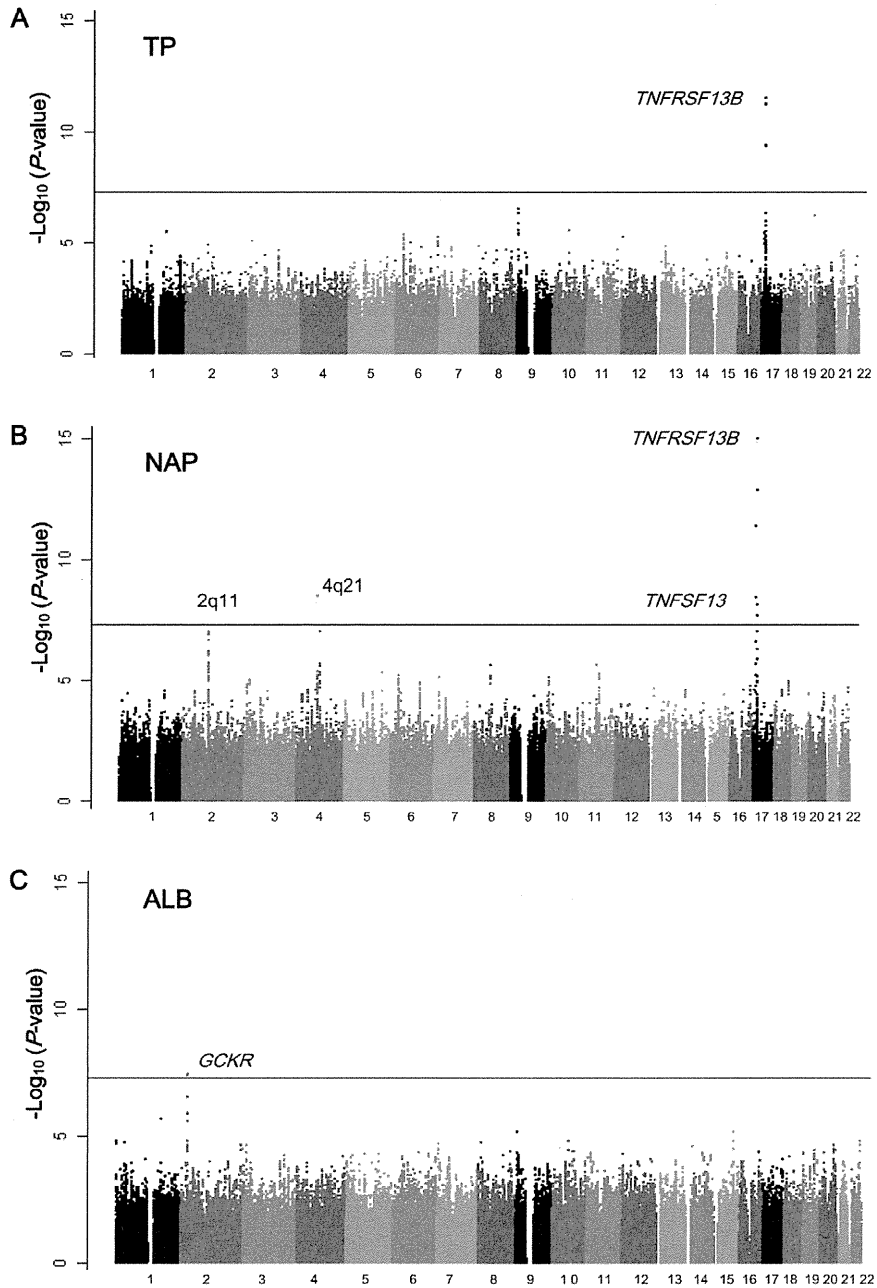

Figure 1. Manhattan plots for the GWAS of (A) TP, (B) NAP, and (C) ALB. SNPs were plotted based on their physical chromosomal positions (horizontal axis) together with their $-\log_{10}$ (*P*-values) in the GWAS (vertical axis). The black horizontal line shows the genome-wide significance threshold of $P = 5.0 \times 10^{-8}$. The SNPs for which *P*-values were smaller than 1.0×10^{-15} are indicated at the upper limit of the plots.
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dependent phospholipid-binding proteins [19]. Annexin A3 was found to be translocated into phagosomes in dendritic cells [20], which are antigen-presenting cells that serve as messengers between the innate and adaptive immune response, and play a key role in allergic, inflammatory, and autoimmune conditions. In addition, annexin A3 was also found to be associated with neutrophil granule membranes [21], where it can play a regulatory role in calcium-dependent granule secretions that contribute to acute inflammation and chronic tissue destruction. The association of rs10007186 with IgA, IgM, and IgE, would suggest additional biological roles of annexin A3 in the immune response.

We also confirmed the association of SNPs in *GCKR* with serum ALB levels (rs1260326, $P_{meta} = 3.1 \times 10^{-9}$). Rs1260326 is a missense variant (T>C, Leu446Pro) and predicted to cause a damaging effect on the protein structure. *GCKR* is a locus frequently associated with several metabolic traits [4,22–24] and rs1260326 has been reported to be associated with serum triglycerides [4].

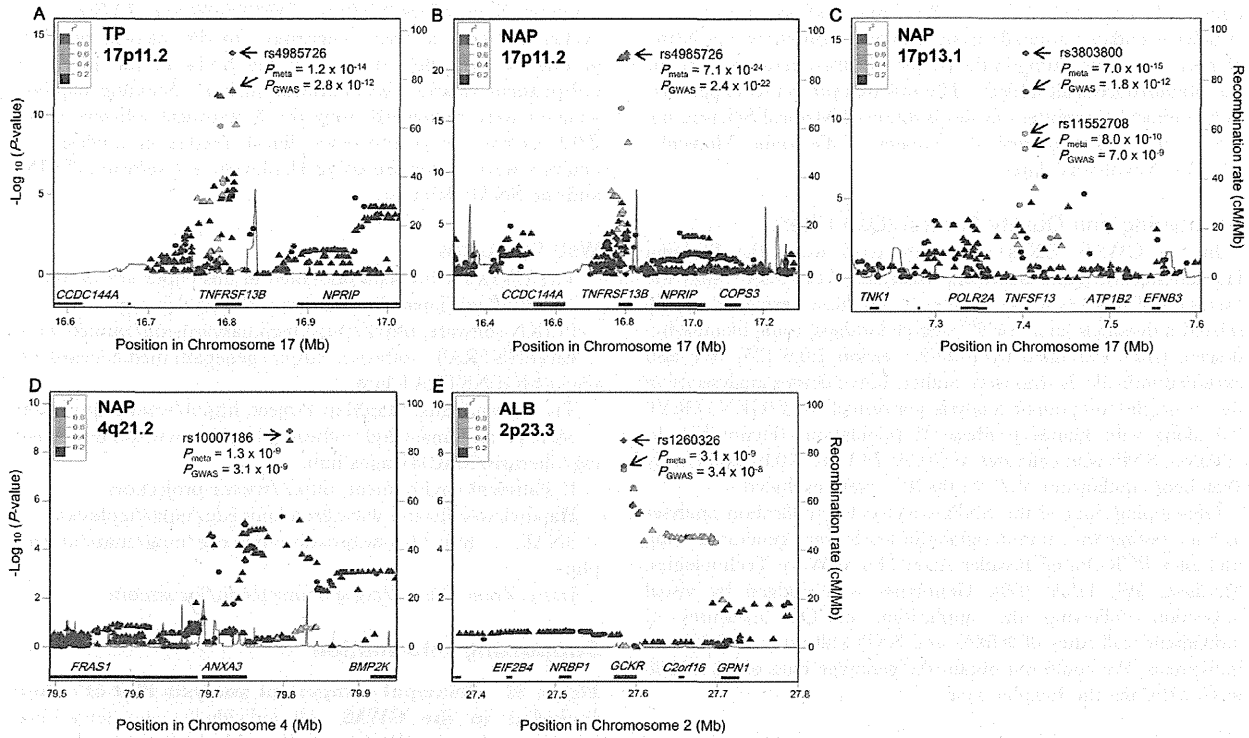

Figure 2. Regional plots for the associations of the SNPs in the GWAS stage of TP, ALB and NAP. SNPs plotted with their $-\log_{10}$ (*P*-values) in the GWAS based on their physical chromosomal positions. Genotyped SNPs are indicated as circles, while imputed SNPs are indicated as triangles. The color scheme indicated the linkage disequilibrium displayed as r^2 values between all SNPs and the top-ranked SNP in each plot. The tested trait, chromosomal locus, and the top-ranked SNPs (in purple color) in the GWAS and combined analyses together with their *P*-values are shown in each plot. The blue lines represent the recombination rates estimated based on HapMap Phase II database. The plots were drawn using Locus Zoom software.
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As a conclusion, the present study identified genetic loci that influence the inter-individual variation in serum levels of TP, ALB, and NAP. The loci associated with NAP encompass genes encoding a TNF-receptor and its ligand, which are implicated in biological roles in the immune system, and their associations with immunoglobulin isotypes were demonstrated here. Our results should add novel insight toward understanding the genetic background contributing to the regulation of the serum levels of NAP and its major components.

Materials and Methods

Study Cohorts

For the GWAS, 9,103 subjects derived from 10 disease cohorts (colorectal cancer, breast cancer, prostate cancer, lung cancer, gastric cancer, diabetes mellitus, peripheral artery disease, atrial fibrillation, ischemic stroke, and myocardial infarction) were selected, and for the replication study, we used data from >1600 independent individuals selected from the BioBank Japan Project [11] (Table 1 and Table S1). For immunoglobulin isotypes analyses, the data from ~1,600 additional individuals in BioBank

Table 3. Association of the SNPs in the GWAS of the NAP with immunoglobulin isotypes.

SNP	Gene	IgG			IgA			IgM			IgE		
		Effect [a] (s.e)	*P* [b]	%EV	Effect [a] (s.e)	*P* [b]	%EV	Effect [a] (s.e)	*P* [b]	%EV	Effect [a] (s.e)	*P* [b]	%EV
rs4985726	*TNFRSF13B*	0.071 (0.022)	1.4×10^{-3}	0.51	0.049 (0.030)	0.099	–	−0.090 (0.032)	5.9×10^{-3}	0.40	0.039 (0.064)	0.54	–
rs3803800	*TNFSF13*	−0.074 (0.024)	2.2×10^{-3}	0.47	−0.086 (0.031)	6.2×10^{-3}	0.39	−0.082 (0.034)	0.018	0.29	−0.117 (0.067)	0.080	–
rs11552708	*TNFSF13*	0.067 (0.022)	2.3×10^{-3}	0.46	0.072 (0.029)	0.013	0.31	0.078 (0.032)	0.014	0.31	0.059 (0.060)	0.33	–
rs10007186	*ANXA3*	−0.018 (0.022)	0.42	–	−0.063 (0.030)	0.036	0.20	−0.078 (0.033)	0.019	0.27	0.200 (0.057)	4.9×10^{-4}	2.02

[a]The effect of the minor alleles on the standardized values.
[b]*P*-values for the associations of SNPs with each normalized immunoglobulin isotype obtained by using a linear regression model.
Abbreviations: s.e: standard error, %EV: percentage of the explanatory variance.
doi:10.1371/journal.pone.0032683.t003

Japan [11] was used (Table 1). The clinical information for the samples is updated annually using a standard questionnaire in the 66 hospitals participating in the project. Written informed consent was obtained from all subjects. The research project was approved by the ethical committees in the Institute of Medical Science, the University of Tokyo, and the Center of Genomic Medicine, RIKEN, Yokohama, Japan.

