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Large-scale genome-wide association studies in east Asians identify new genetic loci influencing metabolic traits

Young Jin Kim[1,14], Min Jin Go[1,14], Cheng Hu[2], Chang Bum Hong[1], Yun Kyoung Kim[1], Ji Young Lee[1], Joo-Yeon Hwang[1], Ji Hee Oh[1], Dong-Joon Kim[1], Nam Hee Kim[1], Soeui Kim[1], Eun Jung Hong[1], Ji-Hyun Kim[1], Haesook Min[1], Yeonjung Kim[1], Rong Zhang[2], Weiping Jia[2], Yukinori Okada[3,4], Atsushi Takahashi[3], Michiaki Kubo[3], Toshihiro Tanaka[3], Naoyuki Kamatani[3], Koichi Matsuda[5], MAGIC consortium[6], Taesung Park[7], Bermseok Oh[8], Kuchan Kimm[9], Daehee Kang[10], Chol Shin[11], Nam H Cho[12], Hyung-Lae Kim[1,13], Bok-Ghee Han[1], Jong-Young Lee[1] & Yoon Shin Cho[1]

To identify the genetic bases for nine metabolic traits, we conducted a meta-analysis combining Korean genome-wide association results from the KARE project (n = 8,842) and the HEXA shared control study (n = 3,703). We verified the associations of the loci selected from the discovery meta-analysis in the replication stage (30,395 individuals from the BioBank Japan genome-wide association study and individuals comprising the Health2 and Shanghai Jiao Tong University Diabetes cohorts). We identified ten genome-wide significant signals newly associated with traits from an overall meta-analysis. The most compelling associations involved 12q24.11 (near *MYL2*) and 12q24.13 (in *C12orf51*) for high-density lipoprotein cholesterol, 2p21 (near *SIX2-SIX3*) for fasting plasma glucose, 19q13.33 (in *RPS11*) and 6q22.33 (in *RSPO3*) for renal traits, and 12q24.11 (near *MYL2*), 12q24.13 (in *C12orf51* and near *OAS1*), 4q31.22 (in *ZNF827*) and 7q11.23 (near *TBL2-BCL7B*) for hepatic traits. These findings highlight previously unknown biological pathways for metabolic traits investigated in this study.

Various metabolic traits that can be measured in blood plasma have been targets for clinical studies because of their usefulness in assessing the risk for a wide range of diseases. The genetic bases for many such traits have been identified in large-scale genome-wide association studies (GWAS) conducted in European-ancestry and Japanese populations[1–3]. To discover new genomic loci associated with these traits, we conducted a two-stage association study in individuals of east Asian ancestry (**Fig. 1** and **Supplementary Table 1**).

In the discovery stage, we combined two Korean GWAS, the Korea Association Resource (KARE) study (n = 8,842)[4] and the Health Examinee (HEXA) shared control study (n = 3,703), for a meta-analysis of nine metabolic traits that can be detected in blood plasma including triglycerides (TG), high-density lipoprotein cholesterol (HDLc), low-density lipoprotein cholesterol (LDLc), fasting plasma glucose (FPG), albumin (ALB), blood urea nitrogen (BUN), gamma-glutamyl transpeptidase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). We performed a meta-analysis for about 2.0 million imputed and genotyped SNPs by a weighted-average method assuming fixed effects with inverse variance (**Supplementary Fig. 1**). The quantile-quantile plots of genome-wide P values showed deviations from the null distribution caused by strong associations observed for each quantitative trait (**Supplementary Fig. 2**). Excluding strong associations, the quantile-quantile plots showed that population stratification effects were negligible. The value of genomic inflation factors (with a range of 1.005–1.050) further supports the validity of ignoring the stratification in our study samples (**Supplementary Table 2**). From the discovery GWAS meta-analysis, we initially chose all lead signals (that is, with pairwise linkage disequilibrium statistics $r^2 < 0.2$ and minor allele frequency (MAF) ≥ 0.05 within a 500-kb window of the genomic region) with association to any of the nine metabolic traits at $P < 5 \times 10^{-8}$ as candidates to carry forward to the replication stage for further validation in the

[1]Center for Genome Science, National Institute of Health, Osong Health Technology Administration Complex, Chungcheongbuk-do, Korea. [2]Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Department of Endocrinology and Metabolism, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China. [3]Center for Genomic Medicine, RIKEN, Kanagawa, Japan. [4]Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan. [5]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. [6]A full list of members is provided in the **Supplementary Note**. [7]Department of Statistics, College of Natural Science, Seoul National University, Seoul, Korea. [8]Department of Biomedical Engineering, School of Medicine, Kyung Hee University, Seoul, Korea. [9]Merck Research Laboratories, External Scientific Affairs, Merck, Sharp & Dohme Korea, Seoul, Korea. [10]Department of Preventive Medicine, Seoul National University College of Medicine, Seoul, Korea. [11]Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Korea University Ansan Hospital, Ansan, Korea. [12]Department of Preventive Medicine, Ajou University School of Medicine, Suwon, Korea. [13]Department of Biochemistry, School of Medicine, Ewha Womans University, Seoul, Korea. [14]These authors contributed equally to this work. Correspondence should be addressed to Y.S.C. (yooncho33@korea.kr).

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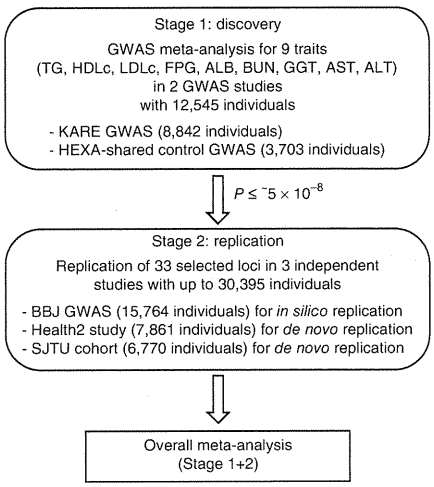

Figure 1 Overall study scheme.

different populations. After excluding imputed SNPs and including one SNP, rs4835265, with a *P* value that approached genome-wide significance ($P = 6.21 \times 10^{-8}$), we selected a total of 33 SNPs for replication (**Supplementary Table 3**).

The replication stage comprised up to 30,395 individuals of east Asian ancestry. We obtained *in silico* replication data for 15,764 individuals from the BioBank Japan GWAS[1] for all traits except FPG. We performed *de novo* replication in the Health2 cohort[4] with up to 7,861 individuals. Additionally, we conducted *de novo* replication in up to 6,770 individuals recruited from the Shanghai Jiao Tong University cohort (**Supplementary Table 1**) for four SNPs that were not genotyped in the Health2 samples for GGT and LDLc.

