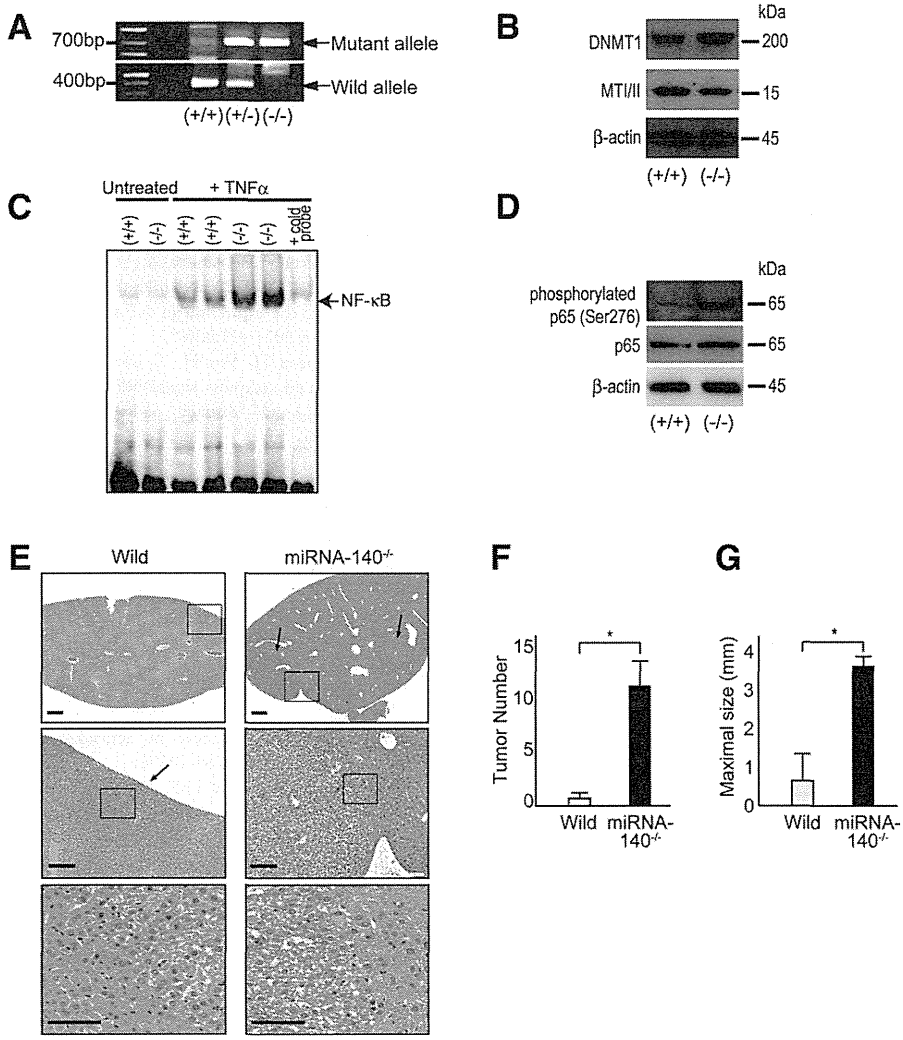

Fig. 4. miRNA-140$^{-/-}$ mice are prone to hepatocarcinogenesis. (A) Representative genotyping of mice with wild-type or mutant alleles. PCR genotyping was performed for miRNA-140 wild-type (419 bp; Wild) and knockout (734 bp; Mutant) alleles. (+/+), wild-type; (+/−), heterozygous; (−/−), knockout. (B) Increased Dnmt1 expression and decreased MTI/II expression in the liver tissues of miRNA-140$^{-/-}$ mice compared with wild-type mice. Western blotting was performed using antibodies against the indicated proteins. (+/+), wild-type; (−/−), miRNA-140$^{-/-}$. The image shown is representative of four independent experiments. (C) NF-κB-DNA binding was assessed via gel-shift assay using equal amounts of liver nuclear extracts from untreated and TNF-α-injected wild-type and miRNA-140$^{-/-}$ mice. (+/+), wild-type; (−/−), miRNA-140$^{-/-}$. Cold probe was added to TNF-α-injected knockout mouse nuclear extract to test assay specificity. A result representative of four independent experiments is shown. (D) Western blotting for phosphorylated p65 expression in the liver at 32 weeks after DEN treatment in miRNA-140$^{-/-}$ mice compared with wild-type mice. A result representative of four independent experiments is shown. (E) Representative histological images of mouse liver at 32 weeks after DEN treatment. Arrows indicate tumors. Higher-magnification images of the highlighted areas in the upper panels are shown in the lower panels. Scale bar, 500 μm. (F) The number (left panel) and size (right panel) of tumors (five random sections per mouse treated with DEN) are presented as the mean ± SD (wild-type mice, n = 8; miRNA-140$^{-/-}$ mice, n = 8). *$P < 0.05$.

components,[22] with subsequent impairment of miRNA function as molecular pathways and possible therapeutic targets for carcinogenesis and other diseases.

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Genome-wide association study identifies a new SMAD7 risk variant associated with colorectal cancer risk in East Asians

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Genome-wide association studies (GWAS) of colorectal cancer (CRC) have been conducted primarily in European descendants. In a GWAS conducted in East Asians, we first analyzed approximately 1.7 million single-nucleotide polymorphisms (SNPs) in four studies with 1,773 CRC cases and 2,642 controls. We then selected 66 promising SNPs for replication and genotyped them in three independent studies with 3,612 cases and 3,523 controls. Five SNPs were further evaluated using data from four additional studies including up to 3,290 cases and 4,339 controls. SNP rs7229639 in the *SMAD7* gene was found to be associated with CRC risk with an odds ratio (95% confidence interval) associated with the minor allele (A) of 1.22 (1.15–1.29) in the combined analysis of all 11 studies ($p = 2.93 \times 10^{-11}$). SNP rs7229639 is 2,487 bp upstream from rs4939827, a risk variant identified previously in a European-ancestry GWAS in relation to CRC risk. However, these two SNPs are not correlated in East Asians ($r^2 = 0.008$) nor in Europeans ($r^2 = 0.146$). The CRC association with rs7229639 remained statistically significant after adjusting for rs4939827 as well as three additional CRC risk variants (rs58920878, rs12953717 and rs4464148)

Key words: Genome-wide association study, GWAS, colorectal cancer, *SMAD7*, genetic susceptibility, single-nucleotide polymorphisms, epidemiology

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

Abbreviations: CHB: Han Chinese in Beijing, China; CI: confidence intervals; CRC: colorectal cancer; EAF: effect allele frequency; GWAS: genome-wide association study; HERPACC-II: Hospital-based Epidemiologic Research Program at Aichi Cancer Center; HWE: Hardy-Weinberg equilibrium; JPT: Japanese in Tokyo, Japan; KCPS-II: Korean Cancer Prevention Study-II; LD: linkage disequilibrium; MAF: minor allele frequency; NCC: National Cancer Center; OR: odds ratio; PCA: principal components analysis; QC: quality control; SCH: Singapore Chinese Study; SMHS: Shanghai Men's Health Study; SNP: single-nucleotide polymorphism; SWHS: Shanghai Women's Health Study

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reported previously in this region. SNPs rs7229639 and rs4939827 explained approximately 1% of the familial relative risk of CRC in East Asians. This study identifies a new CRC risk variant in the *SMAD7* gene, further highlighting the significant role of this gene in the etiology of CRC.

What's new?

An estimated 35% of colorectal cancer (CRC) risk may be due to inherited factors, about 10% of which have been identified in genome-wide association studies (GWAS), though those studies have focused mainly on populations of European ancestry. This GWAS of CRC focused on individuals of East Asian ancestry, with SNP rs7229639 in the *SMAD7* gene found to be associated with CRC risk, independent of rs4939827, which was reported previously in a European population. The findings highlight the role of *SMAD7* in the etiology of CRC and suggest that genetic risk variants may differ by study populations.

Colorectal cancer (CRC) is the third-most common cancer and second-leading cause of cancer death worldwide.[1] Although environmental factors are believed to play an important role in the etiology of CRC, it is estimated that approximately 35% of CRC risk may be attributable to inherited factors.[2] To date, approximately 19 common genetic susceptibility loci for CRC have been identified in genome-wide association studies (GWAS).[3–13] However, these genetic variants, along with high-penetrance germline mutations in known CRC susceptibility genes, including *APC*, the DNA mismatch repair genes, *SMAD4*, *AXIN2*, *BMPR1A*, *TGFBR2*, *POLD1*, *STK11* and *MUTYH*,[14,15] explain less than 15% of excess familial risk of CRC.[3–15]

Most previous GWAS for CRC were conducted in European-ancestry populations. Given the potential difference in genetic architectures between East Asians and European ancestry populations, it is possible that some genetic risk variants for CRC identified in European descendants may not be generalizable to East Asians. Also, GWAS conducted in East Asians could possibly identify genetic risk variants unique to this population. In 2009, we initiated a GWAS in East Asians, the Asia Colorectal Cancer Consortium, and identified three novel genetic susceptibility loci for CRC.[16] In this article, we reported additional findings from this consortium regarding the identification of a new risk variant for CRC in the *SMAD7* gene.

Material and Methods

Study populations

This study, conducted as part of the Asia Colorectal Cancer Consortium, included 8,891 CRC cases and 10,547 cancer-free controls of East Asian ancestry recruited in eight centers located in China, Korea, Japan and Singapore (Table 1). Specifically, Stage 1 consisted of four studies: Shanghai Study 1 (Shanghai-1, $n = 982$), Shanghai Study 2 (Shanghai-2, $n = 553$), Guangzhou Study 1 (Guangzhou-1, $n = 1{,}666$) and Aichi Study 1 (Aichi-1, $n = 1{,}439$). Stage 2 consisted of seven studies: Guangzhou Study 2 (Guangzhou-2, $n = 2{,}892$), Korean-National Cancer Center Study (Korea-NCC, $n = 2{,}721$), Seoul Study (Korea-Seoul, $n = 1{,}522$), Korean Cancer Prevention Study-II (KCPS-II, $n = 1{,}302$), the Japan-BioBank Study (Japan-BioBank, $n = 3{,}498$), Singapore Chinese Study (SCH, $n = 2{,}000$) and Aichi Study 2 (Aichi-2, $n = 863$). Summary descriptions of these 11 participating studies from eight centers are provided in Supporting Information. Study protocols were approved by relevant institutional review boards for all study sites.

Laboratory procedures

Genomic DNA was extracted from either blood or saliva samples according to standard protocols. In Stage 1, genotyping was performed using Affymetrix Genome-Wide Human SNP Array 6.0 (Affy 6.0, 906,602 SNPs) for Shanghai-1 cases and controls; Illumina HumanOmniExpress BeadChip (Illumina OmniExpress, 729,462 SNPs) for Shanghai-2 cases and controls, Guangzhou-1 cases and Aichi-1 cases; Illumina Human610-Quad BeadChip (620,901 SNPs) for Guangzhou-1 controls and Illumina Infinium HumanHap610 BeadChip (592,044 SNPs) for Aichi-1 controls. Genotype calling was performed using Birdseed algorithm for Affymetrix 6.0 or GenomeStudio software for Illumina GWAS platforms based on manufacturer's protocols.