Genotyping and Quality Control (Q.C) Filters

In the GWAS, SNPs were genotyped using the Illumina HumanHap610-Quad BeadChip (Illumina, CA, USA). After the exclusion of samples with call rates of <0.98, we excluded closely related individuals (in 1st or 2nd degree kinships) using identity-by-descent (IBD) evaluated by PLINK version 1.0.6 [25]. We also excluded individuals who were outliers in the cluster analysis using the principle component analysis performed by EIGENSTRAT 3.0 along with HapMap Phase II populations (Figure S1). In addition, SNPs with call rates of <0.99, MAF of <0.01 and Hardy Weinberg equilibrium of $P<1.0\times10^{-7}$ were excluded.

Genotyping data of the SNPs selected for replication analyses and for testing with immunoglobulin levels were generated using multiplex PCR- based Invader Assay (Third Wave Technologies, Madison, WI, USA) [26]. Genotypes were judged by visual inspection, following the application of QC measures of individuals' call rates of >98% and SNPs call rates of >99% of individuals. We could not obtain the genotype data of rs3817588 in *GCKR* using the Invader assay.

Whole-genome Imputation of Genotypes

We performed whole-genome imputation of the GWAS subjects in a two-step procedure, as described elsewhere [27]. HapMap phase II Japanese (JPT) and Han Chinese (CHB) individuals (release 24) were adopted as reference panels. We excluded the imputed SNPs with MAF of <0.01 or *Rsq* of <0.7. As a result, a total of 2,178,644 SNPs on autosomal chromosomes were used for the GWAS.

Statistical Analysis

We obtained the non-transformed values of TP, ALB and NAP (mg/dl) for the subjects from the clinical information stored in BioBank Japan [11], and adjusted them in linear regression models with age, gender, body mass index (BMI), smoking, drinking status, and affection status of the disease as covariates. The residuals were then normalized as Z scores and subjects with Z scores of <-4 or >4 were removed from each trait analysis. The associations of the SNPs with Z scores were evaluated in linear regression models assuming additive effects of allele dosages, using mach2qtl software. The same methods of data normalization and statistical models were applied for the replication analyses and for testing the association with common log-transformed values of immunoglobulin isotypes (IgG IgA, IgM, and IgE). Meta-analyses of the GWAS and the replication study were performed using the inverse-variance method assuming a fixed-effects model.

The significance level used was 5×10^{-8} in the GWAS stage. For the replication stage, we considered 0.05 as significant for the association of rs4985726 with TP and rs1260326 with ALB. For the association of SNPs rs4985726 in TNFRSF13B, rs3803800 and rs11552708 in TNFSF13 with NAP, 0.017 (0.05/3) was considered to be significant. These significance levels represent the Bonferroni correction for multiple statistical tests. In addition, we set a level of 0.05 to consider the association of the selected SNPs with immunoglobulin isotypes as significant.

The haplotype analyses were performed using the Haplo Stats package (version 1.4.0) implemented in *R* statistical software. Epistatic effects of the SNPs in *TNFRSF13B* and *TNFSF13* were evaluated using a linear regression model incorporating the product of the allele dosages of the SNPs in the loci as an independent variable. All statistical analyses including haplotype analyses were performed using the *R* statistical software version 2.9.1 except for genome-wide linear regression analyses. LD analyses were performed using Haploview 4.2 software, PLINK, and the SNAP database.

Web Resources

The URLs for the data presented in this paper are as follows:

The BioBank Japan Project, http://biobankjp.org/

PLIKN software, http://pngu.mgh.harvard.edu/purcell/plink/

EIGENSTRAT software, http://genepath.med.harvard.edu/reich/EIGENSTRAT.htm

The International HapMap Project, http://www.hapmap.org/

MACH and mach2qtl software, http://www.sph.umich.edu/csg/abecasis/MaCH/index.html

R statistical environment, http://www.r-project.org/

Haploview software, www.broad.mit.edu/mpg/haploview/

SNAP, http://www.broadinstitute.org/mpg/snap/ldsearch.php

Locus Zoom, http://csg.sph.umich.edu/locuszoom/

Supporting Information

Figure S1 Principal component analysis Plot of cohorts included in the GWAS. All individuals who were finally incorporated in the GWAS together with the four populations in the HapMap Phase II database (Japanese: JPT; Han Chinese: CHB; Africans: YRI, and European: CEU) were plotted based on the first two eigenvectors.
(PDF)

Figure S2 Quantile-Quantile (Q-Q) plots for the GWAS of (A) TP, (B) NAP, and (C) ALB. The inflation factor, λ_{GC}, for the analysis is shown in the legend of each plot. The SNPs for which *P*-values were smaller than 1.0×10^{-15} are indicated at the upper limit of the plots.
(PDF)

Figure S3 Relationship between the genotypes of SNPs identified in the study and the levels of tested proteins: (A) rs4985726, (B) rs3803800, (C) rs11552708, (D) rs10007186, and (E) rs1260326. For each box plot, the bold line indicates the median value which is the 50th quartile. The limits of each box are the 25th and 75th quartiles.
(PDF)

Table S1 Characteristics of the GWAS cohorts.
(DOC)

Table S2 SNPs showed suggestive associations with each examined trait ($P<1.0\times10^{-6}$).
(DOC)

Table S3 Haplotype analysis of rs3803800 and rs11552708 in *TNFSF13* in association with NAP.
(DOC)

Table S4 Haplotype analysis of rs1260326 and rs3817588 in *GCKR* in association with ALB.
(DOC)

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Author Contributions

Conceived and designed the experiments: YN KM YO YK MK WO. Performed the experiments: WO MK. Analyzed the data: YO YK WO. Contributed reagents/materials/analysis tools: YN KM YO MK. Wrote the paper: WO YO YN. Summarized the whole results: WO.

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nature genetics

A genome-wide association study identifies two susceptibility loci for duodenal ulcer in the Japanese population

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Through a genome-wide association analysis with a total of 7,035 individuals with duodenal ulcer and 25,323 controls from Japan, we identified two susceptibility loci at the *PSCA* gene (encoding prostate stem cell antigen) at 8q24 and at the *ABO* blood group locus at 9q34. The C allele of rs2294008 at *PSCA* was associated with increased risk of duodenal ulcer (odds ratio (OR) = 1.84; $P = 3.92 \times 10^{-33}$) in a recessive model but was associated with decreased risk of gastric cancer (OR = 0.79; $P = 6.79 \times 10^{-12}$), as reported previously[1]. The T allele of rs2294008 encodes a translation initiation codon upstream of the reported site and changes protein localization from the cytoplasm to the cell surface. rs505922 at *ABO* was also associated with duodenal ulcer in a recessive model (OR = 1.32; $P = 1.15 \times 10^{-10}$). Our findings demonstrate a role for genetic variants in the pathogenesis of duodenal ulcer.

Duodenal ulcer is one of the most common gastrointestinal disorders, with a lifetime prevalence of 4–15% (refs. 2,3). *Helicobacter pylori* infection is a major cause of duodenal ulcer and is observed in 70–90% of individuals with this condition[2] as well as in individuals with gastric ulcer and cancer[3]. Eradication of *H. pylori* by antibiotics can effectively cure duodenal ulcer[4], showing the causal role of *H. pylori* in disease pathogenesis. Because of the high prevalence of *H. pylori* infection in the Japanese population, the incidence of peptic ulcer and gastric cancer is much higher in Japanese individuals than in individuals of European descent[5,6]. Among Europeans and non-Japanese Asian populations, duodenal ulcer is more common than gastric ulcer[5,7], while gastric ulcer is more common among Japanese and Japanese-Americans[6,8]. In addition, individuals with duodenal ulcer are well known to have a lower risk for gastric cancer[9]. These heterogeneities in disease susceptibility are influenced by both bacterial and host factors[10]. Smoking and nonsteroidal anti-inflammatory drugs have been shown to increase the risk of peptic ulcer[11]. In addition, in a Finnish twin study in which the concordance rate between probands was 23.6% in monozygotic twins and 14.8% in dizygotic twins, 39% of the liability to peptic ulcer was explained by genetic factors[12]. To identify genetic susceptibility factors for duodenal ulcer, we performed a genome-wide association study (GWAS).

We genotyped DNA samples from 1,043 individuals with duodenal ulcer (cases) and 21,694 controls without peptic ulcer from BioBank Japan[13]. (The characteristics of each cohort and the study design are shown in **Table 1** and in **Supplementary Fig. 1**.) After performing a standard quality control procedure, we had genotyping results for 480,327 SNPs and performed logistic regression analysis by including age and gender as covariates. In the GWAS stage, we calculated the minimum *P* value under three genetic models (additive, recessive and dominant). The genomic inflation factor λ was calculated to be 1.014, indicating minimal evidence of population stratification (**Supplementary Fig. 2**). The association analyses identified two significantly associated loci on chromosomes 8q24 and 9q34 ($P = 2.84 \times 10^{-19}$ and 2.27×10^{-8}, respectively; **Fig. 1**, **Table 2** and **Supplementary Table 1**). Overall, a total of 101 SNPs from 42 distinct genomic regions showed suggestive evidence of association ($P < 5 \times 10^{-5}$).

In the replication stage, we selected the 42 SNPs that had the lowest *P* values in each genomic region and genotyped them in 5,992 independent duodenal ulcer cases and 3,629 controls. We performed logistic regression analysis, adjusting for age and gender, and observed significant associations at rs2294008 on 8q24 (OR = 1.73 and $P = 6.60 \times 10^{-16}$ in a recessive model) and at rs505922 on 9q34 (OR = 1.23 and $P = 1.78 \times 10^{-4}$ in a recessive model) after applying a Bonferroni correction ($P < 0.05/42 = 1.19 \times 10^{-3}$; **Table 2** and **Supplementary Table 2**). A meta-analysis of the GWAS and replication stages identified significant associations for rs2294008 and rs505922 without any heterogeneity between the two stages (OR = 1.84; $P = 3.92 \times 10^{-33}$ and OR = 1.32; $P = 1.15 \times 10^{-10}$, respectively; **Table 2**). Under an additive model, rs2294008 showed equivalent association with duodenal ulcer ($P = 1.79 \times 10^{-33}$) as in the recessive model. Logistic regression analysis of various risk factors showed that the two SNPs, age, gender and smoking are independent risk factors for duodenal ulcer (**Supplementary Table 3**).

