Junttila TT, Kapanen AI, Tommola S, Soini Y, Helin H, Salo J, Joensuu H, Sihvo E, Elenius K, Isola J. Amplification of HER-2 in gastric carcinoma: association with Topoisomerase IIalpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. *Ann Oncol* 2005; **16**: 273-278
10 **Samuels Y**, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004; **304**: 554
11 **Lee JW**, Soung YH, Kim SY, Lee HW, Park WS, Nam SW, Kim SH, Lee JY, Yoo NJ, Lee SH. PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. *Oncogene* 2005; **24**: 1477-1480
12 **Li VS**, Wong CW, Chan TL, Chan AS, Zhao W, Chu KM, So S, Chen X, Yuen ST, Leung SY. Mutations of PIK3CA in gastric adenocarcinoma. *BMC Cancer* 2005; **5**: 29
13 **Velho S**, Oliveira C, Ferreira A, Ferreira AC, Suriano G, Schwartz S, Duval A, Carneiro F, Machado JC, Hamelin R, Seruca R. The prevalence of PIK3CA mutations in gastric and colon cancer. *Eur J Cancer* 2005; **41**: 1649-1654
14 **Ligresti G**, Militello L, Steelman LS, Cavallaro A, Basile F, Nicoletti F, Stivala F, McCubrey JA, Libra M. PIK3CA mutations in human solid tumors: role in sensitivity to various therapeutic approaches. *Cell Cycle* 2009; **8**: 1352-1358
15 **Barbi S**, Cataldo I, De Manzoni G, Bersani S, Lamba S, Mattuzzi S, Bardelli A, Scarpa A. The analysis of PIK3CA mutations in gastric carcinoma and metanalysis of literature suggest that exon-selectivity is a signature of cancer type. *J Exp Clin Cancer Res* 2010; **29**: 32
16 **Kataoka Y**, Mukohara T, Shimada H, Saijo N, Hirai M, Minami H. Association between gain-of-function mutations in PIK3CA and resistance to HER2-targeted agents in HER2-amplified breast cancer cell lines. *Ann Oncol* 2010; **21**: 255-262
17 **Weidlich S**, Walsh K, Crowther D, Burczynski ME, Feuerstein G, Carey FA, Steele RJ, Wolf CR, Miele G, Smith G. Pyrosequencing-based methods reveal marked inter-individual differences in oncogene mutation burden in human colorectal tumours. *Br J Cancer* 2011; **105**: 246-254
18 **Kang GH**, Lee S, Kim WH, Lee HW, Kim JC, Rhyu MG, Ro JY. Epstein-barr virus-positive gastric carcinoma demonstrates frequent aberrant methylation of multiple genes and constitutes CpG island methylator phenotype-positive gastric carcinoma. *Am J Pathol* 2002; **160**: 787-794
19 **Kusano M**, Toyota M, Suzuki H, Akino K, Aoki F, Fujita M, Hosokawa M, Shinomura Y, Imai K, Tokino T. Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein-Barr virus. *Cancer* 2006; **106**: 1467-1479
20 **Chang MS**, Uozaki H, Chong JM, Ushiku T, Sakuma K, Ishikawa S, Hino R, Barua RR, Iwasaki Y, Arai K, Fujii H, Nagai H, Fukayama M. CpG island methylation status in gastric carcinoma with and without infection of Epstein-Barr virus. *Clin Cancer Res* 2006; **12**: 2995-3002
21 **Hino R**, Uozaki H, Murakami N, Ushiku T, Shinozaki A, Ishikawa S, Morikawa T, Nakaya T, Sakatani T, Takada K, Fukayama M. Activation of DNA methyltransferase 1 by EBV latent membrane protein 2A leads to promoter hypermethylation of PTEN gene in gastric carcinoma. *Cancer Res* 2009; **69**: 2766-2774
22 **Gori S**, Sidoni A, Colozza M, Ferri I, Mameli MG, Fenocchio D, Stocchi L, Foglietta J, Ludovini V, Minenza E, De Angelis V, Crinò L. EGFR, pMAPK, pAkt and PTEN status by immunohistochemistry: correlation with clinical outcome in HER2-positive metastatic breast cancer patients treated with trastuzumab. *Ann Oncol* 2009; **20**: 648-654
23 **Nosho K**, Kawasaki T, Ohnishi M, Suemoto Y, Kirkner GJ, Zepf D, Yan L, Longtine JA, Fuchs CS, Ogino S. PIK3CA mutation in colorectal cancer: relationship with genetic and epigenetic alterations. *Neoplasia* 2008; **10**: 534-541
24 **Baba Y**, Nosho K, Shima K, Hayashi M, Meyerhardt JA, Chan AT, Giovannucci E, Fuchs CS, Ogino S. Phosphorylated AKT expression is associated with PIK3CA mutation, low stage, and favorable outcome in 717 colorectal cancers. *Cancer* 2011; **117**: 1399-1408
25 **Rudd ML**, Price JC, Fogoros S, Godwin AK, Sgroi DC, Merino MJ, Bell DW. A unique spectrum of somatic PIK3CA (p110alpha) mutations within primary endometrial carcinomas. *Clin Cancer Res* 2011; **17**: 1331-1340
26 **Miyake T**, Yoshino K, Enomoto T, Takata T, Ugaki H, Kim A, Fujiwara K, Miyatake T, Fujita M, Kimura T. PIK3CA gene mutations and amplifications in uterine cancers, identified by methods that avoid confounding by PIK3CA pseudogene sequences. *Cancer Lett* 2008; **261**: 120-126
27 **Oda K**, Okada J, Timmerman L, Rodriguez-Viciana P, Stokoe D, Shoji K, Taketani Y, Kuramoto H, Knight ZA, Shokat KM, McCormick F. PIK3CA cooperates with other phosphatidylinositol 3'-kinase pathway mutations to effect oncogenic transformation. *Cancer Res* 2008; **68**: 8127-8136
28 **Gymnopoulos M**, Elsliger MA, Vogt PK. Rare cancer-specific mutations in PIK3CA show gain of function. *Proc Natl Acad Sci USA* 2007; **104**: 5569-5574
29 **Corso G**, Velho S, Paredes J, Pedrazzani C, Martins D, Milanezi F, Pascale V, Vindigni C, Pinheiro H, Leite M, Marrelli D, Sousa S, Carneiro F, Oliveira C, Roviello F, Seruca R. Oncogenic mutations in gastric cancer with microsatellite instability. *Eur J Cancer* 2011; **47**: 443-451
30 **Berns K**, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, Linn SC, Gonzalez-Angulo AM, Stemke-Hale K, Hauptmann M, Beijersbergen RL, Mills GB, van de Vijver MJ, Bernards R. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell* 2007; **12**: 395-402
31 **Wang L**, Zhang Q, Zhang J, Sun S, Guo H, Jia Z, Wang B, Shao Z, Wang Z, Hu X. PI3K pathway activation results in low efficacy of both trastuzumab and lapatinib. *BMC Cancer*