Quality control (QC) protocols were applied to exclude samples and SNPs from all four studies in Stage 1 as described previously,[17–19] including (1) genotype call rate per sample <95%, (2) genetically identical (PI_HAT > 0.9) or duplicated samples, (3) genetic sex inconsistent with survey/clinical data, (4) samples with close relative (PI_HAT > 0.25), (5) population structure inconsistent with HapMap Asians (see Statistical analysis), (6) genotype call rate per SNP < 95%, (7) minor allele frequency (MAF) < 0.05, (8) genotyping concordance <95% in QC samples, (9) Hardy–Weinberg equilibrium (HWE) $p < 1 \times 10^{-5}$ in controls or (10) SNPs not in autosomes. After these QC procedures, 580,086 SNPs for 971 individuals (474 cases and 497 controls) remained in the Shanghai-1 dataset; 515,701 SNPs for 485 individuals (254 cases and 231 controls) remained in the Shanghai-2 dataset; 522,096 SNPs for 641 cases and 435,925 SNPs for 972 controls remained in the Guangzhou-1 dataset; and 478,246 SNPs for cases and 443,065 SNPs for controls remained in the Aichi-1 dataset.

Stage 2 genotyping was performed using the Sequenom MassARRAY platform (Sequenom, San Diego, CA) for the promising 66 SNPs selected from Stage 1. These SNPs were

Epidemiology

Table 1. Descriptions of participating studies and subjects included in this analysis

Study	Population	Sample size[1]		Mean age (years)[2]		Female (%)[2]		Genotyping platform
		Genotyped	After QC	Cases	Controls	Cases	Controls	
Stage 1								
Shanghai-1	Chinese	481/501	474/497	60.02	60.20	73.84	72.64	Affymetrix 6.0
Shanghai-2	Chinese	296/257	254/231	61.16	60.75	54.72	56.71	Illumina OmniExpress
Guangzhou-1	Chinese	694/972	641/972	54.86	47.40	36.51	27.06	Illumina OmniExpress/Human610-Quad
Aichi-1	Japanese	497/942	404/942	59.43	47.88	37.38	47.77	Illumina OmniExpress/HumanHap610
Subtotal		1,968/2,672	1,773/2,642					
Stage 2								
Guangzhou-2[3]	Chinese	1,371/1,521	1,371/1,521	58.22	54.64	37.96	39.25	Sequenom
Korea-NCC[3]	Korean	1,392/1,329	1,392/1,329	58.19	55.59	37.64	38.60	Sequenom
Korea-Seoul[3]	Korean	849/673	849/673	59.05	57.19	40.99	47.85	Sequenom
KCPS-II[4]	Korean	325/977	325/976	51.38	41.27	27.08	43.34	Affymetrix 5.0
Japan-BioBank[4]	Japanese	1,595/1,903	1,583/1,898	58.37	52.49	39.17	36.22	Illumina HumanHap 610K/550K
SCH[4]	Chinese	1,000/1,000	991/993	69.99	69.40	42.99	43.00	Affymetrix 6.0
Aichi-2[3]	Japanese	391/472	391/472	59.87	60.08	36.06	35.17	Sequenom
Subtotal		6,923/7,875	6,902/7,862					
Total		8,891/10,547	8,675/10,504					

[1]Number of cases/controls. QC, quality control.
[2]Among samples remained after QC exclusion.
[3]Studies for direct genotyping.
[4]Studies for in silico replication.

genotyped in Guangzhou-2, Korea-NCC and Korea-Seoul studies. Again in Stage 2, standard QC protocols were applied to exclude SNPs, including (1) genotype call rate per SNP < 95%, (2) unclear genotyping cluster, (3) genotyping concordance <95% in QC samples and (4) HWE $P < 7.7 \times 10^{-4}$ (0.05/65) in controls. After these QC procedures, the number of eligible SNPs was 65 in Guangzhou-2, 64 in Korea-NCC and 60 in Korea-Seoul studies. Five of these SNPs were taken forward for *in silico* replication in KCPS-II ($n = 5$), Japan-BioBank ($n = 5$) and SCH ($n = 2$; rs7229639 and rs2143619). Genotyping was conducted using either Affymetrix Genome-Wide Human SNP Array 5.0 in KCPS-II or Illumina HumanHap 610K and 550K in Japan-BioBank or Affymetrix Genome-Wide Human SNP Array 6.0 for SCH. Details of the QC procedures and data processing for samples included in these three studies have been previously reported elsewhere.[11,20,21] Finally, SNP rs7229639 was further genotyped in Aichi-2 using Sequenom along with SNPs for other projects.

Statistical analysis

Genome-wide imputation for samples in four Stage 1 studies was performed using program MACH 1.0[22] based on data from the 90 CHB (Han Chinese in Beijing, China) and JPT (Japanese in Tokyo, Japan) samples included in the HapMap project (release 22). After exclusion of imputed SNPs with MAF < 0.05 and RSQ < 0.50, 1,695,815 genotyped or imputed SNPs remained for meta-analyses.

To evaluate the population structure and identify potential genetic outliers, we performed principal components analysis (PCA) using EIGENSTRAT, version 2.5.[23] We selected a set of ~6,000 uncorrelated SNPs (closest distance between two SNPs >200 kb, MAF > 0.2, $r^2 < 0.1$, and call rate >99%) shared among all 4,415 samples included in Stage 1 and the HapMap Project using PLINK version 1.07.[24] Genotype data of these SNPs from the four Stage 1 studies were pooled together with HapMap data (release 23a) to generate the first ten principal components. Samples were removed from the final analysis if they were more than 6σ away from the means of PC1 and PC2.

Associations of SNPs with CRC risk in each of the four studies included in Stage 1 were assessed by assuming a log-additive effect of the allelic dosage of the SNPs. Odds ratios (ORs) and 95% confidence intervals (CIs) were generated from logistic regression models, adjusted for age, sex and the first ten principal components. We coded 0, 1 or 2 copies of the effect alleles as dosage for genotyped SNPs and used the expected number of copies of the effect alleles as dosage score for imputed SNPs to account for imputation uncertainty.[25] The meta-analysis was performed using an inverse-

variance method assuming fixed-effects, with a Cochran's Q-statistic to test for heterogeneity[26] and I^2 statistic to quantify heterogeneity[27] across studies. Summary statistics of genome-wide meta-analyses were generated using the METAL program.[28] Similar to Stage 1, we evaluated associations of CRC risk with SNPs in each of the studies included in Stage 2 using logistic regression models with adjustment for age and sex. Summary estimates in Stage 2, all studies combined, and subgroups by populations (Chinese, Korean and Japanese) and sex (male and female) were also obtained using a fixed-effects meta-analysis with METAL. SNPs showing an association at $p < 5 \times 10^{-8}$ in the combined analysis of all studies were considered genome-wide significant. We conducted haplotype association analysis for two SNPs in 18q21.1 using SAS Genetics v9.3 with logistic regression models. The familial relative risk (λ) to offspring of an affected individual due to a single locus is estimated using formula: $\lambda=(pr^2+q)/(pr+q)^2$, where p is the frequency of the risk allele, $q = 1 - p$ is the frequency of the reference allele and r is the per-allele relative risk.[29] The proportion of the familial relative risk explained by a locus, assuming a multiplicative interaction between markers in the locus and other loci, is calculated as $\ln(\prod_i \lambda_i)/\ln(\lambda_0)$, where λ_0 is the overall familial relative risk, which is assigned to be 2.2 for CRC estimated from a previous meta-analysis.[30] Assuming that the risks associated with each locus combine multiplicatively, the combined contribution of the familial relative risks from multiple loci is equal to $(\prod_i \lambda_i)/\ln(\lambda_0)$.

To visualize population substructure, we drew a PCA plot using data from the 4,415 Stage 1 samples and 270 subjects from HapMap based on the first two principal components using R version 2.13.0 (http://www.r-project.org/). We also used R package to generate a forest plot to display the association of rs7229639 with CRC risk across studies. We generated regional association plots using the website-based software LocusZoom, version 1.1.[31]. Haploview version 4.2[32] was used to infer linkage disequilibrium (LD) structure.

Results

A total of 19,179 samples are included in the current analysis (Table 1). Cases and controls were reasonably well matched by age and sex in most of the participating studies. All samples in Stage 1 showed a clear East Asian origin and none of them were more than 6σ away from the means of PC1 and PC2 (Supporting Information Fig. S1). Cases and controls in each of the four studies were in the same cluster compared with East Asians in HapMap. After standard QC filter, a total of 1,695,815 SNPs were finally included in the association analyses. Using Stage 1 data, we evaluated association of CRC risk with the 26 previously reported SNPs. Of the 22 SNPs initially identified from GWAS conducted in European-ancestry populations, rs6691170 and rs16892766 are monomorphic in East Asians. One SNP, rs5934683 in Chromosome X, was excluded from analyses in this study. All other 19 SNPs showed an association with CRC risk in the same direction as reported initially (Supporting Information Table S1). Nine of 19 the SNPs showed a statistically significant association with CRC risk at $p < 0.05$. Of the four SNPs initially identified in East Asians, three (rs647161, rs10774214 and rs2423279) were also significantly associated with CRC in our Stage 1, and one (rs7758229) was not associated with CRC risk in these data. To identify new genetic risk variants for CRC, we selected the 66 most promising SNPs for replication in Stage 2 using the following criteria: (1) MAF > 0.05 in each of the four Stage 1 studies, (2) RSQ > 0.70 in all four studies, (3) $p_{meta} < 5.5 \times 10^{-5}$, (4) no heterogeneity ($p_{heterogeneity} > 0.05$ and $I^2 < 25\%$), (5) uncorrelated with SNPs in known CRC loci or with each other ($r^2 < 0.20$) and (6) data available in all four Stage 1 studies. These 66 SNPs were not evaluated in our previously published study.[16]

Of the 66 SNPs selected for Stage 2 replication, 59 SNPs were successfully genotyped in 3,612 cases and 3,523 controls from three studies (Guangzhou-2, Korea-NCC and Korea-Seoul) included in Stage 2 (Supporting Information Table S2). Five SNPs (rs7923556, rs1539213, rs7229639, rs7247381 and rs2143619) from five different regions (10q21.2, 14q21.3, 18q21.1, 19q12 and 20p12.2) showed an association with CRC risk at p-value <0.05 in the same direction as observed in Stage 1 (Supporting Information Table S2). For these five SNPs, we conducted *in silico* replication using data from three additional studies (KCPS-II, Japan-BioBank and SCH) with 2,899 cases and 3,867 controls. SNP rs7229639 was further genotyped in Aichi-2 including 391 cases and 472 controls. Of all the 59 SNPs evaluated in Stage 2, only rs7229639 was consistently associated with CRC risk across all seven studies, showing strong evidence of replication, with p-value 3.39×10^{-8}. Joint analysis of samples in Stages 1 and 2 yielded per-allele OR (95% CI) 1.22 (1.15–1.29) and p-value 2.93×10^{-11} (Table 2), which is substantially lower than the genome-wide significance level of 5×10^{-8}. This association was consistent across all 11 studies in Stages 1 and 2 (Fig. 1), with little evidence of between-study heterogeneity (p for heterogeneity =0.726, $I^2 = 0\%$). Stratification analysis did not reveal any apparent heterogeneity across Chinese (OR = 1.20), Korean (OR = 1.21) or Japanese (OR = 1.23) subjects (p for heterogeneity =0.958), or between men (OR = 1.28) and women (OR = 1.19) (p for heterogeneity =0.268) for this SNP (Table 2). Finally, using genotype data from four studies included in Stage 2, we found that the risk of CRC was increased in a dose–response manner with the number of minor allele (A) of rs7229639 (p for trend = 4.78×10^{-7}), with ORs of 1.21 (95% CI: 1.10–1.34) and 1.62 (95% CI: 1.26–2.08) for heterozygotes and homozygotes, respectively. These data support an additive model rather than dominant (OR = 1.25, 95% CI: 1.14–1.38; $p = 4.39 \times 10^{-6}$) or recessive model (OR = 1.52, 95% CI: 1.19–1.96; $p = 9.95 \times 10^{-4}$).

