Table 1. Global methylation level of leukocyte DNA in Japanese women according to factors used for adjustment

Factor	Level	Crude		Multivariate-adjusted*						
		n	Methylation level (%)	Methylation level (%)	95% CI		Effect	95% CI		P-value
Age, years	<40	25	69.9	70.4	68.7	72.1				
	40–49	109	70.0	70.6	69.5	71.7				
	50–59	129	70.3	70.9	69.8	72.1				
	60–69	96	70.4	71.2	70.0	72.4				
	≥70	25	69.7	70.5	68.8	72.1				
	Trend						0.15	−0.2	0.49	0.403
Body mass index (quartile category)	≤20.8	93	70.1	70.8	69.6	71.9				
	20.9–22.5	97	71.0	71.7	70.5	72.9				
	22.6–24.8	97	70.1	70.8	69.6	71.9				
	≥24.9	97	69.5	70.2	69.1	71.4				
	Trend						−0.26	−0.57	0.05	0.104
Smoking	Never	354	70.1	70.5	69.9	71.1				
	Past	8	70.8	70.7	68.3	73.1				
	Current	20	70.7	71.3	69.7	72.9				
	Trend						0.36	−0.40	1.13	0.351
Physical activity	No	231	69.9	70.4	69.3	71.4				
	≤2 days per week	33	70.9	71.4	70.0	72.8				
	≥3 days per week	120	70.5	70.8	69.7	71.9				
	Trend						0.25	−0.14	0.63	0.208

*Adjusted for age (continuous), body mass index (continuous), smoking (never smoker, past smoker, current smoker), alcohol drinking (non-drinker, occasional drinker, regular drinker of <150 g ethanol/week, regular drinker of ≥150 g ethanol/week), and physical activity in the past 5 years (no, ≤2 days/week, ≥3 days/week). Model for each factor listed in the table did not include the corresponding variable as adjustment. CI, confidence interval.

global methylation level significantly decreased by 0.70% (95% CI, 0.28–1.12) per quartile category for folate intake among non-drinkers, whereas no association was observed among drinkers (0.08% [95% CI, −0.40–0.55]). Additional analysis by the four categories of alcohol drinking used in Table 2 also found a statistically significant interaction ($P_{interaction} = 0.002$). As stated above, we observed an inverse association among non-drinkers. In contrast, the global methylation level significantly increased by 1.32% (95% CI, 0.22–2.42) per quartile category for folate intake among regular drinkers of more than 150 g ethanol/week, but no association was seen among occasional drinkers and regular drinkers of less than this amount (data not shown). No statistically significant interactions were observed for vitamin B2, B6, or B12 intake, alcohol consumption, or five SNPs of *MTHFR*, *MTR*, and *MTRR*.

Discussion

In this cross-sectional study among Japanese women, we found that higher folate intake was significantly associated with a lower level of global methylation of peripheral blood leukocyte DNA. Subgroup analysis suggested that alcohol drinking modified the association between folate intake and global methylation level. Because of the cross-sectional nature of the study, we were not able to determine if higher dietary folate intake leads to global hypomethylation of leukocyte DNA. Considering the role of folate in one-carbon metabolism, however, our findings suggest that dietary folate intake might modulate the global methylation level of leukocyte DNA.

Our findings appear to contradict at least some previous studies of the association between folate level and global methylation level of peripheral blood DNA. Two intervention studies showed decreased methylation of leukocyte DNA in a folate-depleted diet group.[14,16] One of these studies provided an average of 118 μg folate per day to 33 postmenopausal women for 7 weeks,[16] and the second provided an average of 56–111 μg folate per day to eight postmenopausal women for 9 weeks.[14] Although these studies differed from our study in their method of methylation analysis (*in vitro* [3H]methyl incorporation assay by SssI CpG methylase) and subjects (postmenopausal or elderly women recruited in the USA), their data indicate that moderate folate depletion induces hypomethylation of leukocyte DNA. Regarding the effect of folate supplementation on the methylation level of leukocyte DNA, a randomized controlled trial of 400 μg folic acid supplementation per day ($n = 15$) or placebo ($n = 16$) for 10 weeks in patients with colorectal adenoma showed an increase in leukocyte DNA methylation level.[15] In contrast, supplementation with 2 mg folic acid and 20 μg vitamin B12 for 12 weeks did not change this variable.[13] These intervention studies suggest that the effect of folate on the methylation level of leukocyte DNA might depend on dose, but that a dose–response pattern might not be straightforward. For instance, it has been suggested that folates act as inhibitors of dihydrofolate reductase,[26] and that high folate levels could have the same functional effect as a low folate status under certain circumstances.[10,27] In fact, several animal studies showed that the effect of isolated folate deficiency on genomic DNA methylation in rodent liver and colon was either a decrease or increase.[28,29]