The development of duodenal ulcer occurs as a multistep process, involving persistent *H. pylori* infection and subsequent inflammation and damage of the duodenal mucosa. To elucidate the physiological

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Table 1 Characteristics of study populations

Stage	Sample type	*H. pylori* status	Source	Platform	Number of samples	Female (%)	Age (mean ± s.d.)
GWAS	Duodenal ulcer		BioBank Japan	HumanHap610K	1,043	247 (23.7%)	63.2 ± 10.6
	Controls		BioBank Japan	HumanHap610K	21,694	10,162 (46.8%)	62.6 ± 12.5
Replication	Duodenal ulcer		BioBank Japan	Invader assay	5,992	1,639 (27.4%)	63.0 ± 12.1
	Controls		BioBank Japan	HumanHap550K	2,722	2,273 (62.6%)	46.3 ± 15.7
			Healthy controls		907		
Gastric cancer	Gastric cancer		BioBank Japan	HumanHap610K	2,346	521 (22.2%)	64.9 ± 9.1
	Controls		BioBank Japan	HumanHap610K	16,882	8,663 (51.3%)	62.6 ± 13.0
H. pylori susceptibility[a]	Duodenal ulcer	+	Aichi Cancer Center	TaqMan assay	37	8 (21.6%)	53.4 ± 13.3
	Healthy controls	+	Aichi Cancer Center	TaqMan assay	284	144 (50.7%)	54.4 ± 14.3
		−	Aichi Cancer Center	TaqMan assay	509	268 (52.7%)	42.0 ± 15.6

[a]Positivity for *H. pylori* infection was defined by plasma levels of immunoglobulin G (IgG) to *H. pylori* that were greater than 10 U/ml.

roles of the variants, we genotyped the two associated SNPs in additional cohorts consisting of healthy controls, with or without *H. pylori* infection, as well as duodenal ulcer cases with *H. pylori* infection (**Table 1**). Neither SNP showed significant association with *H. pylori* infection in healthy controls (**Supplementary Table 4**). However, we found significant association of rs2294008 ($P = 0.021$; OR = 2.59) and marginal association of rs505922 ($P = 0.076$; OR = 1.90) with duodenal ulcer susceptibility among *H. pylori* carriers (**Supplementary Table 5**). In addition, both SNPs showed significant association with duodenal ulcer risk in individuals with gastric cancer ($P = 4.7 \times 10^{-4}$ and 0.041; OR = 2.88 and 1.62, respectively; **Supplementary Table 5**), of whom nearly 80% were infected with *H. pylori*. Taken together, the data indicate that these SNPs are likely to be associated with duodenal ulcer development after *H. pylori* infection and not with susceptibility to persistent *H. pylori* infection *per se*.

In both the GWAS and replication studies, we used individuals with various other diseases as controls. To exclude the possibility of confounding effects caused by the varied backgrounds of the control samples, we conducted an association analysis using only healthy volunteers as controls. In this analysis, we found strong association of rs2294008 ($P = 2.97 \times 10^{-15}$; OR = 1.75) and rs505922 ($P = 0.006$; OR = 1.17) with duodenal ulcer (**Supplementary Table 6**). In addition, the allelic frequencies of rs2294008 and rs505922 were not significantly different between controls with other disease and healthy controls ($P = 0.19$ and 0.46, respectively; **Supplementary Table 7**). Therefore, the use of controls with other diseases is not likely to have affected the association results in our study.

rs2294008 is located within a genomic region that encodes *ARC*, *JRK*, *PSCA*, *LY6K*, *C8orf55*, *SLURP1*, *LYPD2*, *LYNX1* and *LY6D*, while rs505922 is in a region encoding *OBP2B*, *ABO*, *SURF6*, *MED22*, *SNORD24*, *RFL7A*, *SNORD36B*, *SNORD36A*, *SNORD36C*, *SURF1* and *SURF2*. To further characterize these two loci, we performed imputation analysis (**Fig. 2a,b**). The regional association plots using genotyped and imputed data show that all strongly associated SNPs are confined to regions around the *PSCA* (encoding prostate stem cell antigen) and *ABO* blood group genes (**Supplementary Fig. 3**). Next, we examined the expression of these genes in multiple human tissues. *ABO* was expressed in the gastrointestinal tract, including in the duodenum, concordant with previous reports[14], whereas *PSCA* was highly expressed in stomach but not in normal duodenum (**Supplementary Fig. 4**). However, as metaplasia consisting of gastric-type mucous-secreting cells has been observed in duodenal ulcer lesions[15], *PSCA* is likely to be expressed in the duodenum in such cells.

PSCA encodes a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein that is involved in cell renewal and proliferation[16]. PSCA was shown to be upregulated in various cancers, including bladder, pancreatic and kidney[17], and PSCA expression has been correlated with higher tumor grade and metastatic properties of prostate cancer[16]. However, PSCA downregulation and growth-suppressive effects in esophageal and gastric cancers have also been reported[17]. Hence, *PSCA* might function as an oncogene in some epithelial cells and as a tumor suppressor gene in others. Although previous studies identified an association between rs2294008 and risk for gastric and bladder cancers[1,18], the only known functional consequence of this variation is reduced transcriptional activity. The C allele of rs2294008 is common in European and African populations, whereas the T allele is dominant in Japanese. The T allele of rs2294008 encodes a translation initiation codon for the *PSCA* gene upstream of the known site, resulting in a PSCA protein with an additional nine amino acids at its N-terminus (long PSCA, 123 amino acids; **Fig. 3a**) relative to the reported PSCA protein (short PSCA, 114 amino acids). According to PSORT II (ref. 19), long PSCA contains an N-terminal signal peptide, which is predicted to be N-glycosylated, with the protein localizing to the plasma membrane, whereas short PSCA is predicted to not be glycosylated and to localize to the cytoplasm. As expected, immunocytochemical analysis showed membrane localization of the long PSCA protein in PC3 and Du145 cells (prostate cancer cell lines that have two T alleles at rs2294008) and cytosolic localization of the short PSCA protein in C42B and NCI-H522 cells (prostate and lung cancer cell lines that have two C alleles at rs2294008) (**Fig. 3b**).

We additionally constructed plasmids expressing short or long PSCA protein (pcDNA3.1/S-PSCA and pcDNA3.1/L-PSCA, respectively; **Supplementary Fig. 5**). HEK293T or MKN1 cells transfected

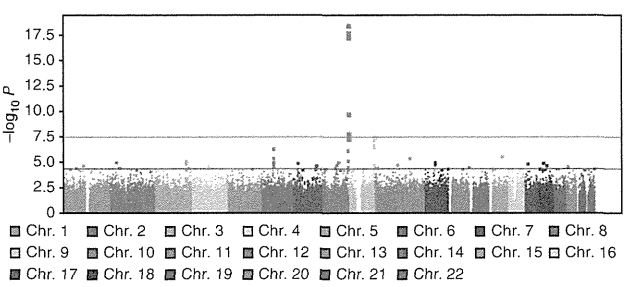

Figure 1 Manhattan plot showing genome-wide *P* values of association. The minimum *P* values under three genetic models (additive, recessive and dominant) were obtained by logistic regression analysis with adjustment for age and gender. The *y* axis shows the $-\log_{10} P$ values of 480,327 SNPs, and the *x* axis shows their chromosomal positions. Horizontal red and blue lines represent the thresholds of $P = 3.47 \times 10^{-8}$ for Bonferroni significance and $P = 5 \times 10^{-5}$ for selecting SNPs for replication, respectively.

Table 2 Results of association analyses of duodenal ulcer

SNP	Chr.	Stage	Allele 1/2[a]	Cases 11	Cases 12	Cases 22	Cases RAF	Controls 11	Controls 12	Controls 22	Controls RAF	Additive[b] P	Additive[b] OR	Dominant[b] P	Dominant[b] OR	Recessive[b] P	Recessive[b] OR	P^{c}_{het}
rs2294008	8q24	GWAS	C/T	252	473	318	0.468	2,984	9,947	8,758	0.367	2.92×10^{-18}	1.49	6.60×10^{-9}	1.50	2.84×10^{-19}	1.99	0.163
		Replication		1,387	2,735	1,866	0.460	503	1,665	1,456	0.369	2.11×10^{-17}	1.36	9.99×10^{-10}	1.38	6.60×10^{-16}	1.73	
		Combined[d]		1,639	3,208	2,184	0.461	3,487	11,612	10,214	0.367	1.79×10^{-33}	1.41	5.81×10^{-17}	1.42	3.92×10^{-33}	1.84	
rs505922	9q34	GWAS	T/C	389	451	203	0.589	6,425	10,758	4,504	0.544	1.60×10^{-5}	1.22	0.24	1.10	2.27×10^{-8}	1.45	0.053
		Replication		1,989	2,805	1,194	0.566	1,055	1,817	757	0.541	1.83×10^{-3}	1.12	0.23	1.08	1.78×10^{-4}	1.23	
		Combined[d]		2,378	3,256	1,397	0.570	7,480	12,575	5,261	0.544	3.43×10^{-7}	1.15	9.55×10^{-2}	1.09	1.15×10^{-10}	1.32	

We analyzed 7,035 duodenal ulcer cases (1,043 in the GWAS and 5,992 in replication) and 25,323 controls (21,694 in the GWAS and 3,629 in replication). Chr., chromosome; RAF, risk allele frequency.

[a]Allele 1, risk allele; allele 2, non-risk allele. [b]*P* values and ORs were calculated by logistic regression analysis, with age and gender as covariates. Non-risk alleles were considered as references in the three genetic models: additive, 1 versus 2; recessive, 11 versus 12 + 22; dominant, 11 + 12 versus 22. [c]Heterogeneity across the two stages was examined by Cochran *Q* test under a genetic model which provided the minimum *P* value in the screening stage. [d]ORs and *P* values were calculated using the Mantel-Haenszel fixed-effects model.

with either of these plasmids were stained with antibody to PSCA, with or without membrane permeabilization. We found that PSCA protein was localized to the plasma membrane only in cells transfected with pcDNA3.1/L-PSCA (**Fig. 3c** and **Supplementary Fig. 6a**). In addition, in protein blots, we observed that the band corresponding to long PSCA was actually 18 kDa rather than the predicted 13 kDa, whereas short PSCA was the predicted size. As the 18-kDa protein band for long PSCA was shifted to approximately 13 kDa by treatment with N-glycosidase (**Fig. 3d**), the modification accounting for its increased size was considered to be N-glycosylation. In addition, short PSCA was degraded through the ubiquitin proteasome pathway, which was inhibited by MG-132 treatment, whereas the long PSCA protein was relatively stable (**Fig. 3e** and **Supplementary Fig. 6b,c**). These findings show that the genetic variation in *PSCA* could have a considerable effect on the biological function of the PSCA protein by altering its subcellular localization and stability.

Of note, when we analyzed rs2294008 and rs505922 in 2,346 individuals with gastric cancer and 16,882 controls (**Table 1**), we found that rs2294008 had opposing effects on gastric cancer and duodenal ulcer risk. Whereas the C allele of rs2294008 increased the risk of duodenal ulcer (OR = 1.84) in a recessive model, it showed a protective effect for gastric cancer, as reported previously[1] ($P = 6.79 \times 10^{-12}$ and OR = 0.79 in an additive model; **Supplementary Table 8**). We also estimated the population attributable risk (PAR) of rs2294008 to be as high as 23.0% for duodenal ulcer (C allele) and 39.2% for gastric cancer (T allele) in the Japanese population.