Our overall meta-analysis combining stage 1 and 2 data identified a total of 33 SNPs reaching genome-wide significance for any of the nine metabolic traits (**Supplementary Table 3**). Among these SNPS, 23 have been detected in the previous studies, whereas 10 are newly identified in this study (**Table 1** and **Fig. 2**). For HDLc association, two SNPs, rs12229654 on 12q24.11 and rs2074356 on 12q24.13, showed strong evidence of association. rs12229654 appears close to the *MYL2* (the myosin, light chain 2, regulatory, cardiac, slow gene) locus ($P_{overall} = 3.41 \times 10^{-23}$, effect size = −0.0280 ± 0.0028). *MYL2* encodes the regulatory light chain associated with the cardiac myosin β (or slow) heavy chain. rs2074356 ($P_{overall} = 6.95 \times 10^{-37}$, effect size = −0.0350 ± 0.0028) is located in *C12orf51*, which has been reported for association with waist-hip ratio[4].

We detected a new variant (rs895636) for FPG on chromosome 2p21 near *C2orf34*, *SIX2* (*SIX homeobox 2*) and *SIX3* (*SIX homeobox 3*) ($P_{overall} = 9.99 \times 10^{-13}$, effect size = 0.0393 ± 0.0055). This locus showed no evidence of association with FPG in the MAGIC consortium. This result might be because of the low allele frequency of the T allele in Europeans (0.20 in the MAGIC consortium and 0.38 in this study), which lowers the statistical power to detect the association even using a *P* value threshold of <0.05 (power = 0.31 in the MAGIC consortium). In addition, inter-ethnic differences in the linkage disequilibrium (LD) pattern we observed by regional association plots and LD plots in this region (**Supplementary Fig. 3a**) could contribute to the failure to detect an association for this variant in the MAGIC consortium. The inter-population variation in LD pattern in the *SIX2-SIX3* locus was further shown by a VarLD plot[5] generated using phase 2 HapMap CHB-JPT and CEU samples (**Supplementary Fig. 3b**). Because the lead signal is possibly a proxy of the causal locus,

Table 1 Association results for metabolic traits that reached genome-wide significance (overall meta $P < 5 \times 10^{-8}$)

							Discovery meta-analysis (KARE+HEXA shared control)			Replication meta-analysis (BBJ+Health2+SJTU)			Overall meta-analysis (Discovery+Replication)			
Trait	Locus	Position	Lead SNP[a]	Candidate gene	Allele[b]	MAF	Effect size[c]	*P*	Sample size	Effect size[c]	*P*	Sample size	Effect size[c]	*P*	Sample size	P_{het}
Plasma lipid traits																
HDLc	12q24.11	109,898,844	rs12229654	*MYL2*	G/T	0.14	−0.0284 ± 0.0041	3.20×10^{-12}	12,394	−0.0277 ± 0.0039	1.17×10^{-12}	13,784	−0.0280 ± 0.0028	3.41×10^{-23}	26,178	9.02×10^{-1}
	12q24.13	111,129,784	rs2074356	*C12orf51*	T/C	0.15	−0.0338 ± 0.0040	2.31×10^{-17}	12,502	−0.0360 ± 0.0038	3.59×10^{-21}	13,784	−0.0350 ± 0.0028	6.95×10^{-37}	26,286	6.90×10^{-1}
Glycemic trait																
FPG	2p21	45,041,857	rs895636	*SIX2-SIX3*	T/C	0.38	0.0392 ± 0.0070	1.87×10^{-8}	11,043	0.0395 ± 0.0090	1.13×10^{-5}	6,574	0.0393 ± 0.0055	9.99×10^{-13}	17,617	6.69×10^{-1}
Renal-function–related traits																
ALB	19q13.33	54,691,821	rs2280401	*RPS11*	A/G	0.17	0.0312 ± 0.0051	6.43×10^{-10}	12,541	0.0267 ± 0.0067	6.82×10^{-5}	9,469	0.0293 ± 0.0041	8.73×10^{-13}	22,010	5.95×10^{-1}
BUN	6q22.33	127,535,304	rs6569474[d]	*RSPO3*	A/T	0.47	−0.0172 ± 0.0032	4.80×10^{-8}	12,510	−0.0044 ± 0.0017	8.93×10^{-3}	22,076	−0.0090 ± 0.0016	1.26×10^{-8}	34,586	4.12×10^{-4}
Liver enzymes																
GGT	4q31.22	147,040,860	rs4835265	*ZNF827*	A/C	0.42	−0.0047 ± 0.0009	6.21×10^{-8}	10,503	−0.0040 ± 0.0007	1.03×10^{-9}	13,874	−0.0043 ± 0.0006	1.01×10^{-14}	24,377	5.39×10^{-1}
	7q11.23	72,615,834	rs12539316[e]	*TBL-BCL7B*	G/A	0.10	0.0081 ± 0.0014	2.41×10^{-8}	10,492	0.0029 ± 0.0010	5.44×10^{-3}	13,902	0.0051 ± 0.0008	5.81×10^{-10}	24,394	2.51×10^{-3}
	12q24.11	109,898,844	rs12229654	*MYL2*	G/T	0.14	0.0132 ± 0.0012	2.23×10^{-26}	10,450	0.0109 ± 0.0009	3.29×10^{-34}	13,840	0.0119 ± 0.0007	8.76×10^{-58}	24,290	1.29×10^{-1}
	12q24.13	111,129,784	rs2074356	*C12orf51*	T/C	0.15	0.0165 ± 0.0012	2.05×10^{-41}	10,505	0.0158 ± 0.0008	6.25×10^{-85}	17,807	0.0161 ± 0.0007	2.88E-126	28,312	6.27×10^{-1}
	12q24.13	111,850,004	rs11066453	*OAS1*	G/A	0.13	0.0105 ± 0.0013	1.55×10^{-15}	10,434	0.0092 ± 0.0008	2.41×10^{-28}	17,852	0.0097 ± 0.0007	6.27×10^{-44}	28,286	3.94×10^{-1}
ALT	12q24.13	111,302,166	rs11066280	*C12orf51*	A/T	0.17	0.0044 ± 0.0008	6.19×10^{-9}	12,241	0.0046 ± 0.0006	2.54×10^{-14}	21,456	0.0045 ± 0.0005	7.62×10^{-22}	33,697	8.36×10^{-1}
AST	12q24.13	111,302,166	rs11066280	*C12orf51*	A/T	0.17	0.0013 ± 0.0002	9.74×10^{-14}	12,241	0.0018 ± 0.0001	1.85×10^{-38}	21,646	0.0016 ± 0.0001	2.77×10^{-63}	33,887	2.53×10^{-2}