SNP rs7229639 is located in intron 3 of the *SMAD7* gene at 18q21.1 (Supporting Information Fig. S2), where three other SNPs (rs4939827, rs12953717 and rs4464148) have

Table 2. Association of rs7229639 in the *SMAD7* gene with colorectal cancerCRC risk in East Asians

	Sample size[1]		EAF[2]		Per-allele association		Heterogeneity	
Study	Cases	Controls	Cases	Controls	OR (95% CI)[3]	*P*-value	$P_{heterogeneity}$[4]	I^2 (%)
Stage 1	1,773	2,642	0.186	0.152	1.32 (1.17–1.50)	1.22×10^{-5}	0.269	24
Stage 2	6,800	7,761	0.176	0.153	1.20 (1.13–1.28)	3.39×10^{-8}	0.897	0
Overall	8,573	10,403	0.178	0.152	1.22 (1.15–1.29)	2.93×10^{-11}	0.726	0
Chinese	3,670	4,204	0.170	0.145	1.20 (1.10–1.32)	6.65×10^{-5}	0.958	0
Korean	2,525	2,889	0.215	0.185	1.21 (1.09–1.33)	1.85×10^{-4}		
Japanese	2,378	3,310	0.151	0.133	1.23 (1.10–1.38)	3.21×10^{-4}		
Male	3,542	4,338	0.190	0.156	1.28 (1.17–1.40)	2.88×10^{-8}	0.268	19
Female	2,456	3,175	0.200	0.174	1.19 (1.07–1.32)	8.13×10^{-4}		

[1]Data from Japan-BioBank and SCH studies were not included in the stratified analysis by sex.
[2]Effect allele (A) frequency of cases and controls.
[3]OR was estimated based on the effect allele (A).
[4]*P* for heterogeneity across studies was calculated using a Cochran's *Q* test.

been reported in previous GWAS conducted in European descendants to be associated with CRC risk.[5] In a fine-mapping analysis, rs58920878 was identified as a potential causal variant in this region.[33] SNP rs7229639 is not correlated with any of four previously reported risk variants in this region in East Asians with r^2 all under 0.05 (data from the 1,000 Genomes Project). In European descendants, rs7229639 was weakly correlated with r4939827 ($r^2 = 0.146$) and not correlated with any of the four other SNPs ($r^2 < 0.07$). Data for these four previously reported SNPs were available in Stage 1, and three of the SNPs showed statistically significant (rs4939827) or marginally significant (rs58920878 and rs12953717) association with CRC risk in our Stage 1 (Table 3, Supporting Information Table S1). Of these previously reported risk variants, rs4939827 showed the strongest association with CRC risk (OR = 0.89, 95% CI, 0.80–0.98, $p = 0.022$) in Stage 1 of our study. Because rs4939827 is in LD with both rs58920878 and rs12953717 in Asians ($r^2 > 0.8$), we selected rs4939827 for additional genotyping in Stage 2 ($p = 0.008$) (data not shown). A combined analysis of samples from both Stages 1 and 2 yielded a per-allele OR (95% CI) of 0.90 (0.85–0.96) ($p = 0.001$) (data not shown).

Conditional analyses were performed to determine whether the observed association with rs7229639 was independent of the other GWAS-identified SNPs in this region (Table 3). The association with rs7229639 remained statistically significant after adjusting for any of these previously GWAS-identified SNPs individually or in combination. Interestingly, adjusting for rs7229639 strengthened the associations of CRC risk with three (rs4939827, rs58920878 and rs12953717) of the four SNPs reported from previous GWAS. Because these three previously reported SNPs were strongly correlated, we selected rs4939827 for further evaluation with rs7229639 in haplotype analysis. Haplotype analysis of rs7229639 and rs4939827 revealed two common haplotypes, A-C and G-T, to be statistically significantly associated with CRC risk (Table 4). For haplotype A-C, the strength of association with CRC (OR = 1.68, 95% CI, 1.37–2.07; $p = 9.92 \times 10^{-7}$) was greater than that of either rs7229639 (OR = 1.22, 95% CI, 1.11–1.33; $p = 3.43 \times 10^{-5}$) or rs4939827 (OR = 0.90, 95% CI, 0.83–0.97; $p = 0.007$) alone. When dominant model was applied, three minor haplotypes were associated with a 1.49-fold (95% CI, 1.29–1.72) increased risk of CRC ($p = 5.01 \times 10^{-8}$).

Discussion

In this two-stage GWAS of CRC including 8,675 cases and 10,504 controls from China, Korea, Japan and Singapore, we identified a new genetic variant (rs7229639) in the *SMAD7* gene to be associated with CRC risk at genome-wide significance level. The association of this SNP with CRC risk remained highly statistically significant after adjusting for all four other risk variants reported previously in this region,[5,33] providing evidence for the presence of multiple genetic risk variants in the *SMAD7* gene for CRC. SNP rs7229639 explained approximately 0.75% of the familial relative risk of CRC in East Asians, while rs4939827, the risk variant identified originally in GWAS, explained about 0.3% of familial relative risk. When haplotypes of these two variants are considered, these variants would explain 1.9% of familial relative risk, approximately six-fold the 0.3% explained by the original risk variant (rs4939827).

SNP rs7229639 (46,450,976 bp, NCBI Human Genome Build 37.3) is located in intron 3 of the *SMAD7* gene at chromosome 18q21.1. In this region, three other variants including rs4939827 (46,453,463 bp), rs12953717 (46,453,929 bp) and rs4464148 (46,459,032 bp) have been found to be associated with CRC risk through GWAS conducted in European-ancestry populations.[5] A subsequent fine-mapping through resequencing 17 kb region of 18q21.1 in a study conducted in European descendants identified a new variant, rs58920878 (46,449,565 bp) as a potential causal variant at this locus.[33] SNP rs58920878 showed the strongest

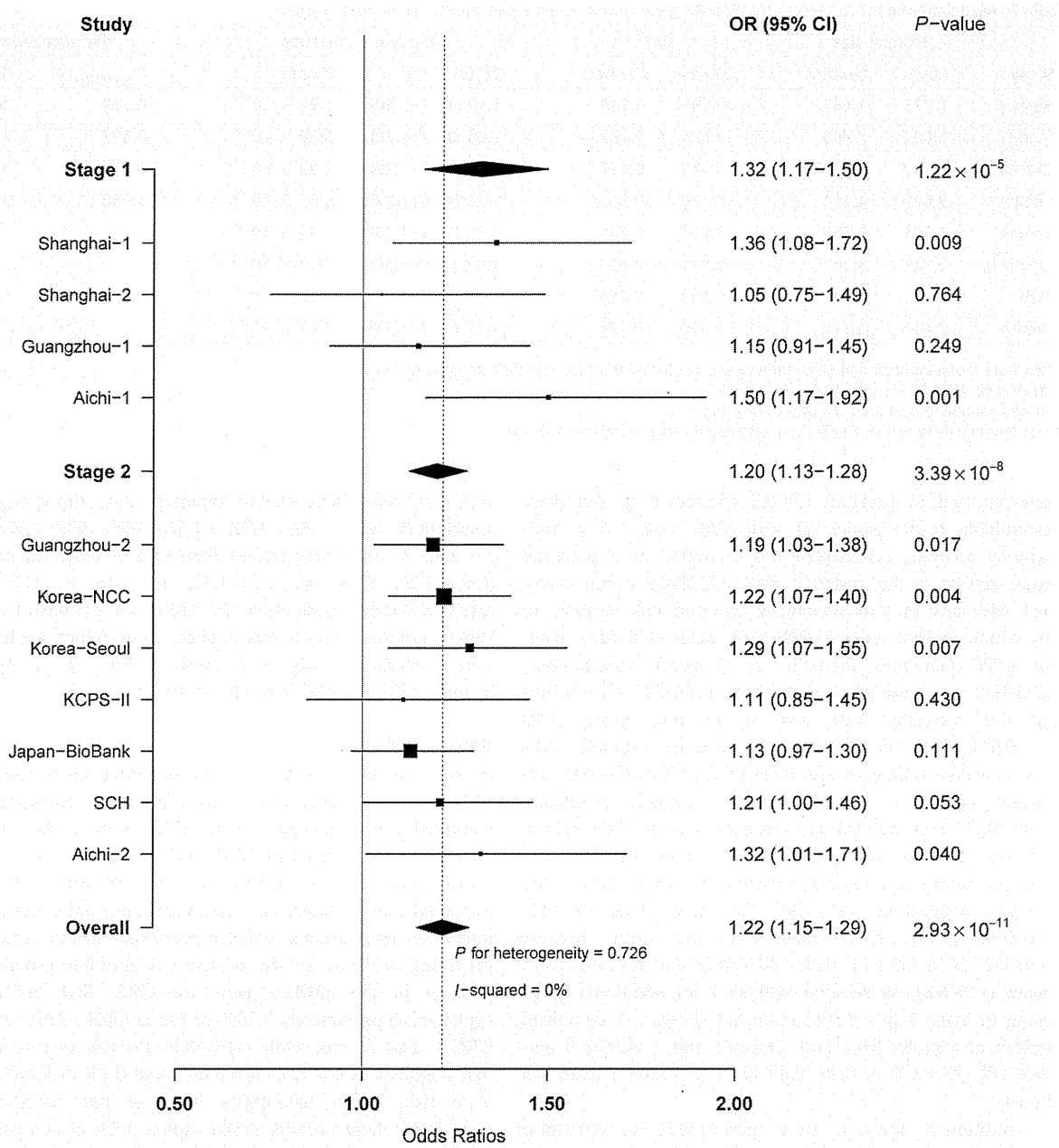

Figure 1. Association of rs7229639 with colorectal cancer risk by studies. Per-allele ORs are presented with the area of the box proportion to the inverse variance weight of the estimate. Horizontal lines show 95% CIs.

association in that study and is correlated with rs4939827 ($r^2 = 0.533$), rs12953717 ($r^2 = 0.927$) and rs4464148 ($r^2 = 0.327$) in European descendants. Although rs58920878 is also in high LD with both rs4939827 ($r^2 = 0.830$) and rs12953717 ($r^2 = 0.785$) in East Asians, it was only weakly associated with CRC risk in our study. The SNP (rs7229639) we identified in this study showed the strongest association with CRC risk and is not correlated with any of the four previously reported SNPs in both East Asian and European populations. Therefore, as expected, adjustment for any or all of these four SNPs did not attenuate the association of rs7229639 with CRC risk in the current study. Haplotype analyses further confirmed the independent association of rs7229639 and rs4939827 with CRC risk in this locus. It is possible that multiple causal variants may be present in this locus among East Asians.