A recent cross-sectional study reported that a dietary pattern characterized by high intake of vegetables and fruits was associated with a lower prevalence of LINE-1 DNA hypomethylation.[30] In contrast, three other studies found no association between dietary folate intake and global methylation level of leukocyte DNA in the control groups of a head and neck cancer case–control study in the US, a bladder cancer case–control study in Spain, and a gastric cancer case–control study in Poland.[6–8] These findings should be interpreted cautiously, however, because the analyses of the bladder and gastric cancer case–control studies were primarily aimed at identifying potential confounders for assessing an association between

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Table 2. Global methylation level according to five dietary factors and five single nucleotide polymorphisms of genes associated with folate metabolic enzymes

Factor	Level	Crude		Multivariate-adjusted*						
		n	Methylation level (%)	Methylation level (%)	95% CI		Effect	95% CI		*P*-value
Folate (μg/day)	≤339.9	96	70.3	71.2	70.0	72.3				
	339.9–419.5	96	70.6	71.4	70.2	72.6				
	419.5–521.7	96	70.1	70.8	69.6	71.9				
	>521.7	96	69.7	70.2	69.0	71.4				
	Trend						−0.36	−0.69	−0.03	0.030
Vitamin B2 (mg/day)	≤1.4	96	69.8	70.6	69.5	71.7				
	1.4–1.6	96	70.0	70.8	69.6	72.0				
	1.6–1.8	96	70.9	71.4	70.2	72.6				
	>1.8	96	70.0	70.7	69.5	71.9				
	Trend						0.08	−0.24	0.39	0.636
Vitamin B6 (mg/day)	≤1.4	96	70.2	71.1	69.9	72.3				
	1.4–1.6	96	70.3	71.1	69.9	72.2				
	1.6–1.8	96	70.1	70.7	69.6	71.9				
	>1.8	96	70.1	70.6	69.4	71.7				
	Trend						−0.19	−0.53	0.15	0.268
Vitamin B12 (μg/day)	≤6.4	96	70.5	71.3	70.1	72.5				
	6.4–8.3	96	69.9	70.6	69.4	71.7				
	8.3–10.6	96	70.3	70.9	69.8	72.1				
	>10.6	96	70.0	70.7	69.5	71.8				
	Trend						−0.14	−0.46	0.17	0.370
Alcohol drinking	Non-drinker	232	69.9	70.4	69.4	71.5				
	Occasional drinker	39	70.9	71.4	70.0	72.8				
	Regular drinker of <150 g ethanol/week	87	70.8	71.3	70.1	72.4				
	Regular drinker of ≥150 g ethanol/week	26	69.9	70.3	68.8	71.9				
	Trend						0.23	−0.11	0.57	0.183
MTHFR rs1801131	AA	254	70.3	70.9	69.9	72.0				
	AC + CC	130	70.0	70.7	69.6	71.8				
	Dominant model						−0.25	−0.98	0.47	0.494
MTHFR rs1801133	CC	112	70.2	70.9	69.7	72.0				
	CT + TT	272	70.1	70.8	69.8	71.9				
	Dominant model						−0.04	−0.80	0.72	0.918
MTR rs1805087	AA	257	70.3	71.0	70.0	72.1				
	AG + GG	126	69.8	70.5	69.4	71.6				
	Dominant model						−0.53	−1.26	0.20	0.156
MTRR rs162049	GG	116	70.2	70.9	69.7	72.0				
	AG + GG	266	70.2	70.8	69.8	71.9				
	Dominant model						−0.05	−0.80	0.70	0.902
MTRR rs10380	CC	302	70.2	70.9	69.8	71.9				
	CT + TT	81	70.2	70.8	69.6	72.0				
	Dominant model						−0.09	−0.93	0.75	0.834

*Adjusted for age (continuous), body mass index (continuous), smoking (never smoker, past smoker, current smoker), alcohol drinking (non-drinker, occasional drinker, regular drinker of <150 g ethanol/week, regular drinker of ≥150 g ethanol/week), and physical activity in the past 5 years (no, ≤2 days/week, ≥3 days/week). CI, confidence interval; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase.