We calculated the minor allele frequency (MAF) of each SNP based on KARE samples. KARE, Korea Association Resource study; HEXA, Health Examinee cohort; BBJ, BioBank Japan study; SJTU, Shanghai Jiao Tong University cohort; P_{het}, heterogeneity *P* value; HDLc, high density lipoprotein cholesterol; FPG, fasting plasma glucose; ALB, albumin; BUN, blood urea nitrogen; GGT, gamma glutamyl transferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
[a]The lead SNPs are defined as SNPs showing the strongest evidence of association with the related trait in the LD block (pairwise LD statistics, $r^2 < 0.2$ and MAF ≥ 0.05 within a 500-kb genomic region window). Information for the SNP ID and chromosomal position is based on NCBI genome build 36 and dbSNP build 129. The alleles are given with respect to the dbSNP orientation. [b]The alleles given are listed as the major allele/minor allele. [c]Effect sizes are reported per copy of the minor allele and are given as β ± s.e.m. The units for the effect sizes are mg/dL for HDLc and FPG, g/dL for ALB, mg/dL for BUN, and IU/L for GGT, ALT and AST. Variables were transformed as natural log for HDLc, common log for BUN, inverse square root for GGT and ALT, and the reciprocal for AST. Proxy SNPs used in the BioBank Japan *in silico* replication are [d]rs2326566 ($r^2 = 1$) and [e]rs2286276 for BBJ ($r^2 = 0.92$).

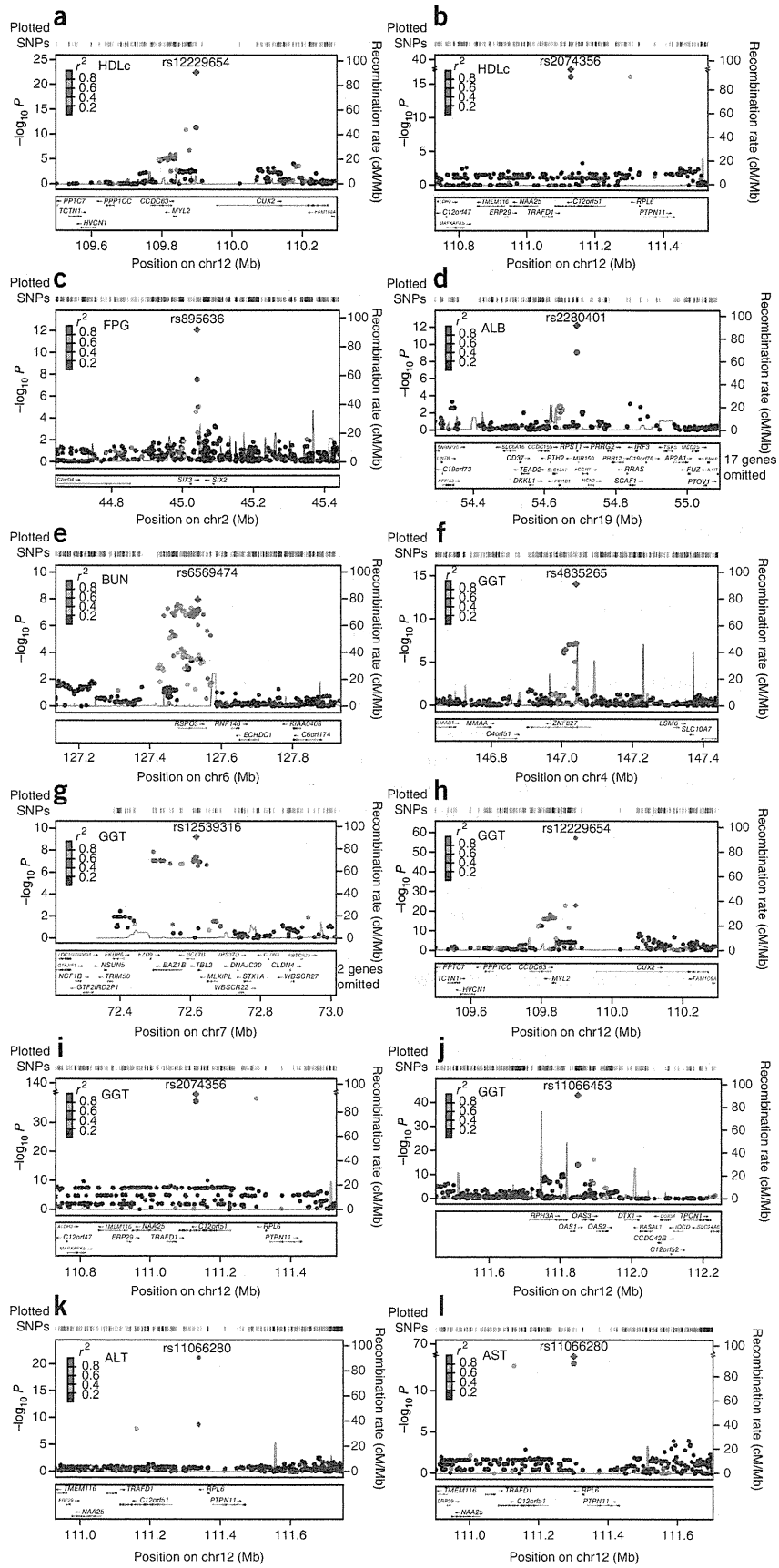

Figure 2 Regional plot of new variants that reached genome-wide significance (overall meta $P < 5 \times 10^{-8}$). (**a**–**l**) The new genome-wide significant loci. In the top panel of each, the association signals scaled by $-\log_{10} P$ (typed or imputed SNPs) at each locus are distributed in a genomic region 400 kb to either side of the lead association signal (typed). Each SNP is plotted as a circle along the chromosomal position, and linkage disequilibrium between the lead SNP and the other SNPs is colored as a scale from low (blue) to high (red) or is colored gray if linkage disequilibrium information was not available in the HapMap phase 2 CHB+JPT samples. The lead SNP is colored purple, and the overall meta-analysis result is shown with a purple diamond. The recombination rate estimated from HapMap phase 2 is plotted in blue. The bottom panels illustrate the locations of known genes. Genetic information is based on NCBI build 36 and dbSNP build 129. HDLc, high density lipoprotein cholesterol; FPG, fasting plasma glucose; ALB, albumin; BUN, blood urea nitrogen; GGT, gamma glutamyl transferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

the different genomic structure supports the different association results observed in east Asians and Europeans.

For plasma albumin, a signal (rs2280401) reaching genome-wide significance mapped to chromosome 19q13.33 ($P_{overall} = 8.73 \times 10^{-13}$, effect size = 0.0293 ± 0.0041). This variant is located in the intronic region of *RRPS11*, the gene encoding the 40S ribosomal protein S11. For BUN association, we detected a variant (rs6569474) on chromosome 6q22.33 in *RSPO3*, which encodes R-spondin 3 ($P_{overall} = 1.26 \times 10^{-8}$, effect size = −0.0090 ± 0.0016).