The *SMAD7* gene is a key member in TGF-β family signaling pathway, which has been shown to play a dual role in

Table 3. Evaluation of independent association of rs7229639 and other four risk variants in the *SMAD7* gene in relation to colorectal cancerCRC risk

Test SNP (allele)	Adjusted SNP(s)	Cases/controls	EAF[1]	OR (95% CI)	*P*-value
rs58920878 (C)	None	1,773/2,642	0.734	0.92 (0.82–1.02)	0.097
rs7229639 (A)	None	4,840/5,925	0.156	1.25 (1.16–1.34)	5.25×10^{-9}
rs4939827 (C)	None	4,840/5,925	0.727	0.90 (0.85–0.96)	8.87×10^{-4}
rs12953717 (C)	None	1,773/2,642	0.733	0.92 (0.83–1.01)	0.085
rs4464148 (C)	None	1,773/2,642	0.059	0.97 (0.80–1.18)	0.770
rs7229639 (A)	rs58920878	1,773/2,642	0.152	1.40 (1.23–1.60)	4.19×10^{-7}
rs7229639 (A)	rs4939827	4,840/5,925	0.156	1.30 (1.20–1.40)	1.87×10^{-11}
rs7229639 (A)	rs12953717	1,773/2,642	0.152	1.40 (1.23–1.60)	3.97×10^{-7}
rs7229639 (A)	rs4464148	1,773/2,642	0.152	1.32 (1.17–1.50)	1.25×10^{-5}
rs7229639 (A)	All four SNPs[2]	1,773/2,642	0.152	1.41 (1.23–1.61)	8.96×10^{-7}
rs58920878 (C)	rs7229639	1,773/2,642	0.734	0.84 (0.76–0.94)	0.002
rs4939827 (C)	rs7229639	4,840/5,925	0.727	0.86 (0.81–0.92)	2.57×10^{-6}
rs12953717 (C)	rs7229639	1,773/2,642	0.733	0.85 (0.76–0.94)	0.002
rs4464148 (C)	rs7229639	1,773/2,642	0.059	1.02 (0.84–1.24)	0.854

Abbreviations: EAF, effect allele frequency; OR, odds ratio; and CI, confidence interval.
[1]Effect allele frequency of the tested SNP in controls.
[2]rs58920878, rs4939827, rs12953717 and rs4464148.

Table 4. Association of colorectal cancerCRC risk with the haplotypes comprising rs7229639 and rs4939827

	Frequency[2]			
Haplotype[1]	Cases	Controls	OR (95% CI)[3]	*P*-value
G-C	0.537	0.582	1.00 (Reference)	
G-T	0.273	0.258	1.41 (1.18–1.67)	1.01×10^{-4}
A-C	0.176	0.149	1.68 (1.37–2.07)	9.92×10^{-7}
A-T	0.013	0.010	1.33 (0.54–3.26)	0.532
G-T/A-C/A-T	0.463	0.418	1.49 (1.29–1.72)	5.01×10^{-8}

[1]Haplotypes of rs7229639 and rs4939827.
[2]Analyses were based on 3,067 cases and 3,283 controls included in Stage 2.
[3]Adjusted for age, sex and study site.

carcinogenesis, including tumor suppressor in early stage and oncogene in advanced stage of cancers.[34] Multiple genes in this pathway are known to be involved in colorectal pathogenesis.[35] *SMAD7* gene encodes Smad family member 7 (Smad7), an inhibitory protein that functions as an antagonist of TGF-β signaling by blocking phosphorylation of receptor-activated Smads or by competitive inhibition of complex formation of receptor-activated Smads with the common-mediator Smad4 in the cytoplasm and nucleus.[36] Smad7 also serves as an important cross-talk mediator of the TGF-β signaling pathway with other signaling pathways including Wnt signaling.[36] Expression of Smad7 was found in both normal colon mucosa and tumor cells,[37] and aberrant Smad7 expression may influence CRC progression.[38] Recently, Smad7 was shown to induce colorectal tumorigenicity through blocking TGF- β-induced growth inhibition and inhibiting apoptosis, and a certain proportion of human colorectal tumors may become refractory to tumor-suppressive actions of TGF-β that might lead to increased tumorigenicity.[39]

A recent study has showed that the previously identified CRC risk variant rs4939827 may be associated with CRC survival.[40]. This SNP was associated with certain characteristics of CRC, including invasiveness of the cancer and *RUNX3* methylation status.[41] No survival data, however, were collected in our study, and thus we could not evaluate these *SMAD7* variants, including the newly identified risk variant rs7229639, with CRC survival in our study.

In summary, this study identified a new CRC risk variant in the *SMAD7* gene among East Asians, which further highlights the significant role of this gene in the etiology of CRC. To date, multiple CRC susceptibility loci have been identified by GWAS in TGF-β pathway genes, including *SMAD7*, supporting an important role of this pathway in the pathogenesis of CRC. Future studies are warranted to investigate these CRC susceptibility loci to identify

causal variants underlying the associations discovered in GWAS.

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Impact of *PSCA* Variation on Gastric Ulcer Susceptibility

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Abstract

Peptic ulcer is one of the most common gastrointestinal disorders with complex etiology. Recently we conducted the genome wide association study for duodenal ulcer and identified disease susceptibility variations at two genetic loci corresponding to the *Prostate stem cell antigen* (*PSCA*) gene and the *ABO blood group* (*ABO*) gene. Here we investigated the association of these variations with gastric ulcer in two Japanese case-control sample sets, a total of 4,291 gastric ulcer cases and 22,665 controls. As a result, a C-allele of rs2294008 at *PSCA* increased the risk of gastric ulcer with odds ratio (OR) of 1.13 (*P* value of 5.85×10^{-7}) in an additive model. On the other hand, SNP rs505922 on *ABO* exhibited inconsistent result between two cohorts. Our finding implies presence of the common genetic variant in the pathogenesis of gastric and duodenal ulcers.

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Introduction

Peptic ulcer is the most common disease in the gastrointestinal tract with symptoms of nausea, vomiting, and abdominal pain, and sometimes causes bleeding and perforation with acute peritonitis. Lifetime prevalence of peptic ulcer is 10–15% in the Japanese and 4–10% in Caucasians [1–3]. Approximately 70% of gastric ulcer patients and 90% of duodenal ulcer patients are associated with *H. pylori* infection [4]. Since eradication of *H. pylori* by antibiotics in combination with proton pomp inhibitor can effectively cure peptic ulcer [5], *H. pylori* is shown to be the major cause of peptic ulcer. Although nearly 50% of individuals on the earth are infected with *H. pylori*, most of them remain asymptomatic indicating that the clinical outcome after the *H. pylori* infection varies substantially between individuals. These inter-individual diversities are affected by various factors including bacteria subtypes, host response, and their interaction. *Duodenal ulcer promoting gene A* (*dupA*) in *H. pylori* was indicated to induce interleukin (IL)-8 that increases the risk of duodenal ulcer and decreases the risk of gastric cancer [6,7]. Nonsteroidal anti-inflammatory drugs (NSAIDs) and smoking are known risk factors for peptic ulcer [8,9]. In addition to these bacterial and environmental factors, host genetic factors had been implicated to have some roles in the risk of peptic ulcer. Proband-wise concordance rate of peptic ulcer in monozygotic twins was as high as 23.6% while that in dizygotic twins was 14.8%. Several candidate gene approaches revealed the possible association of genetic variations in *IL-6*, *IL-8*, *IL-10* [10], *TNF*, *LTA* [11], and *COX1* [12] with peptic ulcer risk.

In our previous genome wide association study (GWAS) of duodenal ulcer using a total of 7,035 cases and 25,323 controls, we identified the significant association of genetic variations at *PSCA* (*prostate stem cell antigen*) and the *ABO* blood group with duodenal ulcer [13]. The C allele of rs2294008 at *PSCA* increased the risk of duodenal ulcer (odds ratio (OR) of 1.84 with *P* value of 3.92×10^{-33}) in a recessive model, while it decreased the risk of gastric cancer (OR of 0.79 with *P* value of 6.79×10^{-12}) as reported previously [14]. Our functional analyses revealed that the T allele of SNP rs2294008 creates an upstream translational initiation codon and add the signal peptide sequences at the N-terminal portion, resulting in alteration of the protein subcellular localization from cytoplasm to cell surface. SNP rs505922 on *ABO* was also associated with duodenal ulcer in a recessive model (OR of 1.32 with *P* value of 1.15×10^{-10}). Since *H. pylori* infection and non-steroidal anti-inflammatory drugs induce gastroduodenal mucosal injury which would cause duodenal and gastric ulcer, we examined the role of variants in the *PSCA* and *ABO* genes on gastric ulcer risk among Japanese population.

Results

A total of 4,291 gastric ulcer cases and 22,665 controls without having the past history of duodenal ulcer or continuous NSAID intake were recruited from the BioBank Japan and the Aichi

Cancer Center (**Table 1**). We then genotyped SNP rs2294008 and rs505922 in two case-control sample sets and examined the association with gastric ulcer in three genetic models (additive, recessive, and dominant model) (**Table 2**). To increase the statistical power of this study, we used subjects with either of 22 diseases as control samples. Therefore we evaluated the confounding effect of disease mix control samples used in this analysis. SNPs rs2294008 and rs505922 did not show significant association between case-mix controls (n = 19,884) and healthy volunteers (n = 2,781) (**Table S1**). In addition, both SNPs did not show the significant deviation from HWE (Hardy-Weinberg equilibrium) in each disease group. Therefore disease mix controls seem not to largely affect the association result in our analysis.

The results of association analyses revealed that gastric ulcer patients had a higher frequency of C allele at rs2294008 than the control group in both sets (39.7% vs 36.9% and 40.1% vs 37.0%, respectively). A meta-analysis of the two studies showed the significant association of rs2294008 in an additive model with no evidence of heterogeneity ($P = 5.85 \times 10^{-7}$ with OR of 1.13), although the association was not statistically significant among Aichi Cancer Center cohort probably due to smaller sample size. Risk alleles (C allele at rs2294008) in the two sample sets were consistent between duodenal ulcer and gastric ulcer, indicating the role of *PSCA* variation as common genetic factors for peptic ulcer. However impact of this variation on gastric ulcer risk was not as strong as those on duodenal ulcer reported previously [13].

On the other hands, SNP rs505922 showed inconsistent results between two cohorts. A T allele of rs505922 increased gastric ulcer risk in all three genetic models in BioBank Japan cohort. However, gastric ulcer patients exhibited lower frequency (53.5%) of a T allele than the healthy controls (55.1%) in the Aichi Cancer Center cohort. Therefore, further association analysis is essential to determine the role of *ABO* variations on gastric ulcer susceptibility.