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Table 3. **Association between mean global methylation level in leucocyte DNA and folate intake by factors related to one-carbon metabolism**

Factor	Level	Number	Multivariate-adjusted*				
			Effect	95% CI		*P*-value for trend	*P*-value for interaction
Alcohol	Non-drinker	232	−0.70	−1.12	−0.28	0.001	
	Drinker	152	0.08	−0.40	0.55	0.749	0.013
Vitamin B2, mg/day	≤1.6	192	−0.32	−0.82	0.18	0.208	
	>1.6	192	−0.67	−1.15	−0.19	0.006	0.304
Vitamin B6, mg/day	≤1.6	192	−0.07	−0.65	0.50	0.803	
	>1.6	192	−0.63	−1.17	−0.10	0.020	0.157
Vitamin B12, μg/day	≤8.3	192	−0.20	−0.64	0.25	0.389	
	>8.3	192	−0.53	−0.99	−0.08	0.022	0.287
MTHFR rs1801131	AA	254	−0.46	−0.86	−0.05	0.028	
	AC + CC	130	−0.18	−0.70	0.33	0.484	0.400
MTHFR rs1801133	CC	112	−0.25	−0.81	0.31	0.384	
	CT + TT	272	−0.41	−0.80	−0.02	0.037	0.627
MTR rs1805087	AA	257	−0.21	−0.61	0.19	0.298	
	AG + GG	126	−0.60	−1.13	−0.08	0.024	0.233
MTRR rs162049	GG	116	−0.49	−1.05	0.07	0.084	
	AG + GG	266	−0.30	−0.68	0.09	0.137	0.555
MTRR rs10380	CC	302	−0.37	−0.73	−0.01	0.042	
	CT + TT	81	−0.32	−1.03	0.39	0.374	0.892

*Adjusted for age (continuous), body mass index (continuous), smoking (never smoker, past smoker, current smoker), alcohol drinking (non-drinker, occasional drinker, regular drinker of <150 g ethanol/week, regular drinker of ≥150 g ethanol/week), and physical activity in the past 5 years (no, ≤2 days/week, ≥3 days/week). CI, confidence interval; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase.

global methylation level and the risk of cancer based on univariate analyses.

The important messages from this and these previous studies may be that: (i) the mechanisms of individual variation in the global DNA methylation level of peripheral blood leukocytes are complex and multifactorial in nature; and (ii) in actual daily dietary life, in Japan, folate intake may not be the major single determinant of global methylation level and may not necessarily confound association analysis between leukocyte global methylation and the risk of cancers that are associated with folate intake. Only a few observational studies have examined associations of dietary and genetic factors related to one-carbon metabolism with global methylation level of leukocyte DNA among healthy individuals based on nutrient intake estimated from the usual diet alone.[6–8] None of the five candidate SNPs examined in this study showed a statistically significant association, although rs1801131 and rs1801133 in *MTHFR*, for instance, have been reported to be linked to altered enzymatic activity[31,32] and folate level.[33,34] Given the present result of rs1801131 in *MTHFR* (AA genotype group: number = 254, mean = 70.25, and SD = 3.3; AC + CC genotype group: number = 130, mean = 70.01, and SD = 3.3), for example, the expected power to detect an association was 10% with a two-sided and error level of 5%. Therefore, we cannot exclude the possibility that the null findings are explained by insufficient power, and additional larger studies are needed to clarify the association between these SNPs and global methylation level.