Newly identified association signals for GGT in the 12q24 region included rs12229654 (12q24.11) near *MYL2* ($P_{overall} = 8.76 \times 10^{-58}$, effect size = 0.0119 ± 0.0007), rs2074356 (12q24.13) in the intronic region of *C12orf51* ($P_{overall} = 2.88 \times 10^{-126}$, effect size = 0.0161 ± 0.0007) and rs11066453 (12q24.13) near *OAS1* (encoding 2′,5′-oligoadenylate synthetase 1) ($P_{overall} = 6.27 \times 10^{-44}$, effect size = 0.0097 ± 0.0007). Variants in *MYL2* and *C12orf51* also showed strong association with HDLc in this study. *OAS1*, encoding an enzyme of the innate immune system, has an antiviral function[6]. It has been reported that polymorphisms of *OAS1* are involved in the progression of disease after hepatitis C virus infection[6] and type 1 diabetes[7]. Other GGT signals included rs4835265 (4q31.22) in *ZNF827*, encoding zinc finger protein 827 ($P_{overall} = 1.01 \times 10^{-14}$, effect size = −0.0043 ± 0.0006), and rs12539316 (7q11.23) near *TBL2*, encoding transducin (beta)-like 2, and *BCL7B*, encoding B-cell CLL/lymphoma 7B ($P_{overall} = 5.81 \times 10^{-10}$, effect size = 0.0051 ±

0.0008). The protein encoded by *ZNF827* may be a transcriptional regulator that affects plasma GGT levels. Deletion of the genomic region comprising *TBL2* and *BCL7B* is commonly observed in Williams-Beuren syndrome[8]. Finally, mutation of *TBL2* is associated with TG level[9,10] and hypertriglyceridemia[11].

For both ALT and AST, we detected the most compelling association ($P_{overall} = 7.62 \times 10^{-22}$, effect size = 0.0045 ± 0.0005 for ALT and $P_{overall} = 2.77 \times 10^{-63}$, effect size = 0.0016 ± 0.0001 for AST) on chromosome 12q24.13 (rs11066280 in *C12orf51*). Notably, this 12q24.13 locus was also associated with HDLc and GGT in this study.

To explore possible functional mechanisms of the ten newly identified variants, we performed expression quantitiative trait locus (eQTL) analysis by examining the association between each SNP and mRNA expression levels of nearby genes. Of the ten variants, one SNP, rs11066453, was highly associated with *OAS1* mRNA expression levels in the Epstein-Barr virus–trasnformed lymphoblastoid cell lines of 210 HapMap phase 2 individuals of European, African and east Asian ancestry ($P_{eQTL} = 1.03 \times 10^{-5}$) (**Supplementary Table 4**). These results indicate that higher *OAS1* transcript levels associated with the rs11066453 G allele are closely related to higher levels of GGT (**Supplementary Fig. 4**).

Despite the fact that 23 known variants for metabolic traits were replicated in our study (**Supplementary Table 3**), many other signals identified in Europeans were not replicated (**Supplementary Table 5**). These discrepancies may be partly caused by allele frequency differences between study populations. For example, minor allele frequencies are considerably lower in the Korean population compared to the European population, as shown by loci in *TTC39B*, *PLTP* and *MVK-MMAB*. In these loci, a significant association with HDLc has been detected in Europeans[12,13] but not in Koreans (**Supplementary Table 5**). Indeed most SNPs showing no evidence of association in this study, although having previously been established in Europeans, have a statistical power ranging from 5–45%, implying an insufficient sample size for these markers to detect association for any of the nine metabolic traits. More sampling may be necessary to detect the evidence of association for these loci in the Korean population.

On the other hand, two SNPs, rs1553318 and rs2925979, that were identified for TG and HDLc, respectively, in Europeans, were not replicated in Koreans, although there was good power (0.8) to detect an association (**Supplementary Table 5**). Comparison of LD indicates that the LD blocks in which these SNPs reside are different between Europeans and east Asians (**Supplementary Fig. 5a,b**), implying that both SNPs may not be proxies for causal variants for the related traits in the Korean population. Further studies of inter-ethnic differences in genetic architecture and environmental factors will be necessary to understand the population specificity of SNPs that are associated with various metabolic traits.

Taking advantage of population-based GWAS of numerous traits, our study was able to catalog pleiotropic loci across the whole genome, and we detected six pleiotropic loci in this study (**Table 2**). It is noteworthy that most of the pleiotropic regions act accordingly on related multiple traits. For example, two loci, 8p21.3 and 11q23.3, reciprocally influence the plasma level of TG and HDLc. This finding is well matched to the negative correlation between plasma HDLc and TG levels[14]. In addition, the effect direction of variants in 7q11.23 for plasma GGT and TG levels coincides with the positive correlation between both of these traits[15]. In total, it is implied that pleiotropic loci might be critical indicators not only to predict diverse blood biochemical conditions but also to unravel underlying molecular mechanisms of physiologically related multiple metabolic traits.

For pleiotropic loci detected in this study, we performed conditional analyses to test whether the association with the other traits is simply secondary to the primary association. Conditional analyses of these loci showed that most association signals for primary traits remained after the adjustment for secondary traits (**Supplementary Table 6**). On the other hand, the association signals on 12q24.13 for ALT and AST were abolished by GGT adjustment, indicating that the multiple effects of this locus on liver enzymes are presumably caused by the secondary effects correlated among physiologically related traits.

In a 2-Mb region on 12q24, multiple SNPs showed evidence of association with HDLc (rs12229654 and rs2074356) and GGT