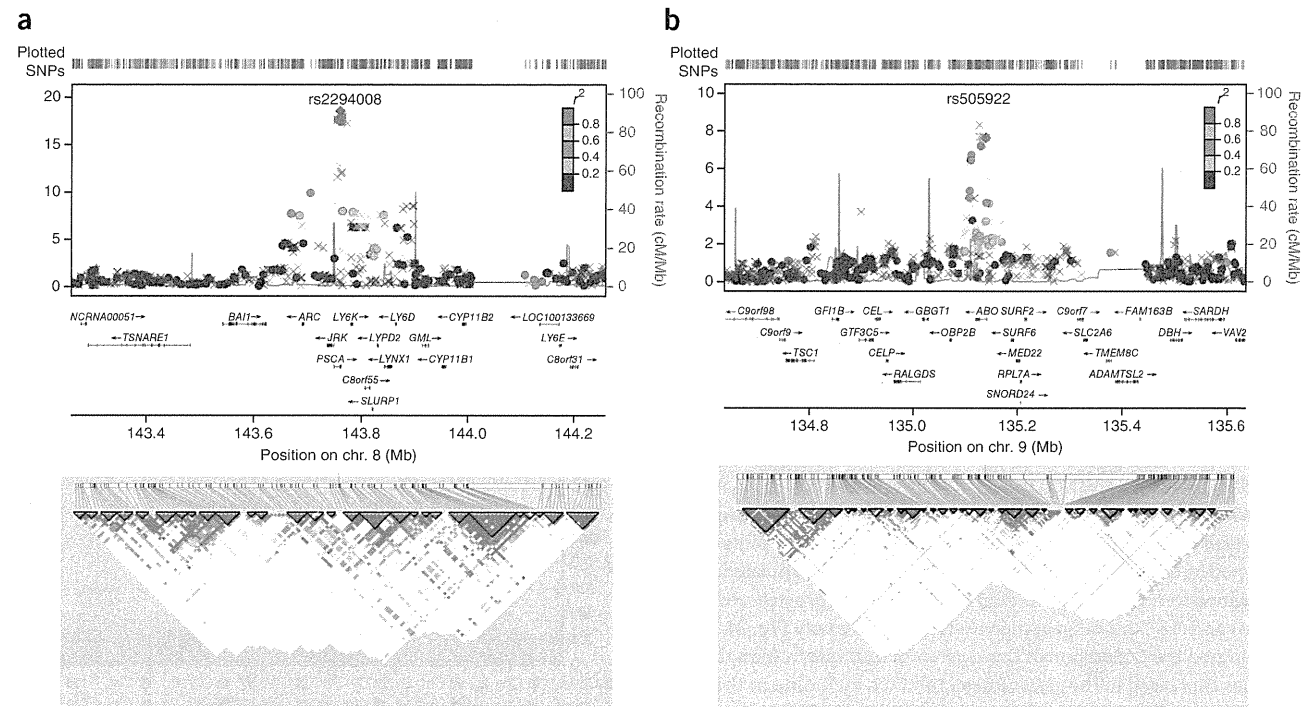

Figure 2 Regional association plots. (**a**,**b**) Data are shown for the associated regions on chromosome 8 including rs2294008 (in *PSCA*) (**a**) and on chromosome 9 including rs505922 (in *ABO*) (**b**). Upper, *P* values of genotyped SNPs (circle) and imputed SNPs (crosses) are plotted (as $-\log_{10} P$) against their physical position on the chromosomes (NCBI Build 36). Through imputation analyses, we obtained genotypes for 527 and 580 SNPs in addition to 155 and 219 genotyped SNPs within the 1-Mb genomic regions surrounding the marker SNPs on chromosome 8 and 9, respectively. Estimated recombination rates from the HapMap Japanese in Tokyo (JPT) population show the local LD structure. The color of each SNP indicates LD with rs2294008 or rs505922 based on pairwise r^2 values from HapMap JPT data. Middle, gene annotations from the UCSC genome browser. Lower, LD map based on *D'* (coefficient of linkage disequilibrium) in the associated regions using genotyping results from 907 healthy control samples from the replication study.

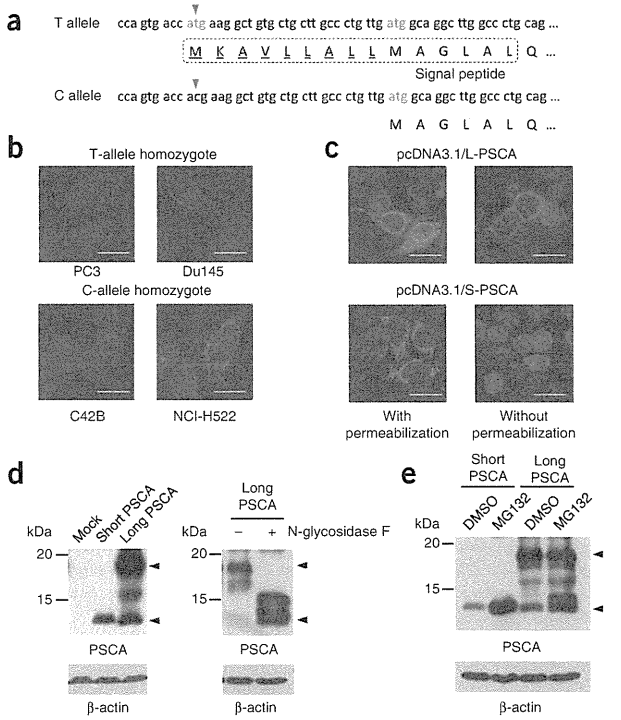

Figure 3 Effects of rs2294008 on subcellular localization and stability of PSCA protein. (**a**) The genomic structure around rs2294008 and corresponding amino-acid sequences. Arrowheads indicate the location of rs2294008. Potential start codons are shown in red. Underlined amino acids are unique to the T allele of rs2294008. (**b**) Representative images of cells stained with antibody to PSCA. PC3 and Du145 cells are homozygous for the T allele, whereas C42B and NCI-H522 cells are homozygous for the C allele of rs2294008. Scale bars, 20 μm. (**c**) HEK293T cells were transiently transfected with the indicated plasmid. Subcellular localization of PSCA protein was evaluated with antibody to PSCA, either with or without membrane permeabilization. Scale bars, 20 μm. (**d**) Left, expression of PSCA protein in HEK293T cells after transfection with the indicated expression plasmids encoding PSCA or with empty vector (mock). β-actin was used to normalize expression levels. Right, lysate from HEK293T cells transfected with pcDNA3.1/L-PSCA treated with N-glycosidase F. (**e**) After transfection with pcDNA3.1/L-PSCA or pcDNA3.1/S-PSCA vector, HEK293T cells were incubated with 10 μM of MG132 for 10 h before harvesting. DMSO was used as control. β-actin was used to normalize expression levels.

In contrast, rs505922 showed no association with gastric cancer in our analysis, which is discordant with previous reports[20,21].

On the basis of our findings, we propose that susceptibility to duodenal ulcer and gastric cancer is influenced by genetic variation in *PSCA* through a growth-promoting effect of the T allele and an effect on T-cell activation by the C allele (**Supplementary Fig. 7**). We hypothesize that, in response to damage in mucosal cells in the duodenum, the tissue repair system is switched on by the aggregation of platelets and the release of growth factors, which is followed by the proliferation and migration of epithelial cells. PSCA is highly expressed in various cancer tissues, and cells treated with small interfering RNA (siRNA) targeting *PSCA* or with antibody against PSCA exhibited a substantially suppressed growth[22,23], indicating a role for cell surface PSCA in cell proliferation. Our findings suggest that individuals with homozygous C alleles might have insufficient epithelial proliferation to counteract the damage because of a lack of functional cell surface PSCA, causing slow recovery from duodenal tissue damage.

A possible alternative mechanism involves antigen presentation. PSCA-derived peptides were reported to be a target of T-cell–based immunotherapy for advanced prostate cancer[24]. Our findings suggest that cytosolic short PSCA protein is likely to be more susceptible to proteasomal degradation than the long PSCA protein at the cell surface. Peptides presented by the human leukocyte antigen (HLA) molecules induce activation of $CD4^+$ and/or $CD8^+$ T cells, which were shown to be involved in peptic ulcer[25] and which also inhibit tumor formation. Therefore, individuals with a C allele might have higher risk for duodenal ulcer and lower risk for gastric cancer as a result of accelerated proteasomal degradation of PSCA protein and subsequent activation of immune responses. Thus, our findings could partially explain why individuals with duodenal ulcer have a low risk for gastric cancer.

We also found that individuals with homozygous T alleles of rs505922 have significantly higher risk for duodenal ulcer. This SNP is located within the *ABO* gene, which encodes a glycosyltransferase. The synthesis of blood group ABH antigens is determined by variations in the *ABO* gene[26]. An analysis of 94 subjects showed that the T allele of rs505922 was in strong linkage disequilibrium (LD; $r^2 = 0.97$) with the *ABO* gene encoding the O blood type, which produces nonfunctional protein due to a single-nucleotide deletion in codon 87 (rs8176719), concordant with a previous report[27]. By using the genotyping results of two tagging SNPs, through which *ABO* alleles can be inferred (rs505922 and rs8176746; **Supplementary Table 9**)[27], we could successfully determine the ABO blood type in 98.6% of samples[28] (**Supplementary Table 10**). Association analysis showed that individuals with blood type O exhibited significantly higher risk for duodenal ulcer than those with blood type A ($P = 2.04 \times 10^{-6}$; OR = 1.43; **Supplementary Table 10**). In contrast, blood type B was associated with a lower risk of intestinal-type gastric cancer than blood type A ($P = 0.019$; OR = 0.85; **Supplementary Table 11**). Even though the extent of association was slightly different between the GWAS (OR = 1.45) and replication (OR = 1.23) samples for rs505922, our findings are consistent with previous epidemiological studies showing an association between the O blood type and duodenal ulcer[29]. Taken together, our data suggest that ABO blood type could be a marker for duodenal ulcer susceptibility.

Recent GWAS have identified association of the *ABO* gene with various diseases, such as pancreatic cancer[30] and myocardial infarction[31]. In addition, the severity of infectious diseases caused by *Escherichia coli* O157 or *Vibrio cholera* has been linked to ABO blood type[32]. ABH antigens are highly expressed in gastrointestinal epithelium[14], and the South American *H. pylori* strain P466 was shown to bind to the H antigen but not to the A antigen[33]. However, the absence of correlation between *H. pylori* infection and ABO blood groups has also been reported[34]. Therefore, further analyses are necessary to fully elucidate the role of the *ABO* gene in the development of duodenal ulcer.

We also investigated the association between previously reported genes and duodenal ulcer using samples from the GWAS stage (**Supplementary Table 12**). Of the 27 SNPs analyzed, 4 at the *VEGFA*, *IL6* and *COX1* loci showed suggestive associations ($P < 0.05$), although these associations were not significant after Bonferroni correction ($P < 0.0019$).

Through the analysis of 7,035 duodenal ulcer cases and 25,323 controls, we have demonstrated a role for two genetic variants in the development of duodenal ulcer. Of note, genotype frequencies for the risk alleles of rs2294008 and rs505922 in the Japanese population are lowest among the 11 HapMap populations (14.2% and 31.8%, respectively; **Supplementary Table 13**). Taken together, our findings provide new

insight into the molecular mechanism responsible for the lower risk of gastric cancer among individuals with duodenal ulcer and the lower incidence of duodenal ulcer in the Japanese population.