Subgroup analyses in the present study showed that alcohol drinking modified the association between folate intake and global methylation level ($P_{\text{interaction}} = 0.01$). The association between folate intake and global methylation level varied by alcohol drinking status: higher folate intake was significantly associated with a lower global methylation level among non-drinkers; no association was observed among occasional and light drinkers; and higher folate intake was significantly associated with a higher global methylation level among relatively heavy drinkers. Alcohol consumption interferes with folate metabolism[35] and decreases levels of serum folate.[36] Although this interaction remained inexplicable, these findings might nevertheless provide hints about its biological mechanism. Furthermore, subgroup analysis by alcohol drinking was based on a relatively small number subjects, particularly with regard to heavy drinkers ($n = 26$), and thus replication of this interaction in a larger study is awaited.

Several limitations of the present study warrant mention. First, misclassifications due to inaccurate measurement would result in null associations. Although dietary intakes in the present study were assessed using a validated FFQ, misclassifications may have been unavoidable. However, as reproducibility of the assay for global methylation level was relatively high in the present study (intra-assay CV, 6.4), measurement errors during laboratory assay might have been minimal. Second, the present study made multiple comparisons, which might have led to false-positive results. In this regard, we observed a statistically significant association between higher folate intake and lower level of global methylation, which might nevertheless be explained by chance. Finally, because the sample size was limited, the study might not have had sufficient statistical power to detect small associations, as mentioned above, and this is one of the possible explanations for the observed absence of associations. In particular, the results of subgroup analysis and interaction tests should be interpreted carefully.

In this cross-sectional study in 384 healthy Japanese women with validated FFQ data, we found that a higher folate intake level was associated with a lower global methylation level of leukocyte DNA. Although the data of this study and others suggest that folate intake can modulate the global methylation level of leukocyte DNA, inconsistencies among the studies have been noted, and may reflect the complex and multifactorial nature of individual variation in the global DNA methylation level of peripheral blood leukocytes.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

BMI	body mass index
CI	confidence interval
CV	coefficient of variation
FFQ	food frequency questionnaire
LUMA	LUminometric Methylation Assay
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
SD	standard deviation
SNP	single nucleotide polymorphism

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Review Article

Genetic factors related to gastric cancer susceptibility identified using a genome-wide association study

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Gastric cancer (GC) is one of the major malignant diseases worldwide, especially in Asia, where Japan and Korea have the highest incidence in the world. Gastric cancer is classified into intestinal and diffuse types. While the former is almost absolutely caused by *Helicobacter pylori* infection as the initial insult, the latter seems to include cases in which the role of infection is limited, if any, and a contribution of genetic factors is anticipated. Previously, we performed a genome-wide association study (GWAS) on diffuse-type GC by using single nucleotide polymorphisms (SNP) catalogued for Japanese population (JSNP), and identified a prostate stem cell antigen (*PSCA*) gene encoding a glycosylphosphatidylinositol-anchored cell surface antigen as a GC susceptibility gene. From the second candidate locus identified using the GWAS, 1q22, we found the Mucin 1 (*MUC1*) gene encoding a cell membrane-bound mucin protein as another gene related to diffuse-type GC. A two-allele analysis based on risk genotypes of the two genes revealed approximately 95% of Japanese population have at least one of the two risk genotypes, and approximately 56% of the population have both risk genotypes. The two-SNP genotype might offer ample room to further stratify a high GC risk subpopulation in Japan and Asia by adding another genetic and/or non-genetic factor. Recently, a GWAS on the Chinese population disclosed an additional three GC susceptibility loci: 3q13.31, 5p13.1 and 10q23. (*Cancer Sci* 2013; 104: 1–8)

Gastric cancer (GC) is one of the major malignant diseases and the second causal of cancer death worldwide.[1] It is usually classified into two types, intestinal and diffuse, a classification which was originally based on histological observation but is recently thought to reflect its pathogenesis.[2] The majority of intestinal-type GC (IGC) arises in the sequence of inflammatory change of the gastric epithelium resulting from bacterial infection; *Helicobacter pylori* (HP) infection – chronic inflammation – intestinal metaplasia – dysplasia – adenocarcinoma. In contrast, de novo diffuse-type GC (DGC) is thought to emerge in a histologically almost normal epithelium as a consequence of some genetic change that occurred in gastric stem cells and/or epithelial precursor cells, although some cases with DGC might represent dedifferentiated stages of IGC, and a contribution of HP is also suggested.[3] In other words, it is apparent that the pathogenesis of IGC is initiated by HP infection, a class I carcinogen acknowledged by WHO, and therefore IGC is essentially a preventable disease by eradicating HP infection. However, DGC might develop earlier in life than IGC,[4] and no definite DGC-specific environmental risk factor has been established. Therefore, so far we have neither solid strategy nor promising theory to envision a consistent diminution of the incidence of DGC.