Since we have genotyping results of 1,862 gastric ulcer cases and 17,482 controls analyzed by Illumina Human Hap610-Quad genechip, we conducted whole genome screening using these sample set. Although 62 SNPs exhibited suggestive associations with *P* values of less than 1×10^{-4}, no SNPs cleared genome wide significant threshold (**Table S2** and **Figure S1**). Thus, our sample set did not have sufficient statistical power to detect gastric ulcer susceptibility loci by GWAS.

We also investigated the association of previously reported genes with gastric ulcer (**Table 3**). We selected 32 SNPs at five gene loci that had been genotyped by Illumina Human Hap610-Quad genechip. As a result, two loci at *LTA* and *PTGS1* indicated suggestive association ($P = 1.64 \times 10^{-3}$ and 0.0376), although these associations were not statistically significant after Bonferroni's correction ($P < 0.00156 = 0.05/32$). Thus further analyses are necessary to elucidate the role of these variations on gastric ulcer.

Discussion

The development of gastric ulcer is determined by the interplay between gastric acid secretion and mucosal resistance, however their underling pathogenesis has not been fully elucidated. Gastric mucus, a gelatinous material secreted by gastric mucous cells, serves as an unstirred layer through which the diffusion of acid and pepsin is reduced. We here found that variation in the *PSCA* gene was significantly associated with gastric ulcer. PSCA was initially identified as a tumor antigen that was highly expressed in prostate, bladder, and pancreatic cancer tissues [15,16]. Since tumor cells treated with anti-PSCA antibody exhibited a growth suppressive effect [17,18], cell surface-PSCA is considered to play an important role in cell proliferation. In contrast, down-regulation of PSCA in gastric and esophageal cancer tissues was also reported [19,20]. Thus the role of PSCA in carcinogenesis is still controversial [21]. These diverse effects of PSCA among various cancer types might be partially explained by the effect of genetic variation. Individuals carrying the T allele at rs2294008 express PSCA proteins with an additional fragment of nine amino acids at the N-terminal portion [13]. On the other hand, individuals carrying the C allele at rs2294008 express a shorter PSCA protein which lacks the signal peptide and is predicted to be localized in the cytoplasm without glycosylation [22]. We also found that the cytosolic shorter PSCA protein was more susceptible to proteasomal degradation than the long PSCA protein at the cell-surface. Since PSCA-derived peptides were reported to be a target of T-cell-based immunotherapy for advanced prostate cancer [23], the shorter PSCA protein would cause the activation of CD4-positive and/or CD8-positive T cells and subsequently promote epithelial mucosal injury [24]. In contrast, the long PSCA protein at the cell surface might facilitate mucosal repair by enhancing epithelial cell proliferation. In addition, T allele of SNP rs2294008 was shown to be associated with higher mRNA and protein expression [25]. Thus the impact of PSCA on gastric ulcer and carcinogenesis could be regulated by the *PSCA* variation.

H. pylori plays an important role in the development of gastritis, peptic ulcers, and gastric cancer, and the eradication of *H. pylori* was shown to reduce the recurrence of gastric ulcer [26] and prevent the onset of gastric cancer [27]. Since vertical transmission during childhood is the major source of infection, family history of *H. pylori* infection or *H. pylori*-related diseases is a risk factor for *H. pylori* infection [28–30]. In addition, recent accumulated evidences

Table 1. Characteristics of study population.

Samples	Source	Platform	Number of samples	Female (%)	Age (mean +/– SD)
Gastric ulcer[a]	BioBank Japan	Illumina HumanHap 610	1,862	32.0 (%)	66.0+/–10.7
		Invader assay	2,004	35.3 (%)	66.5+/–11.7
	Aichi Cancer Center	TaqMan	425	48.7 (%)	55.6+/–12.3
Control[a]	BioBank Japan[b]	Illumina HumanHap 610	17,482	54.1 (%)	62.2+/–13.0
	BioBank Japan[b]	Illumina HumanHap 550	3,309	66.0 (%)	43.8+/–16.2
	Aichi Cancer Center	TaqMan	1,874	38.7 (%)	53.7+/–14.6

[a]Subjects with a history of gastric cancer or duodenal ulcer were excluded from cases and controls.
[b]Control samples consist of patients with colon cancer, breast cancer, diabetes, arteriosclerosis obliterans, atrial fibrillation, brain infarction, drug response, amyotrophic lateral sclerosis, liver cancer, liver cirrhosis, osteoporosis, fibroid, cervical cancer, chronic hepatitis B, ovarian cancer, pulmonary tuberculosis, keloid, drug eruption, hematological cancer, uterus cancer, heat cramp, endometriosis, and 907 healthy volunteers.
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Table 2. Association of PSCA and ABO SNPs with gastric ulcer.

SNP		Case			Control			Additive				Recessive				Dominant			
Chr		CC	CT	TT	RAF[a]	CC	CT	TT	RAF[a]	P [b]	OR [b] (95% C.I.)	P_{Het} [c]	P [b]	OR [b] (95% C.I.)	P_{Het} [c]	P [b]	OR [b] (95% C.I.)	P_{Het} [c]
rs2294008 8q24/*PSCA*	BBJ	##	###	###	#####	###	9577	###	#####	2.55×10^{-6}	### (1.07–1.18)		6.12×10^{-4}	1.18 (1.07–1.30)		3.52×10^{-5}	### (1.08–1.25)	
	ACC	70	201	154	#####	235	917	722	#####	9.09×10^{-2}	### (0.98–1.33)	#####	3.10×10^{-2}	1.38 (1.03–1.84)	#####	0.380	### (0.89–1.37)	0.657
	meta[d]									5.85×10^{-7}	### (1.08–1.18)		8.94×10^{-5}	1.20 (1.09–1.31)		2.60×10^{-5}	### (1.08–1.24)	
rs505922 9q34/*ABO*	BBJ	##	###	###	#####	###	#####	###	#####	9.39×10^{-4}	### (1.03–1.14)	6.91×10^{-2}	7.72×10^{-4}	1.13 (1.05–1.22)		5.26×10^{-2}	### (1.00–1.19)	7.90×10^{-2}
	ACC	97	201	127	#####	379	925	570	#####	0.407	### (0.81–1.09)		0.829	0.97 (0.77–1.23)	#####	0.232	### (0.67–1.10)	
	meta[d]									3.88×10^{-3}	### (1.02–1.12)		1.75×10^{-3}	1.12 (1.04–1.20)		0.141	### (0.98–1.15)	

We analyzed 4,291 gastric ulcer cases and 22,665 controls.

[a]rs2294008 (C allele) and rs505922 (T allele).

[b]*P* values were obtained using chi-square test. To calculate odds ratios (OR), non risk alleles were considered as references.

[c]Heterogeneity across two stages was assessed by Cochran Q test.

[d]OR and P values were obtained using the Mantel-Haenszel fixed-effects model in the meta analysis.

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revealed a number of risk factors of gastric cancer (T allele at rs2294008, blood type A, decreased gastric acid, intake of proton pump inhibitor/H_2 blocker, and *CagA* in *H. pylori* [31]) or peptic ulcer (C allele at rs2294008, blood type O, NSAID intake, *dupA* in *H. pylori*) [32]. In addition, *CYP2C19* genotype was associated with the response to triple anti-*H. pylori* therapy including proton pump inhibitor [33]. However, our previous analysis revealed that SNP rs2294008 and rs505922 did not associated with *H. pylori* prevalence [13]. Taking the above information into account, the estimation of disease risk and drug efficacy would enable us to determine the appropriate treatment protocol for *H. pylori* carriers.

Here we found that *PSCA* variant was significantly associated with gastric ulcer. In our previous analysis, PSCA variation did not associate with *H. pylori* prevalence [13]. Since *H. pylori* infection was associated with many diseases such as MALT lymphoma [34], idiopathic thrombocytopenic purpura [35], atrophic gastritis [36], and NSAID-induced gastric ulcer, it is very interesting to evaluate the effect of *PSCA* variation on these diseases. We hope our findings would contribute to the elucidation of disease pathogenesis as well as to the establishment of personalized medical treatments in the future.

Methods

Ethics Statement

This research project was approved by the ethical committees at the University of Tokyo, RIKEN, and Aichi Cancer Center. All participants provided written informed consent as approved by the ethical committees of the University of Tokyo and Aichi Cancer Center.

Study participants

The demographic details of study participants are summarized in Table 1. A total of 3,866 gastric ulcer patients, and 20,791 gastric ulcer negative controls were obtained from BioBank Japan that was initiated in 2003 with the funding from the Ministry of Education, Culture, Sports, Science and Technology, Japan [37]. In the BioBank Japan Project, DNA and serum of patients with 47 diseases were collected through collaborating network of 66 hospitals throughout Japan. The list of participating hospitals is shown in the following website (http://biobankjp.org/plan/member_hospital.html). A total of 425 gastric ulcer cases and 1,874 healthy controls were obtained from the Aichi Cancer Center. The diagnosis of gastric ulcer was based on clinical, endoscopic, and histological features. List of disease-mix control samples used in this study was shown in **Table S1**. We excluded patients with duodenal ulcer or gastric cancer from both cases and controls. Deregulation of PSCA was reported in many types of malignancy such as prostate, pancreatic, lung, bladder, gastric, cholangiocarcinoma, and esophageal cancer [14–16,20,38,39]. In addition, *ABO* locus was previously shown to be associated with various diseases such as myocardial infarction and pancreatic cancer [40,41]. Therefore, we excluded subjects with these diseases from case mix controls. We also excluded the subjects with continuous NSAID intake.

SNP Genotyping

Genotyping platforms used in this study are shown in Table 1. A total of 1,862 gastric ulcer cases and 20,791 gastric ulcer negative control samples were genotyped with Illumina Human Hap610-Quad or with Human Hap550v3. The other samples were genotyped by the Invader assay system (Third Wave Technologies, Madison, WI) or Taqman assay.

Table 3. Association of variations on candidate genes with Gastric ulcer.