2011; **11**: 248

32 **Crawford A**, Nahta R. Targeting Bcl-2 in Herceptin-Resistant Breast Cancer Cell Lines. *Curr Pharmacogenomics Person Med* 2011; **9**: 184-190

33 **Murakami D**, Tsujitani S, Osaki T, Saito H, Katano K, Tatebe S, Ikeguchi M. Expression of phosphorylated Akt (pAkt) in gastric carcinoma predicts prognosis and efficacy of chemotherapy. *Gastric Cancer* 2007; **10**: 45-51

34 **Oki E**, Baba H, Tokunaga E, Nakamura T, Ueda N, Futatsugi M, Mashino K, Yamamoto M, Ikebe M, Kakeji Y, Maehara Y. Akt phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer. *Int J Cancer* 2005; **117**: 376-380

35 **Rojo F**, Tabernero J, Albanell J, Van Cutsem E, Ohtsu A, Doi T, Koizumi W, Shirao K, Takiuchi H, Ramon y Cajal S, Baselga J. Pharmacodynamic studies of gefitinib in tumor biopsy specimens from patients with advanced gastric carcinoma. *J Clin Oncol* 2006; **24**: 4309-4316

36 **Min Y**, Adachi Y, Yamamoto H, Imsumran A, Arimura Y, Endo T, Hinoda Y, Lee CT, Nadaf S, Carbone DP, Imai K. Insulin-like growth factor I receptor blockade enhances chemotherapy and radiation responses and inhibits tumour growth in human gastric cancer xenografts. *Gut* 2005; **54**: 591-600

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