URLs. BioBank Japan Project participating hospitals (in Japanese), http://biobankjp.org/plan/member_hospital.html; R, http://www.r-project.org/; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/; Primer3, http://frodo.wi.mit.edu/; LocusZoom, http://csg.sph.umich.edu/locuszoom/.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

C.T., Y.N. and K. Matsuda conceived and designed the study. Y.U., K. Matsuo and M.K. performed genotyping. A.T. and N.K. performed quality control analysis for the GWAS. Y.N., K. Matsuda and M.K. managed DNA samples belonging to BioBank Japan. H.I. and K.T. managed DNA samples from the Aichi Cancer Center. C.T. analyzed and summarized all the results. C.T., Y.N. and K. Matsuda wrote the manuscript. Y.N. obtained funding for the study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Sample collection. We obtained DNA from 7,035 subjects with duodenal ulcer from the BioBank Japan Personalized Medicine Project of the Ministry of Education, Culture, Sports, Science and Technology of Japan[35]. In the BioBank Japan Project, DNA and serum of subjects were collected through a collaborating network of 66 hospitals throughout Japan (see **Supplementary Note**). Clinical information on subjects, including drug history, was obtained from clinical records of each participating hospital. More than 200,000 individuals with 47 diseases, irrespective of prior treatment, were enrolled in this project from 2003 through 2009. A list of participating hospitals is provided at the BioBank Japan website (see URLs). A total of 25,323 control samples without duodenal ulcer were obtained from BioBank Japan or from healthy volunteers from the Midosuji Rotary Club in Osaka, Japan. Controls for the GWAS included subjects with colon, breast, prostate, lung, pancreatic or liver cancer, diabetes, myocardial infarction, brain infarction, arteriosclerosis obliterans, atrial fibrillation, cholangiocarcinoma, drug eruption, liver cirrhosis and amyotrophic lateral sclerosis. Controls for the replication analysis included healthy volunteers and individuals with chronic hepatitis B or C, cervical, esophageal, hematological or ovarian cancer, pulmonary tuberculosis, keroidosis and endometriosis. Controls for gastric cancer included subjects with diabetes, myocardial infarction, brain infarction, arteriosclerosis obliterans, atrial fibrillation, drug eruption, liver cirrhosis and amyotrophic lateral sclerosis. A total of 37 individuals with *H. pylori*–positive duodenal ulcer or 793 healthy controls were randomly selected from non-cancer outpatient visitors to the Aichi Cancer Center Hospital in Nagoya, Japan between January 2001 and November 2005 (ref. 36). *H. pylori* infection status was confirmed by determining plasma levels of IgG to the bacterium with a commercially available direct ELISA kit (Eiken Kagaku). Positivity for *H. pylori* infection was defined by *H. pylori*–specific IgG levels of greater than 10 U/ml in serum. We excluded subjects with gastric cancer or gastric ulcer from all controls. We excluded individuals treated with nonsteroidal anti-inflammatory drugs or steroid hormones from both duodenal ulcer cases and controls. We excluded subjects with a history of any cancer or peptic ulcer from the controls for gastric cancer. All subjects were Japanese and provided written informed consent. The clinical and demographic details of the samples are summarized in **Table 1**. This research project was approved by the ethical committees of The University of Tokyo, RIKEN and the Aichi Cancer Center.

SNP genotyping and quality control. In the GWAS stage, 1,043 duodenal ulcer and 21,694 controls were genotyped using Illumina HumanHap610-Quad BeadChip arrays. From the 101 SNPs from 42 distinct genomic regions with $P < 5 \times 10^{-5}$ in the GWAS stage, we selected the most strongly associated SNPs from each genomic region for the replication stage. Because we failed to design probes for rs1051631 and rs16896391, we selected rs10516733 and rs7775478 instead. In the replication stage, we genotyped 5,992 independent duodenal ulcer cases and 3,629 controls without peptic ulcer using the multiplex PCR-based Invader assay (Third Wave Technologies) and Illumina HumanHap550v3 BeadChip arrays, respectively. Because we failed to obtain genotype data for rs2240458 with Illumina HumanHap550v3, the genotyping results for rs2240458 in the replication control samples were imputed using a hidden Markov model. *H. pylori*–positive duodenal ulcer cases and healthy controls from the Aichi Cancer Center were genotyped by TaqMan assay. We observed 100% concordance between results from direct sequencing and those from Invader assays, Illumina BeadChips and TaqMan assays when we analyzed 94 samples for the two significantly associated SNPs (rs505922 and rs2294008). We performed a standard quality control procedure to exclude SNPs with low call rate (<99%), *P* value for the Hardy-Weinberg equilibrium test of $<1.0 \times 10^{-7}$ for controls and minor allele frequency (MAF) of < 0.01. In total, we obtained 480,327 SNPs for analysis in the screening stage.

Statistical analysis. The association of SNPs with the phenotype in the GWAS, replication and combination analyses was tested by multivariate logistic regression analysis with adjustment for age at recruitment and gender, by assuming an additive, dominant or recessive model using PLINK[37]. At the GWAS stage, the genomic inflation factor λ was derived by applying *P* values from logistic regression in an additive model for all the tested SNPs. Quantile-quantile plots were drawn using the R program. Odds ratios were calculated by considering the non-risk allele as a reference, unless otherwise stated. Significance levels after Bonferroni correction for multiple testing were $P = 3.47 \times 10^{-8}$ $(0.05/(480{,}327 \times 3))$ in the GWAS stage and $P = 1.19 \times 10^{-3}$ (0.05/42) in the replication stage, under a genetic model that provided the minimum *P* value in the screening stage. Heterogeneity across two stages was examined by the Cochran *Q* test[38]. We calculated the population attributable risk by $\mathrm{PAR} = (f_{\mathrm{homo}}(\mathrm{OR}_{\mathrm{homo}} - 1) + f_{\mathrm{hetero}}(\mathrm{OR}_{\mathrm{hetero}} - 1))/(1 + f_{\mathrm{homo}}(\mathrm{OR}_{\mathrm{homo}} - 1) + f_{\mathrm{hetero}}(\mathrm{OR}_{\mathrm{hetero}} - 1))$, where *f* is the frequency in the control subjects. PAR is defined as the reduction in incidence that would be achieved if the population had been entirely unexposed.

Imputation analysis. Sequences from the GWAS samples or replication controls that were not genotyped were imputed using a hidden Markov model in MACH[39] and HapMap JPT data (release 27). We applied the same quality control procedure as in the GWAS stage. For all SNPs that passed the quality control criteria, association was tested by logistic regression analysis with adjustment for age and gender.

Cell culture and transfections. Cell lines were purchased from the American Type Culture Collection, Lonza Biologics or the Japanese Collection of Research Bioresources. Cells were transfected with plasmids using FuGENE6 (Roche). For the analysis of proteasomal degradation of PSCA protein, cells were treated with 10 μM of proteasome inhibitor MG132 (Calbiochem) for 10 h before harvest.

Plasmid construction. cDNA fragments of *PSCA* were amplified and cloned into the pcDNA3.1 vector (Invitrogen). Plasmid structure is shown in **Supplementary Figure 5**. The primer sequences for cloning are given in **Supplementary Table 14**.

Quantitative RT-PCR. PolyA RNA or total RNA for normal tissues was purchased from Calbiochem and BioChain. Most of these samples were pooled RNA from multiple individuals; only duodenal tissue was derived from a single individual carrying homozygous C alleles at rs2294008 and homozygous C alleles at rs505922 (estimated from cDNA sequence at rs2294008 and rs8176719). cDNA was synthesized with the SuperScript Preamplification System (Invitrogen). Quantitative RT-PCR was conducted using the SYBR Green I Master on a LightCycler 480 (Roche). The primer sequences are given in **Supplementary Table 14**.

Protein blotting. To prepare whole-cell extracts, cells were collected and lysed in SDS sample buffer with sonication and centrifuged at 16,000*g* for 15 min. For treatment with N-glycosidase, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP-40 and 1 mM PMSF) and incubated with 5 U of N-glycosidase F (Calbiochem) at 37 °C for 20 h. Samples were subjected to SDS-PAGE, and immunoblotting was performed using standard procedures.

Antibodies. Polyclonal antibodies to PSCA (NB100-91938 and ab64919) were purchased from Novus Biologicals and Abcam, respectively. Monoclonal antibody to β-actin (clone AC15) was purchased from Sigma.

Immunocytochemistry. To select cell lines for immunocytochemistry, we analyzed *PSCA* mRNA expression and genotype at rs2294008 in a total of 44 cell lines (40 lung cancer and 4 prostate cancer cell lines). From these, we selected seven (three lung cancer and four prostate cancer cell lines) that were homozygous (or hemizygous) for the T or C allele at rs2294008 and expressed high levels of *PSCA* mRNA. We detected PSCA protein in four cell lines (PC3, Du145, C42B and NCI-H522) by immunocytochemistry and have presented the result for these cell lines. Immunocytochemistry was performed as previously described[40]. For staining of endogenous PSCA protein, cells were fixed using methanol. For cell surface staining, cells were labeled under nonpermeabilizing conditions using buffer without Triton X-100.

ABO blood type. In this study, we examined three SNPs at the *ABO* gene (rs505922, rs8176719 and rs8176746). These SNPs have been shown

to be tagging SNPs for the ABO blood type in the Japanese population[21]. Single-nucleotide deletion in exon 6 (rs8176719) affecting at amino-acid position 87 results in the O blood type, and a C or A at position 796 in exon 7 (rs8176746) distinguishes the B from the A blood type, respectively. We also confirmed strong LD between rs505922 and rs8176719 ($r^2 = 0.97$) by genotyping both SNPs in 94 individuals. Thus, we can deduce ABO blood types based on the genotypes of rs505922 and rs8176746 (**Supplementary Table 9**).

Software. For general statistical analysis, we employed the R statistical environment version 2.6.1 or PLINK-1.06. Haploview software version 4.2 (ref. 41) was used to calculate LD and to draw Manhattan plots. The Primer3 v0.3.0 web tool was used to design primers. We employed LocusZoom to plot regional association plots.

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Serum level of adiponectin and the risk of liver cancer development in chronic Hepatitis C patients

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Obesity and metabolic syndrome are recognized risk factors for development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C (CHC). Dysregulation of adipokines, particularly the decreased secretion of adiponectin, appears to play a key role. To investigate the association between adiponectin and hepatocarcinogenesis, we conducted a large-scale retrospective cohort study. We enrolled 325 patients with CHC (146 men, 179 women; mean age 58.0 ± 10.3 years) whose serum samples were collected between January 1994 and December 2002. Subjects were divided into two groups according to their serum adiponectin levels. We evaluated the association between adiponectin level and the risk of subsequent HCC development using univariate and multivariate Cox proportional hazard regression. Because average serum adiponectin level was higher in females than males, each gender was analyzed separately. Patients with CHC had significantly higher adiponectin levels than healthy controls. During the follow-up period (mean: 9.0 years), HCC developed in 122 subjects. Unexpectedly, subjects with higher serum adiponectin levels had a higher incidence of HCC (males: $p = 0.032$; females: $p = 0.01$; log-rank test). Multivariate analysis revealed that a high serum adiponectin level was independently associated with HCC development (hazard ratio [HR] = 2.07; $p = 0.031$ in females and HR = 1.82; $p = 0.05$ in males). Isoform analysis revealed that middle- and low-molecular-weight isoforms contributed to the risk of HCC. In conclusion, Patients who had CHC with high serum adiponectin levels had a higher risk of liver cancer development. Adiponectin may thus be tumorigenic or indicate a liver disease state independently of other clinical parameters.