The incidence of GC has strong geographical and ethnical characteristics. For example, it is one of the rare cancers in North America and Europe, while its incidence is significantly high in Japan and Korea. This can be explained roughly by the difference in regional prevalence of HP infection.[4] However, Japan has a high incidence of GC (age-standardized incidence rate 62.7/100 000) but lower HP seroprevalence (39.3%) than, for example, Bangladesh (92%) and India (79%), which have a much lower GC incidence, 1.6/100 000 and 5.7/100 000, respectively, suggesting the contribution of some other factor in the carcinogenesis of gastric epithelial cells.[5] Moreover, *Helicobacter* and Cancer Collaborative Group reported that HP infection was not associated with the overall risk of GC developing in the cardia of the stomach.[6]

Genome-wide association study (GWAS) of genetic factors for GC development

Genome-wide association study has been successful in exploring genetic susceptibility factors of a number of polygenic or so-called lifestyle-related diseases based on a common disease – common variant hypothesis.[7,8] The current choice of polymorphic markers in GWAS is single nucleotide polymorphisms (SNP), and the spectrum and frequency of SNP depend on each ethnic population. Japan preceded other countries in the preparation for conducting GWAS, because SNP in the Japanese population (JSNP) were already catalogued in the early 2000s by Dr Yusuke Nakamura at the Institute of Medical Science, The University of Tokyo. The JSNP database led to a number of fruitful harvests in the area of GWAS on genetic factors for common diseases in the late 2000s.[9] As a part of the so-called Millennium Project in Japan, GWAS on GC was performed with two steps of the association study.[10,11] The first step was performed on 85 576 SNP using 188 DGC cases and 752 references, and the second step was performed on 2753 selected SNP with 749 DGC cases and 750 controls. Finally, it listed the top 10 SNP related to DGC with statistical significance, which included four SNP located in chromosome 8q24.3 and two SNP in 1q22 (Table 1).[12] The subsequent linkage–disequilibrium (LD) analyses revealed two genes in the LD block at 8q24.3 and five genes at 1q22.[12,13]

In the 8q24.3 locus, prostate stem cell antigen (*PSCA*) gene was identified as a DGC susceptibility gene, with a significant association between DGC and two SNP, rs2976392 and rs2294008, in the gene (rs2976392: 926 cases, 1397 controls, allele-specific odds ratio = 1.71, 95% confidence interval = 1.50–1.94, $P = 1.5 \times 10^{-16}$; rs2294008: 925 cases, 1396 controls, allele-specific odds ratio = 1.67, 95% confidence interval = 1.47–1.90, $P = 2.2 \times 10^{-15}$).[12] The association was replicated in

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Table 1. Gastric cancer susceptibility loci identified using a genome-wide association study

Locus	Representative SNP (major/minor allele)	Odds ratio (95% CI)	*P*-value	Ethnicity	Cancer type	Nearest gene	Primary report
1q22	rs2070803 (G/A)	1.63 (1.33–1.98)†	1.2×10^{-6}†	Japanese	Diffuse	*MUC1*	Sakamoto *et al.*[12]
3q13.31	rs9841504 (C/G)	0.76 (0.69–0.83)‡	1.7×10^{-9}‡	Chinese	Non-cardia	*ZBTB20*	Shi *et al.*[23]
5p13.1	rs13361707 (T/C)	1.41 (1.32–1.49)‡	7.6×10^{-29}‡	Chinese	Non-cardia	*PRKAA1*	Shi *et al.*[23]
8q24.3	rs2976392 (A/G)	1.62 (1.38–1.89)†	1.1×10^{-9}†	Japanese	Diffuse	*PSCA*	Sakamoto *et al.*[12]
10q23	rs2274223 (A/G)	1.31 (1.19–1.43)†	8.40×10^{-9}†	Chinese	Cardia, non-cardia	*PLCE1*	Abnet *et al.*[24]