SNP	Gene	relative loc	Chr	Position	Gastric ulcer		
					[a] *P*	[b] OR (95% C.I.)	
rs3024505	*IL10*	1044	1	2.05E+08	0.817	###	(0.71–1.31)
rs3024498	*IL10*	0	1	2.05E+08	0.569	###	(0.55–3.01)
rs1554286	*IL10*	0	1	2.05E+08	0.911	###	(0.93–1.08)
rs3021094	*IL10*	0	1	2.05E+08	0.178	###	(0.98–1.12)
rs3024490	*IL10*	0	1	2.05E+08	0.902	###	(0.93–1.07)
rs2222202	*IL10*	0	1	2.05E+08	0.975	###	(0.74–1.33)
rs1800896	*IL10*	−1058	1	2.05E+08	0.766	###	(0.87–1.20)
rs2844484	*LTA*	−3869	6	31644203	4.41×10^{-2}	###	(1.00–1.15)
rs2009658	*LTA*	−1849	6	31646223	0.453	###	(0.94–1.14)
rs2844482	*LTA*	−326	6	31647746	0.484	###	(0.94–1.13)
rs1800683	*LTA*	−22	6	31648050	1.64×10^{-3}	###	(0.83–0.96)
rs2229094	*LTA*	0	6	31648535	0.163	###	(0.98–1.16)
rs2229092	*LTA*	0	6	31648736	0.295	###	(0.90–1.42)
rs1041981	*LTA*	0	6	31648763	1.75×10^{-3}	###	(0.83–0.96)
rs3093662	*TNF*	0	6	31652168	0.220	###	(0.91–1.51)
rs3093668	*TNF*	383	6	31654474	0.335	###	(0.87–1.51)
rs833068	*VEGFA*	0	6	43850505	0.427	###	(0.96–1.10)
rs833069	*VEGFA*	0	6	43850557	0.401	###	(0.96–1.10)
rs3025010	*VEGFA*	0	6	43855555	0.976	###	(0.93–1.07)
rs3025033	*VEGFA*	0	6	43859053	0.282	###	(0.96–1.14)
rs3025035	*VEGFA*	0	6	43859337	0.841	###	(0.93–1.09)
rs6900017	*VEGFA*	4261	6	43866463	9.77×10^{-2}	###	(0.99–1.15)
rs2069837	*IL6*	0	7	22734552	0.728	###	(0.93–1.12)
rs2066992	*IL6*	0	7	22734774	0.953	###	(0.93–1.09)
rs1554606	*IL6*	0	7	22735232	0.813	###	(0.61–1.86)
rs10242595	*IL6*	2611	7	22740756	0.799	###	(0.87–1.11)
rs1236913	*PTGS1*	0	9	1.24E+08	0.389	###	(0.74–1.12)
rs1213266	*PTGS1*	0	9	1.24E+08	0.263	###	(0.81–1.06)
rs4836885	*PTGS1*	0	9	1.24E+08	0.964	###	(0.82–1.23)
rs6478565	*PTGS1*	0	9	1.24E+08	0.318	###	(0.78–1.08)
rs10306163	*PTGS1*	0	9	1.24E+08	0.119	###	(0.80–1.03)
rs10306202	*PTGS1*	1540	9	1.24E+08	3.76×10^{-2}	###	(0.75–0.99)

We analyzed 1,862 gastric ulcer cases and 17,482 controls in this analysis. Chr.,chromosome; Postion in the NCBI Build 36.3.
[a]P values were calculated by Cochran Armitage trend test.
[b]OR, odds ratio was calculated by considering the major allele as the reference.
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Statistical Analysis

The association of SNPs rs2294008 and rs505922 with gastric ulcer was tested by chi-square test. The Odds ratios were calculated by considering the protective allele as the reference allele. The association of SNPs genotyped by Illumina Human Hap610-Quad with gastric ulcer was tested by multivariate logistic regression analysis upon adjusting for age at recruitment and gender using PLINK [42]. Heterogeneity across two stages was examined by Cochran Q test [43].

Supporting Information

Figure S1 Manhattan plot showing the genome-wide P values of association. The P values were obtained by logistic regression analysis upon adjustment for age and gender. The y-axis represents the –log10 P values of 480,566 SNPs, and their chromosomal positions are shown on x-axis.
(TIF)

Table S1 Genotype frequency of two SNPs in disease mix controls.
(DOCX)

Table S2 The result of association analysis of Gastric ulcer in GWAS.
(DOCX)

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Author Contributions

Conceived and designed the experiments: CT K. Matsuda YN. Performed the experiments: CT K. Matsuo MK. Analyzed the data: CT AT NK HI. Contributed reagents/materials/analysis tools: HT YY KT KY. Wrote the paper: CT YN K. Matsuda.

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Review

Cell PRESS

Putting the brakes on anticancer therapies: suppression of innate immune pathways by tumor-associated myeloid cells

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Accumulating evidence has revealed that immunogenic cell death triggered by particular chemotherapeutic agents plays a critical role in harnessing antitumor immunity to clinical responses. However, negative regulatory pathways exist which suppress the induction of effective immune responses by a broad spectrum of anticancer therapies including 'non-immunogenic' regimens. Tumor-associated myeloid cells are unique in that they are capable of manipulating responses to anticancer drugs by utilizing negative regulatory factors of innate immune pathways, including damage-associated molecule-mediated pattern recognition and tolerogenic phagocytosis. Further elucidation of the molecular mechanisms regulating innate immune responses of tumor-associated myeloid cells under cellular stress should enhance the development of new molecular targeting therapies for patients with treatment–refractory cancers.

Tumor microenvironments determine the directions of chemotherapeutic responses

Various intrinsic mechanisms, such as genetic alterations, chromatin modifications, and enrichment of cancer stem/initiating cells, negatively regulate the responses to anticancer therapeutics. Conversely, accumulating evidence has revealed that the interactions of tumor cells with non-transformed cells such as stromal cells, endothelial cells, and inflammatory cells are a determining factor in manipulating tumorigenic activities and tumor responses to anticancer drugs [1–3]. In addition, tumor-associated stromal cells may modify the biological properties of tumorigenic cells, thereby contributing to the acquisition of anticancer drug resistance [4,5]. For example, genotoxic stresses triggered by chemotherapeutic agents stimulate paracrine secretion of interleukin (IL)-6 and a tissue inhibitor of metalloproteinases-1 (TIMP-1) from thymic endothelial cells in a p38-dependent manner and impede therapeutic responses to the chemotherapeutic agent doxorubicin [6]. Tumor-associated stromal fibroblasts facilitate the release of hepatocyte growth factor (HGF) and Wnt16B, which attenuates the therapeutic effects of

Glossary

Chemoresistant niche: although cell intrinsic processes play a major role in chemotherapeutic responses, non-transformed cells, such as fibroblasts, myeloid cells, etc., in tumor microenvironments contribute to support tumor cell survival following the administration of chemotherapeutic agents. In this case, coordinated activation of genotoxic stress signals and inflammation specifically triggered by chemotherapy serves as a driving force to activate non-transformed cells, leading to the release of various soluble mediators and the upregulation of molecules that contribute to chemoresistance.
Damage-associated molecular patterns (DAMPs): danger signals initiate and activate innate immune signals in the non-infectious, sterile inflammatory response. DAMPs are composed of various types of endogenous proteins that are normally sealed in the cytoplasm or nucleus of cells. Necrotic cell death and various cellular stresses facilitate extracellular release of DAMPs, leading to interactions with pattern recognition receptors such as TLRs and stimulation of innate immune signals.
ER stress response: the ER senses various processes of post-translational modifications of proteins, including biosynthetic pathways, protein folding, intracellular trafficking, etc. Cellular stresses, including genotoxic insults by chemotherapy, disturb ER homeostasis and initiate the unfolded protein signals mediated by RNA-dependent protein kinase (PKR)-like ER kinase (PERK), inositol requiring kinase 1 α (IRE1α), and activating transcription factor 6 (ATF6) to establish ER homeostasis and cellular survival. Chemotherapeutic agents regulate mitochondrial stress pathways such as cell death signals and ROS responses by regulating unfolded protein signals.
Phagocytosis receptors: dying cells induced by cytotoxic agents are subjected to phagocytosis through pattern recognition of various types of receptors via molecular markers expressed on dying cells, such as PS and CD47. The phagocytosis of dying cells stimulates or silences inflammation and antigen-specific immunity, which is dependent on the repertoires of receptors or environments where dying cells are recognized.
Tumor-associated myeloid cells: myeloid cells are derived from bone marrow precursors of the granulocytic lineage and consist of various types of cells including monocytes, macrophages, DCs, and granulocytes. Tumor microenvironments recruit myeloid cells and educate them to change their phenotypic and functional properties in support of tumorigenicity and chemoresistance.
Tumor microenvironments: tumors are composed of not only tumor cells but also normal cells surrounding the tumors, such as fibroblasts, myeloid cells, lymphocytes, and endothelial cells. In addition, tumors contain the extracellular matrix and collagens, which support the integrity and homeostasis of tumors. These compositions create tumor microenvironments, in which cellular and extracellular factors communicate with each other and determine tumorigenic activities and responsiveness to anticancer therapeutics.

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BRAF-targeted therapy against melanomas and chemotherapy against prostate cancers, respectively [7,8]. Therefore, tumor-resident normal cells render tumor cells capable of acquiring chemoresistant phenotypes through various tumorigenic mediators that contribute to tumor survival.

Recent studies have revealed that tumor-associated myeloid cells (see Glossary) play a critical role in tumor progression and metastasis by promoting the secretion of soluble factors, angiogenesis, and matrix remodeling [9]. Furthermore, tumor-associated myeloid cells powerfully modulate responsiveness to various anticancer modalities through multiple molecules and signaling pathways in tumor microenvironments. Herein, we highlight the impact that tumor-mediated regulation of myeloid innate immune pathways plays in creating the chemoresistant niche.

Tumor-associated myeloid cells generate chemoresistant niches

The immune system provides both pro- and antitumorigenic effectors in the course of tumor progression. Likewise, tumor microenvironments have a profound impact on the biological behaviors of tumor-infiltrating leukocytes on responses to anticancer agents. In particular, myeloid cells display phenotypic plasticity when encountering different tumor microenvironments. Macrophage polarization is tightly regulated through various transcription factors such as interferon regulatory factor (IRF), signal transducer and activator of transcription (Stat), nuclear factor-κB (NF-κB), and peroxisome proliferator-activated receptor (PPAR). The epigenetic changes mediated by histone acetylase and methylase also influence macrophage differentiation and function. In particular, tumor-associated macrophages (TAMs) with M2-type characteristics have protumorigenic and immunosuppressive properties. This phenotypic diversity of macrophages may serve as a critical factor in regulating tumorigenic activities and anticancer therapeutic responses [10,11]. For example, the concomitant depletion of M2-type macrophages and activation of M1-type macrophages are responsible for eliciting antitumor responses by taxane and trabectedin [12,13].

Monocytes are composed of various subsets that contribute to tumorigenic activities, such as those expressing CCR2 and the angiopoetin-2 receptor Tie2 [14,15]. For example, $CCR2^+$ inflammatory monocytes serve as a major precursor of protumorigenic macrophage which may negatively regulate antitumor responses of chemotherapy [9,14]. Moreover, Tie2-expressing monocytes suppress the therapeutic effects of vascular-disrupting agents through chemokine (C–X–C motif) ligand 12 (CXCL12)–CXCL4-mediated recruitment to hypoxic tumor microenvironments [16]. Thus, circulating monocytes serve as proangiogenic mediators that contribute to the resistance to antiangiogenic agents.

Dendritic cells (DCs) are potent immunogenic sentinels that link innate and adaptive immune responses. Tumor microenvironments support the differentiation of immunosuppressive and protumorigenic DCs through the regulation of various immune regulatory mediators and transcriptional factors [17]. DCs positively regulate tumor immunosurveillance at early tumor stages, but they support tumor growth and metastatic activities during later stages [18]. Moreover, vascular endothelial growth factor-A (VEGF-A) and β-defensin facilitate the recruitment of DCs, which enhance tumor angiogenesis, into tumor tissues [19]. Tumor-associated DCs also repress innate antitumor immune responses to DNA vaccine and chemotherapy through interactions between T cell immunoglobulin mucin domain protein-3 (TIM-3) and high mobility group box1 (HMGB1) [20]. Yet, ATP released from dying tumor cells after treatment with anthracyclin promotes the recruitment of bone marrow-derived myeloid cell precursors into tumor tissues, which differentiate into $CD11c^+CD11b^+Ly6C^{high}$ inflammatory DCs through interaction with purinergic receptor P2Y2. The inflammatory DCs serve as a major population to activate antigen-specific T lymphocytes in local tumor environments and trigger antitumor responses by anthracyclin [21]. Taken together, DCs serve as a major player in manipulating antitumor responses induced by anticancer agents.