Table 2 Pleiotropic loci detected from GWAS for various traits

	This study								
Locus	SNP ID	Position (bp)	Candidate gene	Affected Trait	Variable transformation	Effect allele (frequency)	Effect size[a]	P^b	Affected trait(s) identified from previous studies
2p23.3	rs780092	27,596,658	*GCKR*	ALB	No	C (0.33)	−0.0235 ± 0.0040	$4.75 \times 10^{-9,c}$	HTG[19], TGC[20], eGFRcrea[21], SUA[22], CRP[23], FI[24], HOMA-IR[24], FPG[24], SU[25]
				TG	Ln	C (0.33)	−0.0500 ± 0.0046	4.58×10^{-27}	
7q11.23	rs12539316	72,615,834	*TBL2-BCL7B*	GGT	1/sqrt	G (0.10)	0.0051 ± 0.0008	5.81×10^{-10}	TG[13], SLE[26], eGFRcrea[21]
	rs2286276	72,625,290		TG	Ln	A (0.10)	−0.0652 ± 0.0082	1.44×10^{-15}	
8p21.3	rs10503669	19,891,970	*LPL*	TG	Ln	A (0.12)	−0.0857 ± 0.0065	6.84×10^{-39}	HTG[19], MCV[1]
				HDLc	Ln	A (0.12)	0.0426 ± 0.0031	8.04×10^{-43}	
9q34.2	rs651007	135,143,696	*ABO*	LDLc	No	A (0.26)	2.2026 ± 0.3841	9.78×10^{-9}	MCHC[1], PC[27], VTE[28], E-selectin[29], P-selectin[30], ICAM-1[30], RBC[1], HB[1], HT[1], ALP[1], ACE[31]
11q23.3	rs11216126	116,122,450	*ZNF259-APOA1/C3/A4/A5-BUD13*	HDLc	Ln	C (0.20)	0.0322 ± 0.0026	2.6×10^{-34}	ATC[32], HDLc[13], HTG[19], Glioma[33], SLE[26]
	rs603446	116,159,645		TG	Ln	T (0.23)	−0.0875 ± 0.0051	2.03×10^{-65}	
12q24.11	rs12229654	109,898,844	*MYL2*	HDLc	Ln	G (0.14)	−0.0280 ± 0.0028	3.41×10^{-23}	HDLc[13]
				GGT	1/sqrt	G (0.14)	0.0119 ± 0.0007	8.76×10^{-58}	
12q24.13	rs2074356	111,129,784	*C12orf51*	HDLc	Ln	T (0.15)	−0.0350 ± 0.0028	6.95×10^{-37}	T1D[34], WHR[4]
				GGT	1/sqrt	T (0.15)	0.0161 ± 0.0007	2.88×10^{-126}	
	rs11066280	111,302,166	*C12orf51*	ALT	1/sqrt	A (0.17)	0.0045 ± 0.0005	7.62×10^{-22}	
				AST	Reciprocal	A (0.17)	0.0016 ± 0.0001	2.77×10^{-63}	

Previous studies were retrieved based on $P < 5 \times 10^{-7}$. Information for SNP ID and chromosomal position is based on NCBI genome build 36 and dbSNP build 129. ALB, albumin; TG, triglyceride; GGT, gamma glutamyl transferase; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; AST, aspartate aminotransferase; HTG, hypertriglyceridemia; TGC, two-hour glucose; eGFRcrea, estimated glomerular filtration rate by serum creatinine; SUA, serum uric acid; CRP, c-reactive protein; FI, fasting insulin; HOMA-IR, insulin resistance; FPG, fasting plasma glucose; SU, serum urate; SLE, serum lupus erythematosus; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; PC, pancreatic cancer; VTE, venous thromboembolism; ICAM-1, intercellular adhesion molecule-1; RBC, erythrocyte count; HB, hemoglobin concentration; HT, hematocrit; ALP, alkaline phosphatase; ACE, angiotensin-converting enzyme activity; ATC, alpha-tocopherol; T1D, type 1 diabetes; WHR, waist hip ratio; Ln, natural log; 1/sqrt, inverse square root.

[a]Effect sizes are listed as β ± s.e.m. [b]All *P* values are the overall meta-analysis (discovery+replication) *P* value except the *P* value given for [c]ALB, which is the discovery *P* value.

(rs12229654, rs2074356 and rs11066453). Thus, we tested the independence of these SNPs for association with HDLc and GGT by conditional analysis. When association was adjusted for the other SNPs in this region, most of the association signals were abolished or the strength of association was substantially diminished (**Supplementary Table 7**). This result indicates that the associated SNPs in this region do not represent independent signals even though they are a long distance away from each other (with a physical separation of 720 kb to 2 Mb) and are in only moderate LD ($r^2 = 0.19$ to $r^2 = 0.59$). Because none of the signals showed evidence of being a representative proxy in the 12q24 region, further efforts (such as fine mapping) might be necessary to elucidate the causal variant for GGT and HDLc.

Results from a previous GWAS indicated that 12q24 shows multiple associations with several traits in Europeans[16]. Researchers from this previous study[16] proposed that the pleiotropic association of 12q24 correlates closely with positive selection in Europeans but not in Asians or Africans. A Japanese GWAS showed an association of the *ALDH2* locus in the 12q24 region with biochemical and hematological traits[1]. In east Asians, modest signatures of recent selection on 12q24.13 have been suggested by the evidence of both haplotype diversity reduction and a selective sweep in the relevant region[17]. Notably, all 12q24 association signals that appeared in the long-range haplotype in Europeans are monomorphic in east Asians, whereas all 12q24 variants in east Asians are monomorphic in Europeans (**Supplementary Table 8**). These findings evoke a notion that the 12q24 region showing association with multiple traits evolved differently between two populations. Indeed, current inspection of phylogeny of haplotypes showed that the association signals in the 12q24 region arose in east Asians independently of Europeans[17]. Taken together, further functional and evolutionary dissection is necessary to identify the molecular biological and physiological consequences distinctly displayed by genetic variants at this genomic region in two different ethnic groups.

Although tremendous efforts have identified considerable numbers of genetic bases responsible for common traits and diseases, the heritability of these traits is not fully understood[18]. GWAS meta-analysis is a critical method for discovering additional common variants not yet identified for common traits. In this context, we believe that our findings contribute to explaining the missing heritability of traits that we investigated in this study.

URLs. BioBank Japan, http://biobankjp.org/; MACH 1.0, http://www.sph.umich.edu/csg/abecasis/mach/; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/; R, http://www.r-project.org/.

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

The study was supervised by J.-Y.L., Y.S.C., T.T., N.K., K.M., W.J., K.K., B.O., H.-L.K. and B.-G.H. Genotyping experiments were designed by Y.S.C., B.O., M.K., C.H., H.-L.K. and J.-Y.L. Genotyping experiments were performed by J.H.O., D.-J.K., M.K., C.H. and R.Z. DNA sample preparation was carried out by E.J.H. and J.-H.K. Phenotype information was collected by N.H.K., S.K., H.M., Y.K., N.H.C., C.S. and D.K. Statistical analysis was performed by M.J.G., Y.K., Y.K.K., J.Y.L., S.K., Y.O., A.T., C.H. and T.P. Bioinformatic analysis was conducted by Y.J.K., C.B.H., M.J.G., C.H., J.-Y.H. and Y.S.C. The manuscript was written by Y.J.K., M.J.G., Y.O. and Y.S.C. All authors reviewed the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study participants. Two independent genome-wide association analyses for metabolic traits included in the discovery meta-analysis were carried out as part of the KARE study and the HEXA shared control GWAS in Korea. The KARE study has previously been described[4]. The participants in the HEXA shared control GWAS were originally selected from one of the Korean Genome Epidemiology Study (KoGES) population-based cohorts, specifically, the Health Examinee (HEXA) cohort, as a control for the Korean cancer and coronary artery disease GWAS. Approximately 3,700 of the 1,200,000 participants aged 40–69 years from the HEXA cohort were randomly selected for genome-wide association analysis.