CI, confidence interval; SNP, single nucleotide polymorphism. †Allelic model. ‡Additive model.

the Korean population (rs2976392: 449 cases, 390 controls, allele-specific odds ratio = 1.90, 95% confidence interval = 1.56–2.33, $P = 8.0 \times 10^{-11}$; rs2294008: 454 cases, 390 controls, allele-specific odds ratio = 1.91, 95% confidence interval = 1.57–2.33, $P = 6.3 \times 10^{-11}$) and it also showed a relatively weak correlation to IGC in both populations from Japan (rs2976392: 599 cases, 1397 controls, allele-specific odds ratio = 1.29, 95% confidence interval = 1.12–1.49, $P = 5.0 \times 10^{-4}$) and Korea (rs2976392: 416 cases, 390 controls, allele-specific odds ratio = 1.37, 95% confidence interval = 1.12–1.68, $P = 0.0017$). Later, the association of rs2976392 or rs2294008 with GC was validated in other Japanese and Korean panels and in Chinese and Caucasian studies (Table 2).[14–21] Intriguingly, *PSCA* was also identified as a gene related to bladder-cancer susceptibility for Caucasians by GWAS.[22]

Our subsequent analyses revealed that the 1q22 region contains 13 SNP with strong LD over five genes, but we have concluded that the Mucin 1 (*MUC1*) gene is responsible for the observed association as the second DGC susceptibility gene; rs2070803 with $P = 2.20 \times 10^{-6}$, adjusted per allele odds ratio = 1.63 (606 cases and 1264 controls), which was replicated in additional Japanese ($P = 3.93 \times 10^{-5}$, odds ratio = 1.81, 304 cases and 1465 controls) and Korean ($P = 2.19 \times 10^{-4}$, odds ratio = 1.82, 452 cases and 372 controls) case-control panels.[13] While rs2070803 was one of the original LD mapping markers, which we found to have an association with DGC, we later identified rs4072037 in the *MUC1* gene as a functional SNP. [13]

In addition, the combined genotype association data of rs2294008 in *PSCA* and rs4072037 in *MUC1*, both of which were shown to have biological functions (discussed in sections *PSCA* gene and *MUC1* gene), revealed that 66.5% of the Japanese control subjects had the risk genotype of rs4072037 (risk allele = A, in a recessive model of the allele effect), 84.6% had the risk genotype of rs2294008 (risk allele = T, in a dominant model) and 55.8% had both, showing an odds ratio = 8.4 (Fig. 1).[13] This suggests approximately 95% of Japanese population possess at least one of the two risk genotypes. The risk allele of rs2294008 is a major allele in the Japanese population, but the allele is minor in some other ethnic populations including Caucasians (Supporting Information Table S1).[12,13] Moreover, Korea has the highest GC incidence almost equivalent to that of Japan,[5] where both the risk alleles are similarly major, and it was estimated that more than 90% of the Korean population has at least one risk genotype of the two SNP.[13] In Japanese population and other ethnic groups, the association was demonstrated between one or two of the risk alleles, and it seems possible that the ethnic prevalence of GC development is influenced by the risk allele frequency of the SNP with proven biological functions.[12,13]

The majority of IGC arises in the chronic inflammatory lesion of the gastric epithelium resulting from HP infection, but some genetic contribution has also been suggested, and, indeed, the association of *PSCA* with IGC was demonstrated.[12] Independent of the DGC GWAS described above, an IGC GWAS was also initiated in Japan. The first screening (1600 cases, 3400 controls, 501 909 SNP) has already been performed by the National Cancer Center in collaboration with RIKEN, in which, intriguingly, rs2294008 and three other SNP in *PSCA* were included in the six SNP showing the most statistically significant association ($P < 1 \times 10^{-6}$, Hiromi Sakamoto, Teruhiko Yoshida and Yusuke Nakamura, unpublished data). In the first screening, even *PSCA*, which showed the strongest association, showed a relatively low odds ratio, for example, rs2294008 with odds ratio = 1.27 for IGC.