Epidemiology

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with an increasing incidence globally.[1,2] Recently, obesity and metabolic syndrome were shown in several epidemiologic studies to increase the risk of HCC.[3–5] Because the prevalence of obesity and metabolic syndrome has been increasing in both Japan and Western nations, a possible association between obesity and hepatocarcinogenesis has attracted considerable attention in recent years.

The mechanism by which obesity and metabolic syndrome promote hepatocarcinogenesis remains not fully understood. However, obesity-induced dysregulation of adipokines, cytokines secreted by adipose tissue, is considered to play a key role.[6,7] Adipose tissue controls the functions of other organs through the secretion of various adipokines such as leptin, adiponectin, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and resistin. Obesity with visceral fat accumulation increases the levels of leptin, TNFα, IL-6, and resistin, and decreases adiponectin levels.[6,7] These adipokines flow directly into the liver through the portal vein and exert a variety of effects on liver diseases.[8]

Adiponectin, one of the major adipokines, possesses anti-inflammatory and insulin-sensitizing properties, and levels typically decline with increasing body weight.[9] Hypoadiponectinemia has been implicated in the development of obesity-related morbidities such as dyslipidemia and cerebrovascular disease.[10–12] In addition, hypoadiponectinemia has been reported to enhance hepatic steatosis, inflammation, fibrosis,

Key words: hepatocellular carcinoma, carcinogenesis, chronic hepatitis C, adiponectin

Abbreviations: AFP: alpha fetoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; CHC: chronic hepatitis C; CI: confidence interval; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HR: hazard ratio; IL-6: interleukin-6

Additional Supporting Information may be found in the online version of this article.

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and hepatocarcinogenesis in animal liver disease models.[13–15] This hypothesis is considered to be applicable to human liver disease, especially nonalcoholic steatohepatitis (NASH). Indeed, reduced adiponectin levels were found in patients with NASH and were associated with increased steatosis and necroinflammation in the liver.[16]

Chronic hepatitis C virus (HCV) infection is a major cause of HCC in the United States, southern European countries, and Japan.[2] Obesity and metabolic syndrome have been found to be associated with hepatocarcinogenesis in chronic hepatitis C (CHC) as well as in NASH,[5,17] and hypoadiponectinemia may be implicated in HCV-related hepatocarcinogenesis. Although some studies reported that the serum adiponectin level was associated with viral load, genotype, response to antiviral therapy, insulin resistance, and liver histology such as steatosis, inflammation, and fibrosis in CHC, such associations remain controversial.[12,18–24] There are also conflicting results as to whether HCV infection itself affects serum adiponectin levels.[22,23] Furthermore, only a few clinical studies were designed to investigate the role of adiponectin in viral hepatitis-related hepatocarcinogenesis.[12,25]

Based on previous reports, we hypothesized that adiponectin may have a role in ameliorating disease severity and that hypoadiponectinemia may be a risk factor for future HCC development in patients with CHC. To examine this hypothesis, we conducted a large-scale retrospective cohort study seeking to elucidate any association between serum adiponectin levels and risk of hepatocarcinogenesis in patients with CHC.

Material and Methods

Patients

Between January 1994 and December 2002, 1428 HCV RNA-positive patients, excluding those with (or with a history of) HCC, visited the liver clinic of the Department of Gastroenterology at the University of Tokyo Hospital. Patients whose serum samples were collected after informed consent was given were enrolled in the study. Exclusion criteria were the following: positivity for hepatitis B surface antigen, presence of infections in addition to HCV, presence of biliary disease, and ongoing interferon therapy at the time of serum collection. Patients who visited the hospital for consultation only were also excluded. Patients' history of interferon therapy and their responses to it were investigated during the follow-up period. Patients who achieved a sustained virologic response, defined by undetectable HCV-RNA at least 24 weeks after the end of therapy, were also excluded. Furthermore, we excluded patients who developed HCC within 1 year of serum collection to rule out the possibility of occult HCC. In total, 325 patients were enrolled, and the association between serum adiponectin levels at entry and the subsequent incidence of HCC was analyzed. Although no information on whether serum samples were taken under fasting conditions was available, the serum adiponectin level has been reported to undergo no meal-related or circadian changes.[26,27] Therefore, we decided that these samples were appropriate for our study. All blood tests were performed at the time of serum collection. HCV RNA was measured using the Amplicor HCV assay version 1 (Roche, Tokyo, Japan) and HCV serotypes was examined using a serotyping assay (SRL, Tokyo, Japan). In patients who did not undergo liver biopsy, clinical cirrhosis was diagnosed based on the presence of clinical and laboratory features of portal hypertension (the presence of esophageal varices and/or collateral circulation at endoscopy and ultrasonography).[28] Control serum samples were collected from 70 age- and gender-matched healthy subjects in whom liver diseases were ruled out, recruited from the Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of the authors' institution.

Follow-up and HCC diagnosis

Each subject was screened for HCC with ultrasonography at or immediately after the first visit, and those in whom HCC was detected were excluded from the study. Afterward, patients were followed-up every 3–6 months at the outpatient clinic, when blood tests including tumor markers and ultrasonography were carried out. Contrast-enhanced computed tomography was performed when HCC was suspected based on ultrasonography, and/or the serum α-fetoprotein (AFP) level showed an abnormal increase. HCC was diagnosed by dynamic computed tomography, and hyperattenuation in the arterial phase with washout in the late phase was considered a definite sign of HCC. When diagnosis of HCC was ambiguous, ultrasound-guided tumor biopsy was performed and a pathologic diagnosis was made based on the Edmondson and Steiner criteria. Time to HCC occurrence was defined as the interval between the date of serum collection and the diagnosis of HCC. Patients were censored at the time of death without HCC development, the last visit when lost to follow-up, or the end of the study period. The last observation in our study was taken on January 31, 2009.

Assay for adiponectin and high-molecular-weight adiponectin

Serum samples were stored at −70°C until required. Adiponectin levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. High-molecular-weight (HMW) adiponectin levels were measured in a commercial laboratory (SRL Inc.). Middle-plus low-molecular-weight (MLMW) adiponectin levels were calculated as the difference between the levels of total adiponectin and HMW adiponectin.

Table 1. Baseline characteristics

Variable	All (n = 325)	Male (n = 146)	Female (n = 179)
Age (years)[1]	60 (52–65)	60 (51–66)	60 (53–65)
Platelet count ($\times 10^3/\mu l$)[1]	147 (106–187)	148 (109–182)	144 (105–193)
Total bilirubin level (mg/dl)[1]	0.7 (0.5–0.9)	0.7 (0.6–0.9)	0.6 (0.5–0.8)
Serum Albumin level (g/dl)[1]	4.0 (3.8–4.2)	4.0 (3.8–4.2)	4.0 (3.8–4.2)
AST level (IU/l)[1]	53 (36–81)	54 (42–76)	52 (32–83)
ALT level (IU/l)[1]	59 (33–96)	65 (47–100)	51 (30.5–92.5)
AFP level ng/ml[1]	5.0 (3.0–11)	6.0 (3.0–11.4)	5.0 (3.5–10.5)
Prothrombin time activity (%)	85.5 (74.3–100)	85.7 (73.8–97.4)	85.1 (74.4–100)
Drinking >50 g/day, n (%)	46 (14.2)	42 (28.8)	4 (2.2)
BMI (kg/m^2)[1]	22.5 (20.4–24.6)	22.7 (20.9–24.6)	22.3 (20.3–24.7)
Diabetes mellitus, n (%)	38 (11.7)	23 (15.8)	15 (8.4)
HCV serotype 1, n (%)	241 (74.2)	113 (77.3)	128 (71.5)
Patients who received IFN, n (%)	49 (15.1)	21 (14.4)	28 (15.6)

[1]Expressed as median (25th–75th percentiles).

Immunohistochemistry

Liver biopsy samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, and then sectioned. For immunohistochemistry, liver biopsy samples were deparaffinized and incubated overnight at 4°C with antiadiponectin antibodies (Abcam, Cambridge, UK). Binding of the primary antibody was detected with antirabbit IgG antibody, followed by visualization with 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO). Expression in the samples was judged as weak or strong depending on the staining intensity assessed by a single observer, blinded to the clinical data.

Statistical analysis

Student's t-test was used to evaluate the differences in serum adiponectin levels between groups. Correlations between variables were analyzed using Spearman's rank correlation coefficient. A p value of less than 0.05 on a two-tailed test was considered significant. Cumulative HCC incidence was estimated using the Kaplan–Meier method, and the differences between groups were assessed with the log-rank test. In the analysis of risk factors for hepatocarcinogenesis, we tested the following variables obtained at the time of entry in univariate and multivariate Cox proportional hazard regression analysis: age, body mass index (BMI), heavy alcohol drinking (alcohol intake > 50 mg/day), serum albumin concentration, total bilirubin concentration, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, prothrombin activity, platelet count, AFP concentration, comorbidity with diabetes mellitus, and serum adiponectin level. To assess the importance of adiponectin isoforms in HCC development, we added HMW adiponectin level and MLMW adiponectin level to variables described above instead of total adiponectin, and performed multivariate Cox proportional hazard regression analysis with a step-wise selection procedure. Diagnosis of diabetes mellitus was based on medical history or a 75 g oral glucose tolerance test.[29] Data processing and analysis were performed using S-PLUS 2000 (MathSoft, Seattle, WA) and SAS Software version 9.1 (SAS Institute, Cary, NC).

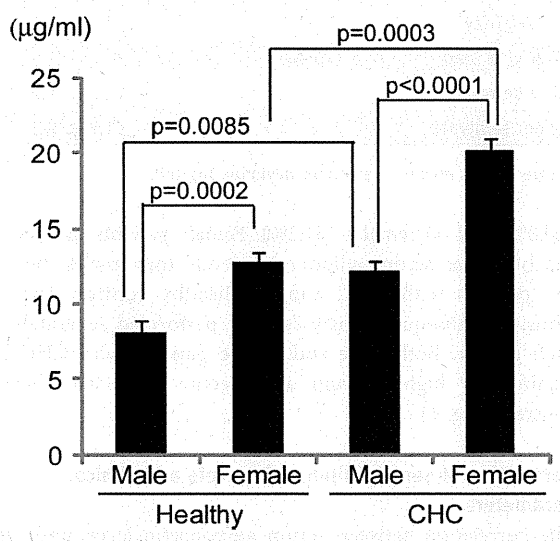

Figure 1. Serum adiponectin levels in healthy subjects (30 males, 40 females) and patients with CHC. Data are expressed as means ± standard error of the mean (SEM).