Replication analyses for SNPs selected from the discovery stage were conducted in three independent study populations. Participants in the BioBank Japan project have been previously described[35]. The BioBank Japan project recruited ~300,000 Japanese individuals representative of 47 diseases (see URLs). Genome-wide association analysis results of *in silico* replication were available from ~16,000 individuals in the BioBank Japan project. Participants in the Health2 study cohorts in Korea have previously been described[4]. Population-based cohorts for the Health2 study included 8,500 participants, among which 7,861 individuals were selected for the *de novo* replication study. A total of 6,770 participants from the Shanghai Diabetes Institute of Shanghai Jiao Tong University, China were selected from the Shanghai Diabetes Study I ($n = 5994$) and II ($n = 5372$) for additional *de novo* replication analysis of some SNPs chosen from the discovery stage (**Supplementary Table 3**). The general characteristics of the study participants are summarized in **Supplementary Table 1**.

Genotyping and quality control. A total of 10,004 KARE study participants were genotyped using the Affymetrix Genome-Wide Human SNP array 5.0, and individual genotypes were called by applying the Bayesian Robust Linear Modeling using the Mahalanobis Distance Genotyping Algorithm. Exclusion criteria for samples and SNPs have been described[4]. After quality control, 8,842 samples and 352,228 SNPs remained for the subsequent association analyses.

A total of 4,302 of the approximately 1,200,000 participants from the HEXA cohort were genotyped with the Affymetrix Genome-Wide Human SNP array 6.0. Genotypes were called using the Birdseed Genotyping Algorithm[36]. Sample quality control was carried out using individual genotype data to exclude samples with genotyping calls <95% ($n = 443$), gender inconsistency ($n = 8$), heterozygosity ($n = 25$), cryptic relatedness (identity-by-state value >0.80; $n = 33$), evidence of non-Asian ancestry ($n = 26$) and any kind of tumor ($n = 64$). SNP marker quality control was also performed to exclude SNPs with a high missing call rate (>5%), low minor allele frequency (<1%) or low Hardy-Weinberg equilibrium P value ($P < 1 \times 10^{-6}$). The 3,703 samples and 646,062 SNPs that remained after quality control were used for subsequent association analyses.

The genome-wide association results used for the *in silico* replication study were generated from genotyping 15,764 individuals from the BioBank Japan project using the Illumina Human610-Quad BeadChip at the Center for Genomic Medicine, RIKEN. Exclusion criteria for samples and SNP quality control of genome-wide scan data have been described[1].

Replication genotyping was carried out using Korean or Chinese samples. Seven of 11 new SNPs that showed the strongest evidence of association with given traits in the discovery stage were genotyped with the TaqMan reaction or GoldenGate assay (Illumina Inc.) using 7,861 samples from the Health2 study in Korea. The quality of genotyping by both methods was assessed by conducting duplicate genotyping of approximately 1–2.5% of the samples. The concordance rates were higher than 99% in the duplicate samples. A genotype success rate of over 98% was achieved.

Additional genotyping with four new SNPs (rs12539316, rs651007, rs4835265 and rs756825) was performed for the *de novo* replication analysis. This was achieved by using 5,942 samples from the Shanghai Diabetes Institute of Shanghai Jiao Tong University in China by primer extension of multiplex products with detection by matrix-assisted laser desorption ionization time of flight mass spectroscopy using a MassARRAY platform (MassARRAY Compact Analyzer, Sequenom)[37]. The concordance rates were over 98% for all SNPs based on 190 duplicates.

Phenotypes. Parameters were measured for fasting plasma glucose, plasma lipids (HDLc, TG and total cholesterol), renal-function-related traits (ALB and BUN) and liver enzymes (GGT, ALT and AST). Biochemical measurements were obtained in the morning before the first meal of the day. Some of the BBJ samples were taken from non-fasting subjects[1]. The concentration of LDLc was calculated with Friedewald's formula[38]. Missing values were assigned for individuals with TG >400 mg/dl. Individuals receiving lipid-lowering therapy were excluded from analysis of dyslipidemia-related traits (LDLc, HDLc and TG). We excluded any participants taking medication likely to influence liver enzyme traits (GGT, AST and ALT). For the association analysis, fasting plasma glucose levels were obtained only from non-diabetic individuals (**Supplementary Table 9**).

Genotyping for *de novo* replication study. In the *de novo* replication study from the Health2 study data, we performed genotype assays using the TaqMan reaction for five SNPs (rs12708980, rs2074356, rs16940212, rs599839 and rs1348637), using the GoldenGate assay (Illumina Inc.) for 20 SNPs (rs11066280, rs603446, rs11066453, rs2393791, rs11216126, rs4820599, rs10503669, rs10830962, rs1799884, rs780092, rs12483959, rs12654264, rs2001945, rs4686914, rs12686004, rs12229654, rs13069049, rs519113, rs2738446 and rs6569474) and using a MassARRAY platform (MassARRAY Compact Analyzer, Sequenom) for 7 SNPs (rs2072134, rs11023241, rs12539316, rs2280401, rs4835265, rs6548311 and rs756825).

Imputation. In the discovery stage, SNP imputation for each individual genome-wide scan was performed using the IMPUTE[39] program. International HapMap (phase 2, release 22, NCBI build 36, and dbSNP build 126) data comprising 2.2 million SNPs from 90 individuals from the JPT and CHB populations were used as a reference panel. After excluding imputed SNPs with low genotype information content (<0.5), posterior probability score <0.90, call rate <0.90, MAF <0.01 and Hardy-Weinberg equilibrium $P < 1 \times 10^{-7}$, a total of 1,573,409 SNPs for the KARE and 1,984,393 SNPs for the HEXA share control GWAS remained for subsequent analysis.

SNP imputation for *in silico* replication was performed by MACH 1.0 (see URLs) for 15,764 samples from the BioBank Japan GWAS[1] based on the International HapMap phase 2, release 22, NCBI build 36. Imputed SNPs with Rsq ≤ 0.3 were excluded for the association analysis.