Myeloid-derived suppressor cells (MDSCs) are generated from bone marrow-derived precursors recruited into tumor tissue. MDSCs play a critical role in suppressing antitumor immune responses and supporting tumor progression [17]. MDSCs also suppress tumor chemosensitivity by regulating innate immune pathways. For example, MDSCs inhibit type I interferon (IFN)-mediated signals in intratumor effector lymphocytes by inducible nitric oxide synthase (iNOS)-dependent pathways [22]. Moreover, certain types of chemotherapeutic agents such as 5-fluorouracil (5-FU) and gemcitabine activate the Nlrp3-mediated inflammasome in MDSCs, leading to production of IL-1β, which in turn triggers the production of IL-17 by $CD4^+$ T cells and attenuates antitumor efficacy of cytotoxic chemotherapy [23]. Collectively, these findings support the importance of MDSCs in controlling responses to anticancer therapies. Taken together, these facts indicate that tumor microenvironments greatly impact therapeutic responses to anticancer agents by negatively regulating the immunogenic properties of myeloid cells.

Myeloid cell derived soluble mediators generate chemoresistant niches

Accumulating evidence has revealed that various repertoires of cytokines, chemokines, and growth factors strongly influence tumor progression and resistance to anticancer agents [24]. Indeed, multiple cytokine networks in tumor microenvironments adopt dedicated strategies to evade therapeutic responses to anticancer agents by enhancing tumor cell survival, angiogenesis, matrix remodeling, and repression of host antitumor immunity [25]. These observations underscore the complex regulatory mechanisms of host immunity by soluble mediators in cancer pathogenesis and therapeutic responses.

$Ly6C^{high}CCR2^+$ inflammatory monocytes are preferentially recruited into chemokine (C–C motif) ligand 2 (CCL2)-enriched tumor microenvironments and differentiate into protumorigenic, immunosuppressive macrophages [9,14]. Therefore, circulating monocytes represent TAM precursors that contribute to tumorigenesis and the resistance to anticancer agents. Moreover, cancer cells are capable of producing several growth factors that facilitate

myeloid cell differentiation and maturation, such as granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF). These cytokines play a critical role in recruiting MDSCs or immature myeloid precursors into tumor tissues and promoting differentiation to TAMs [17,26]. The blockade of M-CSF or its receptor (CSF1R) attenuates tumorigenic activities by suppressing angiogenesis and oncogenic signals related to metastatic phenotypes [27]. Encouraged by positive preclinical data, CSF1R kinase inhibitors have recently been introduced into clinical trials where they exhibited potent antitumor activities and were shown to overcome resistance to cytotoxic chemotherapy in patients with advanced breast cancer [28]. Taken together, the regulation of myeloid cell recruitment and differentiation through the dynamic interplay of cytokine and chemokine networks greatly contributes to the formation of chemoresistant niches.

Escape from cell death signals following treatment with anticancer agents is a major hallmark of tumorigenicity and chemoresistance. In particular, NF-κB serves as a key transcriptional factor for activating Bcl2-mediated antiapoptotic signals and suppressing p53 pathways [29]. Several cytokines that are subject to NF-κB-mediated regulation, such as tumor necrosis factor-α (TNF-α) and IL-6, are produced at higher levels by myeloid cells infiltrating tumors than by those in normal tissues. These cytokines protect tumor cells from genotoxic insults in cooperation with other transcriptional factors such as Stat3 and/or inflammatory regulators such as prostaglandin E2 (PGE2), VEGF, and CXCL12 [6,30,31]. Cathepsin families have important roles in lysosomal degradation of proteins and contribute to pathological inflammation such as pancreatitis. Treatment with taxol induced recruitment of macrophages into breast tumor tissue, in which cathepsins B and S are activated and contribute to the protection of tumors from taxol-induced cell death [32]. Thus, upon encountering cell death signals induced by anticancer therapeutics, myeloid cells support tumor cell survival through multiple complex networks of cytokines, chemokines, and proteases.

Myeloid cell derived cytokines also play critical roles in controlling stem and progenitor cells, which have emerged as a major tumorigenic population mediating resistance to various anticancer agents [33]. For example, well-differentiated leukemic or breast cancer cells are converted into stem cell like progenitors by inflammatory pathways such as IL-6–Stat3 and NF-κB signal cascades [34,35]. Furthermore, IL-6 produced by TAMs augments self-renewal activities and anticancer drug resistance of colon or lung cancer stem-like cells through coordinated activation of Stat3 and sonic Hedgehog pathways [36,37]. Transforming growth factor-β (TGF-β) is an immune regulatory cytokine produced by tumor-associated myeloid cells, and its signaling pathways are well correlated with chemoresistance in breast and ovarian tumors [38]. TGF-β produced by leukemic cells and their stromal components plays a critical role in maintaining leukemic stem cells through FoxO-dependent pathways, and treatment with a TGF-β inhibitor improves therapeutic responses to the BCR–ABL inhibitor imatinib for chronic myeloid leukemia [39]. Together, myeloid cell derived cytokines may serve as key sentinels linking tumor inflammation with stem cell associated signals.

Inflammatory responses manifest as a major protumorigenic element to consolidate tumorigenicity and resistance to anticancer therapies. In particular, IL-23 predominantly produced by myeloid cells is a major growth mediator that contributes to T helper 17 (Th17) cell differentiation thereby enhancing angiogenesis and inflammation in tumor microenvironments [40]. By contrast, IL-17 produced by γδ T cells positively regulates chemosensitivity by generating tumor-specific T lymphocytes by IL-1β-dependent mechanisms, whereas Th17-derived IL-17 also stimulates chemotherapy-induced antitumor responses in cooperation with IFN-γ-mediated induction of CXCL9 and CLCL10 [41,42]. Bimodal activities of IL-17 highlight the diverse role on pro- and antitumor activities of effector cells and their derivatives, which have a huge impact on chemotherapy-mediated antitumor responses.

Tumor angiogenesis exploits multiple strategies for enhancing tumorigenic activities and chemoresistance, and myeloid cells coordinate cytokines and proteinases to promote tumor angiogenesis. Matrix metalloproteinases (MMPs) are produced at high levels by TAMs and regulates tissue integrity and homeostasis. MMP-2 and MMP-9 negatively regulate therapeutic responses to doxorubicin by maintaining the integrity of tumor vessels and pericyte coverage within the vasculature [43]. Moreover, MMP-2 and MMP-9 regulate the release of VEGF-A and TGF-β from stromal cells and the extracellular matrix, further fostering tumorigenic and chemoresistant phenotypes [43,44]. Thus, myeloid cell derived MMP-2 and MMP-9 promote chemoresistance by regulating angiogenesis and tissue remodeling.

Taken together, these data suggest that tumor-associated myeloid cells serve as major sources of various soluble factors that mediate inflammation and tissue remodeling in tumor microenvironments, thus creating better conditions for tumor aggressiveness and chemoresistance (Figure 1).

Damage-associated molecular pattern-mediated innate immune pathways regulate chemoresistant niches

Although the vast majority of cancers arise from *de novo* oncogenic and epigenetic alterations, most tumors manifest continuous inflammatory signal activation as a result of smoldering inflammation. This occurs even in the absence of infection or autoimmunity and causes tumor progression and resistance to anticancer therapies [2]. In particular, endogenous inflammatory mediators, termed damage-associated molecular patterns (DAMPs), are released mainly from stressed or injured cells and constitute critical sentinels that connect pattern recognition receptor (PRR)-mediated innate signals with sterile inflammatory reactions and greatly impact both cancer pathogenesis and autoimmunity [45,46].

Toll-like receptors (TLRs) serve as PRRs that recognize several DAMPs and activate innate immune signals. TLR stimulation breaks the tolerogenic status of myeloid cells and triggers the innate and adaptive arms of effector responses. This may license myeloid cells to stimulate host immunity and antagonize tumorigenicity [47]. Indeed, TLR4 on myeloid cells stimulates tumor-specific immune

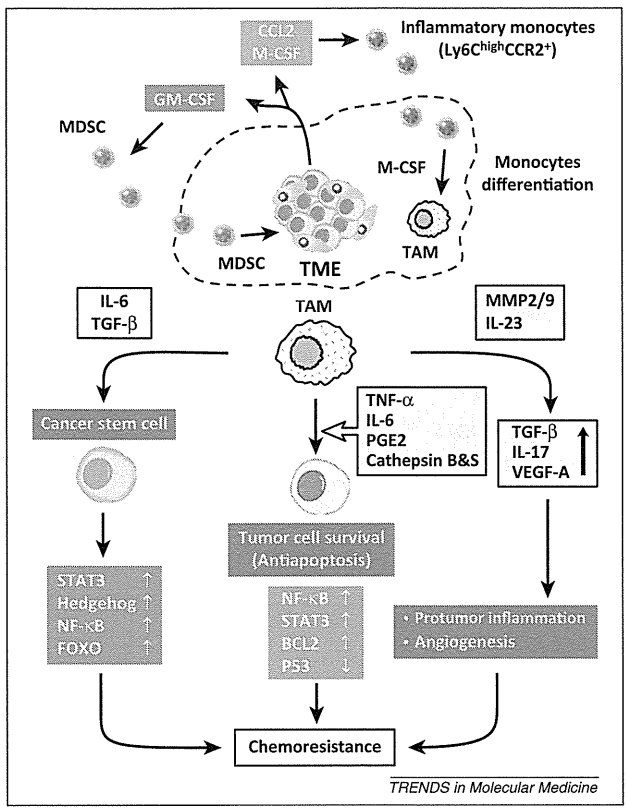

Figure 1. Myeloid cell derived cytokines regulate chemoresistant niche. Myeloid cells regulate anticancer drug responses through a complex network of various soluble mediators, including cytokines, chemokines, and cysteine/metalloproteases in tumor microenvironments (TMEs). Bone marrow-derived myeloid precursors are recruited to TMEs where they produce various cytokines (M-CSF, GM-CSF) and chemokines (CCL2, CXCL12). The myeloid-derived soluble factors promote tumor aggressiveness and chemoresistance by regulating tumor cell survival (TNF-α, IL-6, PGE2, cathepsin B/S, etc.), stem cell activities (IL-6, TGF-β, etc.), protumorigenic inflammation, and angiogenesis (MMP-2/9, IL-23, TGF-β, IL-17, VEGF-A, etc.). Abbreviations: M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; CXCL12, chemokine (C–X–C motif) ligand 12; CCL2, chemokine (C–C motif) ligand 2; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; PGE2, prostaglandin E2; TGF-β, transforming growth factor-β; MMP, matrix metalloproteinase; VEGF-A, vascular endothelial growth factor-A.

responses by recognizing HMGB1 released from damaged tumor cells and thereby improves antitumor responses to chemotherapy and radiotherapy [48]. By contrast, TNF-α stimulates TLR2-mediated innate pathways through NF-κB-dependent inflammatory signals, creating protumorigenic environments and promoting tumor metastasis [49]. Additionally, TLR4 promotes skin tumorigenesis through immune and tumor cell intrinsic mechanisms [50], and TLR7/8 upregulate antiapoptotic Bcl-2 family molecules, contributing to tumor cell survival and chemoresistance [51]. Thus, TLR signals are involved in creating a diverse milieu for providing protumorigenic or antitumorigenic environments. Moreover, tumor microenvironments may divert myeloid cells to activate antitumor host responses or promote protumorigenic inflammation by regulating the various modes of interaction between TLRs and DAMPs. Taken together, it is critical to determine the molecular mechanisms responsible for DAMP-mediated sterile inflammation in tumor microenvironments.