Besides the Japanese study, two GC GWAS were recently conducted on the Chinese population; one unveiled 3q13.31 and 5p13.1 as a GC-related chromosomal region, in addition to 1q22 and 8q24, and the other 10q23 (Table 1).[23,24] The 5p13.1 includes eight genes in the vicinity of rs13361707, and the susceptibility gene is yet to be identified in this region. Recombination hotspot analyses suggested *PLCE1*, a member of the phospholipase C family, in the 10q23, and *ZBTB20*, encoding zinc finger and BTB domain-containing protein 20, in the 3q13.31, are likely to be the causal for the association in the GWAS.[23,24]

PSCA gene

As mentioned above, *PSCA* was identified as a DGC susceptibility gene by the Japanese GWAS, although it was originally reported as the gene upregulated in prostate cancer.[25] It is also upregulated in many types of other cancers including urinary bladder cancer, renal cell carcinoma, hydatidiform mole, ovarian mucinous tumor, pancreatic cancer, non-small-cell lung cancer and glioma (Table 3).[26–32] In those cancers, PSCA can act to promote tumor progression and it was actually demonstrated that suppression of the gene with siRNA resulted in growth inhibition of prostate cancer cells.[33] In contrast, downregulation of the gene was reported only in esophageal and gastric cancers.[12,34] Recently, we have reported that the gene is also downregulated in gallbladder cancer.[35] Intriguingly, both gallbladder cancer and IGC develop in a similar sequence of chronic inflammation, intestinal metaplasia, dysplasia and cancer. Moreover, PSCA is downregulated in intestinal metaplasia in both gallbladder and gastric epithelia.[12,35] There could be other cancers in which PSCA is silenced during carcinogenesis.

It was demonstrated that PSCA has growth inhibition activity on GC cells, which is concordant with the finding of frequent downregulation in the cancer.[12] In the stomach, PSCA is expressed in the isthmus/neck region, a middle portion of the gastric epithelium, in which rapidly amplifying pre-pit cells are present to support the rapid turnover of mucus-secreting pit cells (Fig. 2). It is speculated that PSCA has a role in regulating cell growth of the pre-pit cells and that reduction of its function predisposes the pre-pit cells to abnormal cell division and carcinogenesis (Fig. 2). It is thought that the initial lesion of DGC arises in the isthmus/neck region, based on a detailed

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Table 2. Association studies of prostate stem cell antigen (*PSCA*) and gastric cancer