Statistical analyses. The association of imputed and genotyped SNPs with any of nine quantitative traits was tested with multiple linear regression analysis in an additive genetic model (with 1 degree of freedom) after adjusting for age, gender and recruitment area. Association analyses were performed using PLINK[40] (see URLs) and SAS (version 9.1; SAS Institute Inc.). Measurements of HDLc, TG, GGT, ALT, AST and BUN were transformed with the natural log, 1/(square root), the reciprocal or the common log to achieve a normal distribution before the association analysis (**Supplementary Table 5**). The meta-analysis was performed using a weighted average method assuming fixed effects with inverse variance[41]. A Cochran's Q test was applied to assess heterogeneity among the studies[42]. All meta-analysis calculations were performed using the R program (version 2.7.1; see URLs). Quantile-quantile plots of genome-wide P values were generated to detect SNPs that showed strong deviations from the null distribution because of strong associations with a related trait. The genomic inflation factor (λ) was estimated from the median of the χ^2 statistic divided by 0.456 (ref. 43).

Expression quantitative trait locus (eQTL) analysis. Gene expression information in the Epstein-Barr-virus–transformed lymphoblastoid cell lines of 210 individuals from HapMap phase 2 (60 from the CEU, 60 from the YRI, 45 from the CHB and 45 from the JPT population) was obtained from NCBI Gene Expression Omnibus. SNP genotype data were derived for each individual from the corresponding HapMap phase 2 dataset. The *cis* association between each significant SNP for the relevant metabolic trait and genes within 1 Mb of the lead SNP was examined by the linear regression analysis for the additive effect of SNP.

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

Toru Arano[1]*, Hayato Nakagawa[1,2]*, Ryosuke Tateishi[1], Hitoshi Ikeda[2], Koji Uchino[1], Kenichiro Enooku[1], Eriko Goto[1], Ryota Masuzaki[1], Yoshinari Asaoka[1], Yuji Kondo[1], Tadashi Goto[1], Shuichiro Shiina[1], Masao Omata[3], Haruhiko Yoshida[1] and Kazuhiko Koike[1]

[1] Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655
[2] Department of Clinical Laboratory Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655
[3] Yamanashi Prefectural Hospital Organization, 1-1-1, Fujimi, Kofu, Yamanashi 400-8506

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

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Correspondence to: Hayato Nakagawa, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Tel.: +81-3-3815-5411, Fax: +81-3-3814-0021, E-mail: n-hayato@yf7.so-net.ne.jp

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).

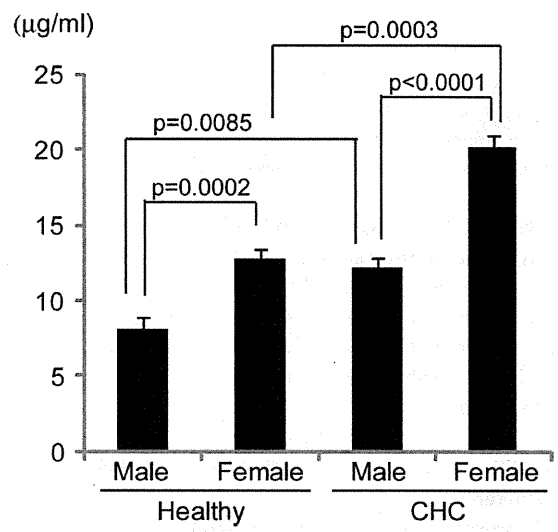

Figure 1. Serum adiponectin levels in healthy subjects (30 males, 40 females) and patients with CHC. Data are expressed as means ± standard error of the mean (SEM).

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

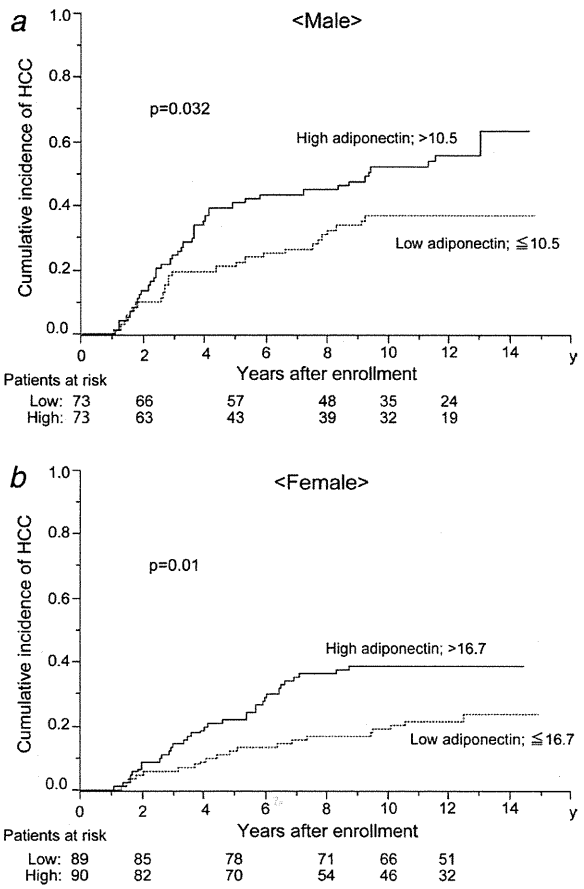

Figure 2. Cumulative incidence of HCC development stratified according to the median value of serum adiponectin for each gender: low (serum adiponectin concentration ≤10.5 μg/ml in males, ≤16.7 μg/ml in females) and high (>10.5 μg/ml in males, >16.7 μg/ml in females). (*a*) Male patients; (*b*) female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

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,

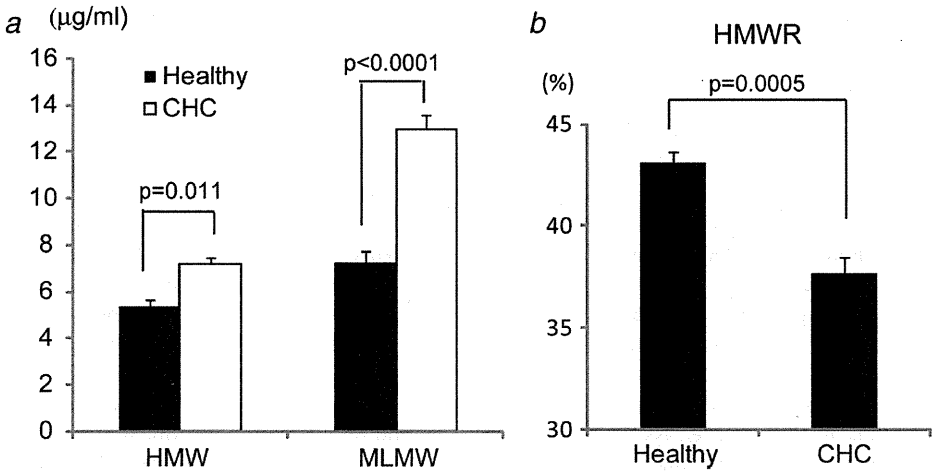

Figure 3. Assessment of adiponectin isoforms in healthy female subjects and patients with CHC. (*a*) Serum HMW and MLMW adiponectin levels; (*b*) ratio of HMW adiponectin to total adiponectin (HMWR). Data are expressed as means ± standard error of the mean (SEM).