Results

Subject profile and serum adiponectin levels

In total, 325 subjects (146 males and 179 females; mean age: 58.0 ± 10.3 years) were included in the study. Detailed demographic data are shown in Table 1. Median BMI was 22.7 for males and 22.3 for females, and diabetes mellitus was present in 15.8% of the male and 8.4% of the female subjects. The subjects diagnosed as having cirrhosis, based on liver biopsy or clinical and laboratory features, were 45 males

Table 2. Correlations between serum adiponectin levels and other parameters

	Male			Female		
Variables	Spearman's rho	adiponectin	*p*	Spearman's rho	adiponectin	*p*
Age	0.325		<0.0001	0.224		0.003
Platelet count	−0.189		0.023	−0.127		0.089
Total bilirubin	0.033		0.77	0.05		0.46
Albumin	−0.152		0.059	−0.077		0.27
AST	0.115		0.17	0.05		0.5
ALT	0.012		0.89	−0.001		0.98
AFP	0.028		0.77	0.087		0.27
Prothrombin time	−0.12		0.12	−0.013		0.77
BMI	−0.392		<0.0001	−0.105		0.16
Diabetes mellitus						
Yes[1]		10.8 ± 8.2	0.22		15.0 ± 11.6	0.04
No[1]		12.4 ± 7.7			20.6 ± 6.5	
Drinking						
> 50 g/day[1]		11.2 ± 7.5	0.25		14.4 ± 9.9	0.28
≤ 50 g/day[1]		12.5 ± 7.8			20.2 ± 11.8	
HCV viral load	−0.049		0.56	0.071		0.35
HCV serotype 1		12.2 ± 7.2	0.83		20.3 ± 11.5	0.77
Other serotypes		11.9 ± 9.3			19.8 ± 12.3	

[1]Expressed as means ± standard deviation (μg/ml).

(30.8%) and 56 females (31.2%). Female patients had significantly higher serum adiponectin levels than males, both in the patients with CHC and the healthy controls (Fig. 1). Thus, all subsequent analyses were performed separately for each gender. Both male and female patients with CHC had significantly higher serum adiponectin levels than healthy controls (Fig. 1).

Correlation of serum adiponectin levels and clinical parameters

The correlation between serum adiponectin levels and other clinical factors was evaluated to elucidate the clinical relevance of serum adiponectin levels in patients with CHC (Table 2). In male subjects, the serum adiponectin level was correlated positively with age and negatively with platelet count and BMI. In female subjects, the serum adiponectin level was positively correlated with age and was lower in patients with diabetes mellitus. Platelet count showed a weak negative correlation. The serum adiponectin level did not correlate with hepatitis C viral factors, such as viral load or serotype.

Incidence of HCC stratified based on serum adiponectin levels

The mean follow-up period was 9 years. During this time, 19 (13.1%) male and 17 (9.5%) female subjects were lost to follow-up. By the end of the study follow-up period, HCC had developed in 122 subjects (67 males and 55 females). The cumulative incidence rates at 5 and 10 years were 31.5% and 42.0% (5.5% per person-year) in male and 17.3% and 29.3% (3.2% per person-year) in female subjects. Subjects were divided into two groups based on serum adiponectin levels, with the median value as the cutoff (10.5 μg/ml in male and 16.7 μg/ml in female subjects). Unexpectedly, both male and female subjects with high serum adiponectin had a significantly higher incidence of HCC (males, $p = 0.032$; females, $p = 0.01$; log-rank test; Fig. 2). In male subjects, the cumulative incidence rates at 5 and 10 years were 21.9% and 37.7% in the low, and 41.1% and 51.0% in the high adiponectin groups, respectively. In female subjects, the cumulative incidence rates at 5 and 10 years were 12.4% and 19.3% in the low, and 22.2% and 39.2% in the high adiponectin groups, respectively.

Risk analyses

Risk factors for HCC development were analyzed separately for each gender. In the univariate analyses, high serum adiponectin levels (> 10.5 μg/ml in males; > 16.7 μg/ml in females) was a significant risk factor for HCC in both male and female subjects (Table 3). Other significant risk factors for HCC included age, AFP level, and laboratory parameters indicative of more advanced liver disease such as serum albumin level. Heavy alcohol consumption and diabetes mellitus were significant risk factors in male subjects only, and higher BMI was a significant risk factor in female subjects only. In a multivariate proportional hazard regression analysis, a high

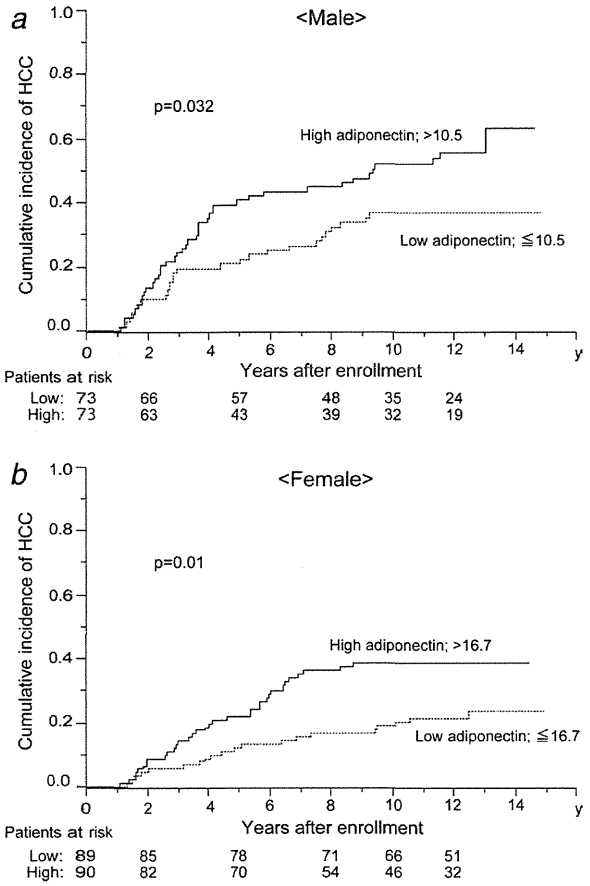

Figure 2. Cumulative incidence of HCC development stratified according to the median value of serum adiponectin for each gender: low (serum adiponectin concentration ≤10.5 μg/ml in males, ≤16.7 μg/ml in females) and high (>10.5 μg/ml in males, >16.7 μg/ml in females). (*a*) Male patients; (*b*) female patients.

adiponectin level was found to be an independent risk factor in female subjects, with a hazard ratio (HR) of 2.07 (95% confidence interval [CI]: 1.06–4.04; $p = 0.031$; Table 3). In male subjects, a high adiponectin level was correlated with HCC development at a borderline significance level, with a HR of 1.82 (95% CI: 1.00–3.33; $p = 0.050$). Age, prothrombin time, and AFP level were independent risk factors in both male and female subjects. Heavy alcohol consumption was an independent risk factor in male subjects only. BMI in females and diabetes mellitus in males were correlated with HCC at a borderline significance level, with HRs of 1.09 (95% CI: 0.99–1.19; $p = 0.061$) and 1.81 (95% CI: 0.96–3.44; $p = 0.066$), respectively.

The above analyses showed that both a higher BMI and higher adiponectin were risk factors for hepatocarcinogenesis, despite their negative correlation, so the relationship among BMI, adiponectin, and hepatocarcinogenesis was complex, especially in females. Thus, we investigated the contribution of adiponectin to hepatocarcinogenesis, stratified by BMI in females. Subjects with a higher adiponectin level had a much higher incidence of HCC in the overweight group (BMI > 25; $p = 0.0036$), whereas the difference was not significant in the non-overweight group (BMI ≤ 25; $p = 0.094$) (Supporting Information Figure 1). These results suggest that adiponectin might play an important role in hepatocarcinogenesis in CHC patients, especially in overweight patients.

Immunohistochemistry

To investigate the localization of adiponectin in the liver, we performed immunohistochemistry for adiponectin using liver biopsy samples. Of the 325 patients enrolled in our study, 64 underwent a liver biopsy around the same time as serum collection. From these, 35 paraffin-embedded samples were available (F 0-2, $n = 9$; F3, $n = 10$; F4, $n = 16$). Adiponectin was stained primarily in hepatocytes, and the staining intensity tended to be higher according to the progression of fibrosis (Supporting Information Figure 2A, B). Additionally, the serum adiponectin level was higher in patients with strong staining for adiponectin than in patients with weak staining (Supporting Information Figure 2C).

Assessment of adiponectin isoforms

Circulating adiponectin exists in several isoforms, including low- (trimer; LMW), middle- (hexamer; MMW), and high-molecular-weight (12- to 18-mer; HMW) forms, each of which may exert distinct functions.[9] Recent evidence suggests that HMW adiponectin is the more biologically active form with regard to insulin sensitivity.[30] In addition, the ratio of HMW adiponectin to total adiponectin (HMWR) was reported to be predictive of insulin resistance, metabolic syndrome, and cardiovascular disease.[31,32] To investigate the composition of adiponectin isoforms, we measured serum HMW adiponectin levels in female subjects with CHC compared to healthy controls because multivariate risk analyses revealed that serum adiponectin as a risk factor was more important in women than in men. Both HMW and MLMW adiponectin levels were significantly higher in patients with CHC than in the healthy controls (Fig. 3*a*) and significantly correlated with total adiponectin (Spearman's rho = 0.928; $p < 0.0001$, Spearman's rho = 0.985; $p < 0.0001$, respectively), whereas HMWR was significantly lower in patients with CHC (Fig. 3*b*). To assess the contribution of each component to HCC development, we reanalyzed the risk factors for HCC development using HMW adiponectin level and MLMW adiponectin level instead of total adiponectin. Patients were divided into two groups based on the median value of each parameter. Whereas high HMW adiponectin (> 5.96 μg/ml) and high MLMW adiponectin (> 10.6 μg/ml) were significant risk factors for HCC in the univariate analysis, only the high MLMW adiponectin level retained significance in a multivariate analysis (HR: 1.96; 95% CI: 1.06–3.60; $p = 0.029$; Supporting Information Table 1).

Epidemiology

Table 3. Risk factors for HCC development: univariate and multivariate analyses

Variable	Univariate analyses Hazard ratio (95% CI)	*p*	Multivariate analyses Hazard ratio (95% CI)	*p*
Male				
Age (per year old)	1.07 (1.04–1.10)	<0.0001	1.05 (1.02–1.09)	<0.0001
Platelet count (per $10^3/\mu l$)	0.987 (0.982–0.992)	<0.0001	0.995 (0.990–1.001)	0.13
Total bilirubin (per 0.1 mg/dl)	1.05 (1.00–1.12)	0.057	0.99 (0.92–1.07)	0.96
Serum albumin level (per 0.1 g/dl)	0.87 (0.81–0.93)	<0.0001	1.01 (0.94–1.09)	0.72
AST level (per 1 IU/l)	1.005 (1.001–1.008)	0.005	1.00 (0.99–1.02)	0.22
ALT level (per 1 IU/l)	1.002 (0.999–1.005)	0.21	0.99 (0.98–1.00)	0.41
AFP level > 10 ng/ml	3.58 (2.19–5.86)	<0.0001	2.99 (1.70–5.24)	0.0001
Prothrombin time activity (per 1%)	0.94 (0.92–0.96)	<0.0001	0.95 (0.93–0.97)	<0.0001
Drinking > 50 g/day	1.76 (1.07–2.89)	0.025	1.88 (1.04–3.37)	0.034
BMI (per 1 kg/m^2)	0.97 (0.90–1.04)	0.44	1.01 (0.93–1.09)	0.76
Diabetes mellitus (yes)	1.89 (1.06–3.37)	0.029	1.81 (0.96–3.44)	0.066
Adiponectin level > 10.5 μg/ml	1.69 (1.03–2.76)	0.034	1.82 (1.00–3.33)	0.050
Female				
Age (per year old)	1.12 (1.07–1.16)	<0.0001	1.11 (1.06–1.17)	<0.0001
Platelet count (per $10^3/\mu l$)	0.976 (0.969–0.982)	<0.0001	0.98 (0.97–0.99)	0.004
Total bilirubin (per 0.1 mg/dl)	1.15 (1.08–1.23)	<0.0001	0.86 (0.76–0.97)	0.015
Serum albumin level (per 0.1 g/dl)	0.82 (0.77–0.89)	<0.0001	0.94 (0.85–1.04)	0.27
AST level (per 1 IU/l)	1.008 (1.004–1.012)	<0.0001	0.99 (0.98–1.00)	0.32
ALT level (per 1 IU/l)	1.004 (1.001–1.007)	0.019	1.00 (0.99–1.01)	0.83
AFP level >10 ng/ml	10.51 (5.96–18.53)	<0.0001	4.85 (2.38–9.90)	<0.0001
Prothrombin time activity (per 1%)	0.91 (0.89–0.93)	<0.0001	0.94 (0.91–0.98)	0.018
Drinking >50 g/day	0.47 (0.17–1.30)	0.15	0.85 (0.33–2.20)	0.74
BMI (per 1 kg/m^2)	1.15 (1.08–1.23)	<0.0001	1.09 (0.99–1.19)	0.061
Diabetes mellitus (yes)	1.78 (0.84–3.77)	0.13	0.84 (0.35–1.99)	0.69
Adiponectin level >16.7 μg/ml	2.02 (1.16–3.50)	0.012	2.07 (1.06–4.04)	0.031