On the basis of this background information, we attempted to identify the factors that modulate innate immune signals and antitumor responses mediated by tumor-derived DAMPs, and identified TIM-3 as one candidate. TIM-3 is preferentially expressed on T lymphocytes during chronic viral infection or malignancy [52]. In addition, DCs express TIM-3 upon stimulation with TLR ligands such as lipopolysaccharide (LPS), and TIM-3 triggers antimicrobial responses through interactions with galectin-9 [53]. However, the role of myeloid cell derived TIM-3 in the regulation of innate immune systems remains largely unclear. In our study, DC-derived TIM-3 suppressed innate immune responses by interacting with major DAMP HMGB1 [20]. The interaction of TIM-3 with HMGB1 interfered with the endocytosis into endosomal vesicles of ligands for TLR3, TLR7, TLR8, and TLR9, and thereby impaired a diverse range of innate immune signals including IRF and NF-κB [20].

Although the role of cytosolic innate sensing systems in the regulation of anticancer drug responses remains largely unknown, therapeutic delivery of double-stranded RNA, an agonist for RIG-I-like helicase (RLH), triggers potent antitumor immunity and leads to tumor eradication [54]. Moreover, RLH activation triggers antitumor responses by activating proimmunogenic and apoptotic gene programs in tumor cells [55]. Consistent with the antitumor properties of RLH-mediated immune regulation, DC-derived TIM-3 impedes innate immune responses mediated by RLH, suggesting that TIM-3-positive myeloid cells may restrain RLH-mediated innate signals in tumor microenvironments. More importantly, blockade of TIM-3 improves the antitumor efficacy of DNA vaccines and cytotoxic chemotherapy [20]. Thus, TIM-3 serves as a negative regulator of innate immunosurveillance systems by counter-regulating tumor-derived DAMPs. Nevertheless, DAMPs released from inflammatory tumor microenvironments may support pro- or antitumor activities depending on the different tumor microenvironments. Under such conditions, HMGB1 activates inflammatory signals, such as NF-κB and Stat3, and stimulates release of proinflammatory cytokines by directly interacting with TLR4 or the receptor for advanced glycan end product (RAGE) on myeloid cells [56]. In this scenario, the HMGB1–TLR4 interaction stimulates antitumor responses by activating immunogenic DCs [48], whereas the HMGB1–RAGE interaction on myeloid cells contributes to tumor metastasis and chemoresistance through NF-κB-mediated activation of MMP-2/9 [57].

Thus, tumor microenvironments manipulate DAMP signals to create an ideal condition for tumor progression in multiple ways: TIM-3 suppresses innate immune sensing mediated by the HMGB1–TLR4 interaction, which is responsible for tumor immunosurveillance. In marked contrast, RAGE utilizes the same HMGB1 to stimulate protumorigenic inflammatory signals such as NF-κB and Stat3. Alternatively, it is possible that TIM-3 may interfere with the antitumor responses exerted by the HMGB1–DNA complex, which might be recognized by receptors other than TLR4 and RAGE on myeloid cells. In this regard, tumor microenvironments may promote protumorigenic inflammation but concomitantly suppress antitumor immunity through the regulation of DAMPs by multiple sets of PRRs on myeloid cells (Figure 2).

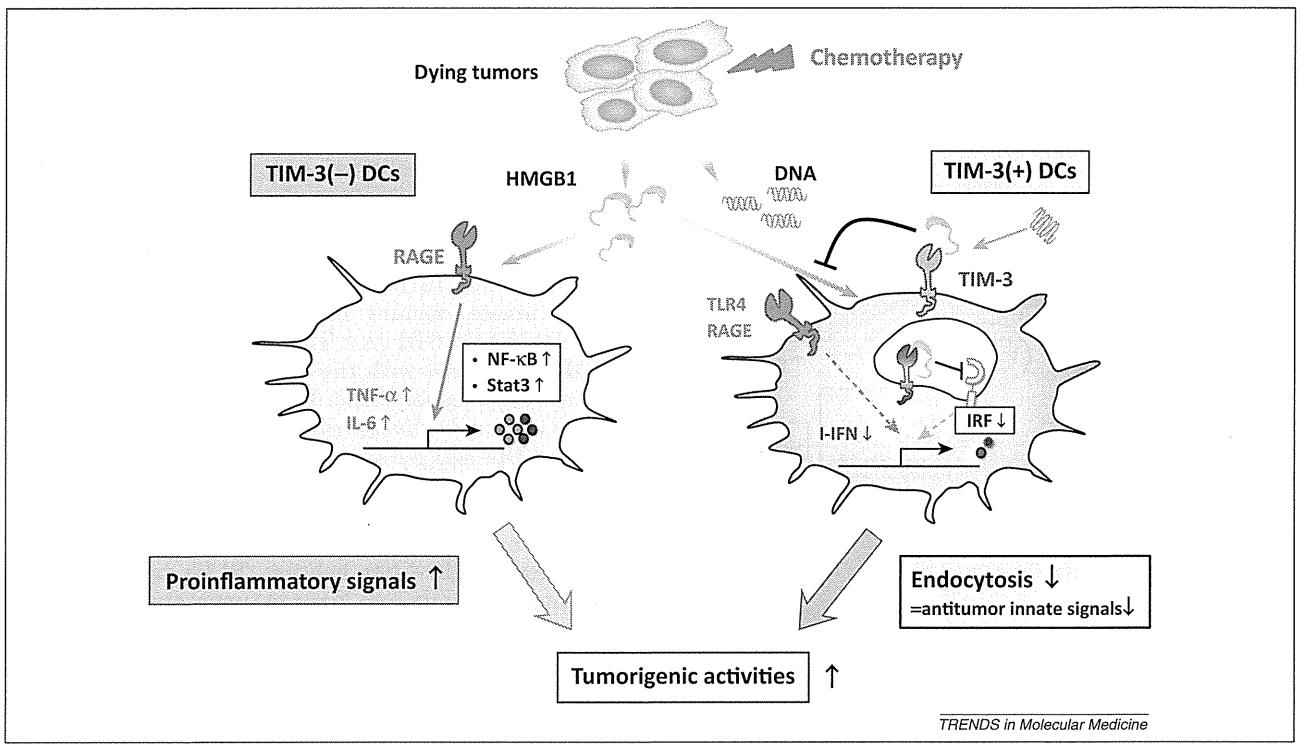

Figure 2. Tumor-associated DCs impede antitumor efficacy of chemotherapy. The release of endogenous nucleic acids from dying tumor cells upon use of cytotoxic therapies has the potential to activate innate immune responses mediated by TLR and RLH-dependent PRR-transduced signals. However, tumor microenvironments modulate DCs to upregulate TIM-3. TIM-3 on DCs interferes with HMGB1-mediated endocytosis of nucleic acids into endosomes and thus impedes tumor immunosurveillance by suppressing IRF signals and type-I interferon responses. By contrast, the RAGE–HMGB1 interaction on DCs stimulates protumorigenic inflammation through NF-κB and Stat3-mediated mechanisms. In total, smoldering inflammation controlled by HMGB1-mediated innate pathways creates an ideal environment for tumorigenicity and chemoresistance. Abbreviations: DCs, dendritic cells; TLR, Toll-like receptor; RLH, RIG-I-like helicase; PRR, pattern recognition receptor; TIM-3, T cell immunoglobulin mucin domain protein-3; IRF, interferon regulatory factor; RAGE, receptor for advanced glycan end product; NF-κB, nuclear factor-κB; HMGB1, high mobility group box1; Stat, signal transducer and activator of transcription.

Inflammasome signals are regulated by endogenous inflammatory components and play a dominant role in the regulation of anticancer drug responses by manipulating T cell differentiation and cytokine profiles in tumor environments. Brunchard *et al.* reported that gemcitabine and 5-FU induced disruption of lysosomal integrity and release of cathepsin B in MDSCs, leading to activation of the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing-3 (NLRP-3), and promoting IL-1β production. The MDSC-derived IL-1β generated IL-17-producing $CD4^+$ T cells, which triggered resistance to cytotoxic chemotherapy [23]. However, immunogenic cell death (ICD) triggered by oxaliplatin or anthracyclin activated the same NLRP-3-mediated inflammasome signals and IL-1β production by promoting the release of ATP and its interaction with the P2X7 purinergic receptor in inflammatory DCs in tumor tissues [58]. In this case, DC-derived IL-1β was responsible for activating antigen-specific $CD8^+$ T cells and regressing tumor growth. Furthermore, chemotherapeutic agents activate autophagic pathways, which are associated with endoplasmic reticulum (ER) stress responses and cause the release of ATP, which may in turn activate the NLRP-3 pathway and enhance antitumor immunity [59].

Thus, the differential immunogenicity of dying cells or the alteration of the inflammatory milieu exerted by therapeutic regimens may impact the biological functions of inflammasome signals by altering effector T cell differentiation as well as crosstalk between tumor cells and myeloid cells in tumor immunosurveillance. In addition, the temporal and/or spacial dynamics of IL-1β secretion might influence their pro- and antitumor impact of cytotoxic chemotherapy in distinct tumor microenvironments. These differences further highlight the pleiotropic actions of myeloid cells and their derivatives in the regulation of antitumor responses triggered by chemotherapy.

Myeloid cell phagocytic pathways regulate chemoresistant niches

Phagocytosis serves as a physiological process for the removal of debris produced by dying cells, which might cause inflammation and autoimmunity [60]. In addition, phagocytosis facilitates the processing and presentation of various repertoires of immunogenic antigens to tumor-specific lymphocytes under certain conditions [61]. Phagocytosis is mainly mediated through PRR-dependent recognition of phosphatidylserine (PS) exposed on the surface of apoptotic cells, and recent studies have identified the phagocytic receptors on myeloid cells that function to regulate tumor immunogenicity.

Milk-fat globule-EGF factor VIII (MFG-E8) is secreted from macrophages, DCs, and tumor cells, and binds PS to promote the ingestion of apoptotic cells by engaging $\alpha_v\beta_3$ integrin on myeloid cells [62]. GM-CSF increases MFG-E8 production by macrophages and MFG-E8-mediated phagocytosis impedes antitumor immunity through the