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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The authors have no conflicts of interest regarding our study.

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INTERVENTIONAL

Mortality and complication rates of percutaneous ablative techniques for the treatment of liver tumors: a systematic review

Luis Calzadilla Bertot · Masaya Sato · Ryosuke Tateishi · Haruhiko Yoshida · Kazuhiko Koike

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Abstract
Objectives Reported rates of major complications and mortality of radiofrequency ablation (RFA), microwave ablation (MWA) and percutaneous ethanol injection (PEI) for the treatment of liver tumours were substantially heterogeneous among studies. The aim was to analyse the mortality and major complication rates of percutaneous RFA, PEI and MWA.
Methods MEDLINE and EMBASE search from January 1982 to August 2010. Randomised clinical trials and observational studies, age >18, more than 50 patients for each technique analysed, studies reporting mortality and major complications were included. Random effects model was performed, with assessment for heterogeneity and publication bias.
Results Thirty-four studies including 9531, 1185, and 1442 patients for RFA, MWA, and PEI, respectively were included. For all ablative techniques pooled proportion mortality rate was 0.16% (95% confidence interval [CI], 0.10–0.24). Pooled mortality rate associated with RFA, PEI and MWA was 0.15% (0.08–0.23), 0.59% (0.14–1.3) and 0.23% (0.0–0.58) respectively. Pooled proportion of major complications was 3.29% (2.43–4.28). Major complication rates associated with RFA, MWA, and PEI was 4.1% (3.3–5.1), 4.6% (0.7–11.8) and 2.7% (0.28–7.4) respectively.
Conclusions Percutaneous RFA, PEI and MWA can be considered safe techniques for the treatment of liver tumours.

Keywords Liver neoplasms · Ablation techniques · Mortality · Complications · Meta-Analysis

Abbreviations

HCC	Hepatocellular carcinoma
RFA	Radiofrequency ablation
PEI	Percutaneous ethanol injection
MWA	Microwave ablation
CI	Confidence interval
CRC	Colorectal cancer
CT	Computed tomography
MRI	Magnetic resonance imaging
SIR	Society of interventional radiology

Introduction

Both hepatocellular carcinoma (HCC) and metastatic liver cancer, mainly from colorectal cancer (CRC), represent the most frequent liver neoplasms [1]. HCC has an estimated global incidence of over 500 000 new cases per year and represents the third largest cause of cancer-related death in the world [2]. Every year, at least one million new patients, especially from Eastern Asian and African countries, are reported [3]. In addition, the liver is the second common site of metastasis from other solid cancers, particularly in patients with colorectal cancer [4].

L. C. Bertot · M. Sato · R. Tateishi (✉) · H. Yoshida · K. Koike
Department of Gastroenterology, Graduate School of Medicine,
The University of Tokyo,
7-3-1 Hongo, Bunkyo-ku,
Tokyo 113-8655, Japan
e-mail: tateishi-tky@umin.ac.jp

Image-guided tumour ablation plays an important role in the treatment of primary and metastatic liver cancer. Radiofrequency ablation (RFA) has gained wide acceptance by showing superior anticancer effects and greater survival benefit compared with the formerly-used percutaneous technique, percutaneous ethanol injection (PEI). RFA is currently considered as the standard loco-regional treatment for HCC worldwide in patients with three or fewer lesions, 3 cm or less in diameter and for selected patients with hepatic metastases of colorectal, breast and endocrine tumours [5–7]. However PEI still plays a role in the treatment of liver tumours when RFA is precluded or not available [6]. Recently other emerging techniques such as microwave ablation (MWA) have attracted interest in clinical practice [8].

However, the performance of image-guided procedures will always entail some risks. Information regarding mortality and complications is absolutely essential for every intervention to permit an accurate assessment of the risks and benefits [9]. Findings in various studies suggest that ablative techniques are relatively safe. RFA-associated mortality and morbidity were reported to be 0.2% to 1.4% and 2.2% to 12.0%, respectively, while PEI was reported to have mortality up to 0.7% and a major complication rate of 3.2–4.6%. However reports from the literature are heterogeneous because of the study design, sample size, different technical approaches, number of centres reporting complications and non-uniform terms as well as different parameters to calculate the rate of complications [10–13].

The aim of this systematic review was to analyse the mortality and major complications rates of RFA, PEI and MWA for the treatment of liver tumours by including both randomised and observational studies.

Materials and methods

Eligibility criteria

Randomised clinical trials and observational studies of percutaneous RFA, PEI and MWA for the treatment of liver tumours in patients aged over 18 were included. In order to exclude small (and probably underpowered) studies, we only considered studies analysing more than 50 patients for at least one technique. All studies reported at least one of the three following procedures: RFA, PEI or MWA

All studies reporting mortality for each procedure were considered for the primary outcome measure. For secondary outcome measure we considered all studies reporting the number of major complications, complication-related deaths and the types of complication for each technique.

Studies that mentioned only procedures using celiotomy and laparoscopic approaches, reporting the number of complications of procedures using percutaneous or celiotomy or laparoscopic approaches all together and did not mention complications due to percutaneous procedures alone, and those that focused only on specific complications and mentioned only procedures under computed tomography (CT) and magnetic resonance imaging (MRI) guidance were excluded from the review.

We categorised death and major complications based on the standardised SIR (Society of Interventional Radiology) grading system (Table 1) [14–16]. The definition of death is self-explanatory and is represented by "SIR classification F". Major complications are events that lead to substantial morbidity, disability, increased level of care, or that may result in hospital admission or substantially lengthened hospital stay, and are represented by "SIR classifications C-E". This includes any case in which a blood transfusion or interventional drainage procedure is required.

Several complications, such as pneumothorax or peritoneal haemorrhage can be either a major or a minor complication, depending on the severity or their course. As for tumour seeding, we categorised them as major complications if the ectopic tumour focus could not be successfully ablated or otherwise treated. We adopted a definition of "treatment" as a completed effort to ablate one or more tumours according to the above-described guideline. Usually a patient with liver tumours has two or more therapeutic opportunities for ablative therapy during clinical course because of tumour recurrence. We reported the complication rates on a per treatment basis rather than on a per patient basis.

Table 1 Society of Interventional Radiology (SIR) Classification system for complications by outcome

Minor complications
A. No therapy, no consequence
B. Nominal therapy, no consequence; includes overnight admission for observation only
Major complications
C. Require therapy, minor hospitalisation (<48 h)
D. Require major therapy, unplanned increase in level of care, prolonged hospitalisation (>48 h)
E. Permanent adverse sequelae
F. Death