Association between serum adiponectin and serum IL-6 levels

Although adiponectin has often been suggested to have anti-inflammatory properties, recent studies have revealed that adiponectin exerts pro-inflammatory effects in immune cells through nuclear factor kappa B (NF-κB) activation and subsequent secretion of IL-6 and TNFα.[33,34] IL-6 is one of the most important pro-inflammatory cytokines in hepatocarcinogenesis,[35] and we previously reported that high serum IL-6 levels were correlated with future HCC development in patients with CHC using the same subject cohort as our study.[36] Thus, we investigated the correlation between serum adiponectin and IL-6 levels in female subjects, but none was found (Spearman's rho = −0.018; $p = 0.81$) (Supporting Information Table 2). In addition, HWM adiponectin and MLMW adiponectin levels showed no significant correlations with serum IL-6 levels (HMW; Spearman's rho = −0.059; $p = 0.42$ and MLMW; Spearman's rho = 0.006, $p = 0.93$, respectively) (Supporting Information Table 2). Multivariate analyses of the risk factors for HCC, including total adiponectin and IL-6, revealed that they were independent risk factors for HCC (adiponectin > 16.7 μg/ml: HR: 2.05; 95% CI: 1.04–4.03; $p = 0.035$ and IL-6: HR: 1.49; 95% CI: 1.03–2.16 per log unit increase; $p = 0.033$) (Supporting Information Table 3). These data suggest that IL-6 is likely not a major mediator of the association of adiponectin with hepatocarcinogenesis.

Discussion

Adiponectin is considered to be important in metabolic syndrome, and hypoadiponectinemia has been reported to be correlated with various diseases related to metabolic syndrome.[10–12] However, we found that patients with CHC having high serum adiponectin levels had a higher risk of developing HCC. To our knowledge, this is the first study reporting a positive association between serum adiponectin levels and future HCC development.

Adiponectin reportedly exerts its effects through interaction with two specific receptors, AdipoR1 and AdipoR2.[37] AdipoR1 is expressed in skeletal muscle and other tissues,

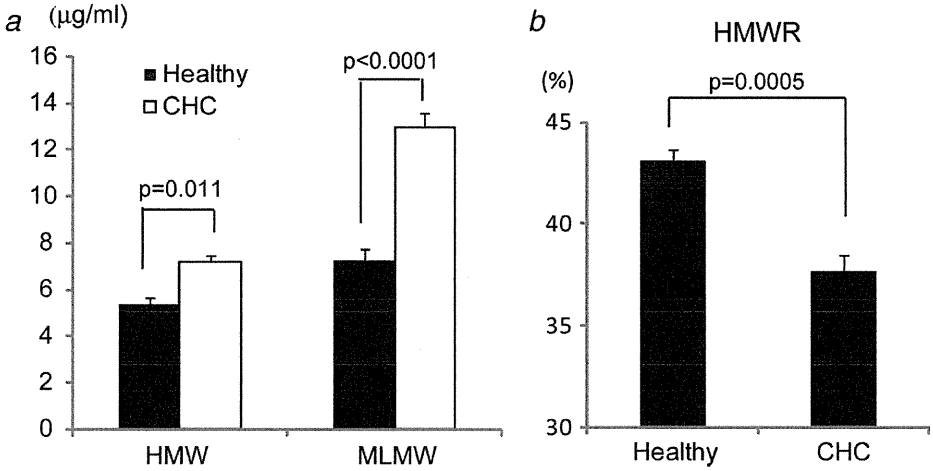

Figure 3. Assessment of adiponectin isoforms in healthy female subjects and patients with CHC. (*a*) Serum HMW and MLMW adiponectin levels; (*b*) ratio of HMW adiponectin to total adiponectin (HMWR). Data are expressed as means ± standard error of the mean (SEM).

whereas AdipoR2 is expressed primarily in the liver. AdipoR1 activates the AMP kinase (AMPK) pathway and AdipoR2 the peroxisome proliferator-activated receptor alpha (PPARα) pathway to increase insulin sensitivity and decrease inflammation.[30] However, recent studies have revealed that different forms of adiponectin may exert distinct functions. For example, HMW adiponectin is considered to play a crucial role in insulin sensitization, whereas MLMW adiponectin transverses the blood–brain barrier and activates AMPK in the hypothalamus, resulting in promotion of food intake.[38] Hui *et al.*[39] reported that serum MMW adiponectin levels were elevated in patients with chronic hepatitis B and declined markedly after antiviral therapy, particularly in patients with a virological response. We showed that both HMW adiponectin and MLMW adiponectin levels were elevated in patients with CHC, and a higher MLMW adiponectin level was an independent risk factor for HCC development. These findings suggest that an elevated MMW or LMW adiponectin level may represent a particular liver disease state, independently of other clinical parameters. However, both the HMW and MLMW adiponectin levels showed strong positive correlations with the total adiponectin level, so measuring the total adiponectin level may be sufficient for assessing the association between adiponectin and hepatocarcinogenesis.

Several studies have reported that serum adiponectin levels in patients with advanced liver fibrosis were elevated.[39] Because adiponectin is largely metabolized by the liver,[40] serum adiponectin concentration may increase due to decreased hepatic degradation. Serum adiponectin may represent a surrogate marker of liver fibrosis, as it was negatively correlated with platelet count in our study. However, the multivariate analysis revealed that high serum adiponectin was an independent risk factor of hepatocarcinogenesis, particularly in female subjects. A recent cross-sectional study reported that high serum adiponectin was independently correlated with HCC in patients with chronic hepatitis B.[25] Additionally, our immunohistochemical analysis revealed that adiponectin accumulated in the fibrotic liver, consistent with a previous report.[39] These data suggest that adiponectin, after accumulation in the fibrotic liver, may possess tumorigenic functions.

Adiponectin is often considered to have anti-inflammatory properties. However, elevated plasma adiponectin levels have recently been reported in several diseases associated with inflammation, such as arthritis,[41] preeclampsia[42] and end-stage renal disease.[43] Furthermore, high serum adiponectin was a significant predictor of progression of chronic kidney disease.[44] In the rodent liver injury model, adiponectin was induced by ischemia–reperfusion and exerted a harmful effect on the liver under certain circumstances.[45] In our study, serum adiponectin level was elevated in patients with CHC as compared to healthy controls, which is consistent with a previous report.[23] On the other hand, serum adiponectin was significantly lower in patients with NASH compared to healthy subjects (Nakagawa H, unpublished observation). Thus, adiponectin may play different roles in inflammatory or infectious diseases and in metabolic diseases, including NASH. We speculate that while hypoadiponectinemia may be the initiator in the pathogenesis of NASH, adiponectin is elevated in CHC due to fibrosis progression and subsequently modulates disease progression. No significant correlation between adiponectin and IL-6 levels in serum was found, although other inflammatory factors (such as TNFα) may contribute to the link between adiponectin and hepatocarcinogenesis. An alternative explanation for the association of high adiponectin levels with HCC development could be adiponectin resistance caused by downregulation of adiponectin receptor. However, a causal relationship between adiponectin and hepatocarcinogenesis was not evaluated here, so further study will be needed.

Recently, two studies described a protective role of adiponectin in HCC progression using HCC cell lines cultured *in*

vitro and an *in vivo* xenograft model, and these results may conflict with ours.[46,47] However, the administration of adiponectin to cancer cell lines or xenograft models can examine the direct effects of adiponectin on cancer cell proliferation, apoptosis, and metastasis, but not the role of adiponectin in the entire carcinogenesis process. Because HCC usually develops after chronic inflammation and fibrosis progression in CHC, the status of background liver disease is very important in carcinogenesis. Although adiponectin may have some protective effects against cancer cells, adiponectin may have potentially tumor-promoting effects, by modulating the surrounding environment, such as the inflammatory process. In fact, the serum adiponectin level has been reported to be positively correlated with histological inflammation of the liver.[19]

During preparation of this article, Nkontchou *et al.*[48] reported that the higher HOMA index but not serum adiponectin level was a risk factor for HCC development in cirrhotic patients with hepatitis C in univariate and multivariate analyses. A major difference in the study design between their study and ours is that all patients are diagnosed as cirrhosis by liver biopsy in their study, whereas patients clinically diagnosed as cirrhosis were about one-third of subjects in our study. Thus, the different results between two studies suggest that adiponectin may be a surrogate marker of severity of liver disease or play some roles in the progression of chronic hepatitis. On the other hand, the correlations of serum adiponectin levels and clinical parameters were similar in the two studies. The serum adiponectin level is higher in females than in males, while incidence of HCC is significantly higher in males than in females; thus, a gender-stratified analysis would show results similar to ours, at least when unadjusted. Besides, from the results of their study and ours, we can at least conclude that hypoadiponectinemia is not a major reason why obesity promotes hepatocarcinogenesis in patients with CHC.

Consistent with previous reports, high BMI and comorbidity with diabetes mellitus were both associated with a risk of HCC development in this patient cohort.[17] Although the mechanism by which obesity and metabolic syndrome promote hepatocarcinogenesis in patients with CHC remains unknown, the data presented herein suggest strongly that hypoadiponectinemia does not have a major role. Other factors such as leptin, TNFα, IL-6, resistin, insulin resistance-induced elevated insulin, and free insulin-like growth factor levels, or hepatic fat accumulation, may be involved in obesity-mediated hepatocarcinogenesis.

Our study has several limitations. First, because liver biopsy samples were obtained from only about one-fifth of subjects, assessing the relationship between serum adiponectin and liver histology was not sufficient. Second, an index of insulin resistance such as homeostatic model assessment (HOMA) was not available. The relationship between adiponectin and insulin resistance in CHC remains controversial and further studies are needed to clarify these points.

In summary, patients with CHC having high serum adiponectin levels had a higher risk of hepatocarcinogenesis. In female patients in particular, a high serum adiponectin level was shown to be an independent risk factor. In the assessment of adiponectin isoforms, an elevated serum MLMW adiponectin level was an important risk factor. Adiponectin may possess certain tumor-promoting functions or reflect a specific liver disease state independently of other clinical parameters.

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