Report	SNP (major>minor)†	Ethnicity	Cases	Controls	Model‡	Reference§	Odds ratio	95% confidence interval	*P* value	Analyzed histology or subclasses
Sakamoto *et al.*[12]	rs2294008 T>C	Japanese	749	750	Per-allele	C	1.58	1.35–1.85	6.3×10^{-9}	Diffuse
			925 (diffuse) 599 (intestinal)	1396	Per-allele	C	1.67/1.29/1.30	1.47–1.90/1.11–1.49/ 1.10-1.52	2.2×10^{-15}/ 5.1×10^{-4}/0.0015	Diffuse/intestinal/ intestinal versus diffuse
					Dominant	CC	4.18/1.59/2.70	2.88–6.21/1.15–2.21/ 1.64–4.50	1.5×10^{-17}/ 0.0041/4.7×10^{-5}	
					Recessive	CC + CT	1.62/1.24/1.35	1.35–1.93/1.01–1.52/ 1.06–1.71	9.4×10^{-8}/ 0.040/0.013	
		Korean	454 (diffuse) 417 (intestinal)	390	Per-allele	C	1.91/1.37/1.39	1.57–2.33/1.12–1.68/ 1.14–1.69	6.3×10^{-11}/ 0.0017/7.9×10^{-14}	
					Dominant	CC	3.61/1.85/1.81	2.41–5.51/1.27–2.71/ 1.17–2.83	3.2×10^{-11}/ 0.0011/0.0066	
					Recessive	CC + CT	1.61/1.22/1.39	1.15–2.26/0.84–1.77/ 1.00–1.94	0.0051/0.31/0.050	
			749	750	Per-allele	G	1.62	1.38–1.89	1.1×10^{-9}	Diffuse
	rs2976392 A>G	Japanese	926 (diffuse) 599 (intestinal)	1397	Per-allele	G	1.71/1.29/1.32	1.50–1.94/1.12–1.49/ 1.13–1.56	1.5×10^{-16}/5.0×10^{-4}/6.0×10^{-4}	Diffuse/intestinal/ intestinal versus diffuse
					Dominant	GG	4.24/1.55/2.73	2.92–6.29/1.13–2.16/ 1.67–4.56	6.4×10^{-18}/ 0.0059/3.3×10^{-5}	
					Recessive	GG + GA	1.66/1.24/1.35	1.39–1.99/1.02–1.52/ 1.07–1.71	1.5×10^{-8}/ 0.035/0.012	
		Korean	449 (diffuse) 416 (intestinal)	390	Per-allele	G	1.90/1.37/1.39	1.56–2.33/1.12–1.68/ 1.14–1.69	8.0×10^{-11}/ 0.0017/9.0×10^{-4}	
					Dominant	GG	3.47/1.86/1.75	2.32–5.27/1.27–2.72/ 1.13–2.74	1.1×10^{-10}/ 0.0010/0.010	
					Recessive	GG + GA	1.64/1.24/1.41	1.17–2.30/0.86–1.80/ 1.01–1.97	0.0036/0.26/0.041	
Wu *et al.*[14]	rs2294008 C>T	Chinese	1020 (non-cardia) 716 (cardia)	1020	CT	CC	1.37/1.28	1.14–1.64/1.05–1.58	0.001/0.017	Non-cardia/cardia
					TT	CC	1.26/0.94	0.90–1.76/0.63–1.39	0.176/0.738	
					Dominant	CC	1.35/1.23	1.13–1.61/1.01–1.49	0.001/0.042	
	rs2976392G>A				GA	GG	1.21/1.09	1.01–1.45/0.89–1.34	0.041/0.402	
					AA	GG	1.14/0.99	0.82–1.59/0.69–1.44	0.430/0.968	
					Dominant	GG	1.20/1.08	1.01–1.43/0.88–1.31	0.041/0.470	
Matsuo *et al.*[15]	rs2294008 T>C	Japanese	708	708	Per-allele	C	1.4	1.19–1.65	3.7×10^{-5}	Gastric cancer
					Dominant	CC	2.07	1.45–2.95	6.4×10^{-5}	
					Recessive	CC + CT	1.31	1.11–1.65	0.003	
	rs2976392 A>G				Per-allele	G	1.4	1.19–1.65	4.1×10^{-5}	
					Dominant	GG	2.09	1.46–2.99	5.7×10^{-5}	
					Recessive	GG + GA	1.36	1.10–1.67	0.004	

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Table 2 (continued)

Report	SNP (major>minor)†	Ethnicity	Cases	Controls	Model‡	Reference§	Odds ratio	95% confidence interval	*P* value	Analyzed histology or subclasses
Lu *et al.*(16)	rs2294008 C>T	Chinese	1053	1100	CT	CC	1.16/1.24/1.17	0.97–1.39/0.89–1.73/0.95–1.42		Gastric cancer/diffuse/intestinal
					TT	CC	1.05/1.21/0.94	0.74–1.47/0.66–2.24/0.64–1.39		
					Dominant	CC	1.14/1.24/1.13	0.96–1.36/0.90–1.70/0.93–1.37		
	rs2976392G>A				GA	GG	1.40/1.34/1.37	1.17–1.67/0.96–1.87/1.12–1.66		
					AA	GG	1.23/1.87/1.03	0.88–1.72/1.10–3.20/0.70–1.52		
					Dominant	GG	1.37/1.43/1.31	1.15–1.62/1.04–1.96/1.09–1.58		
Ou *et al.*(17)	rs2294008C>T	Tibetan	196	246	Per-allele	C	1.34	1.00–1.79	0.049	Gastric cancer
					CT	CC	1.5	1.01–2.23	0.042	
					TT	CC	1.55	0.77–3.15	0.221	
	rs2976392G>A				Per-allele	G	1.07	0.80–1.45	0.645	
					GA	GG	1.09	0.74–1.61	0.650	
					AA	GG	1.13	0.50–2.54	0.776	
Lochhead *et al.*(18)	rs2294008C>T	Caucasian	312	383	CT	CC	1.9/2.9/1.6	1.2–2.9/1.0–10.1/1.0–2.6	0.003/0.028/0.040	Gastric cancer/diffuse/intestinal
					TT	CC	1.9/3.7/1.6	1.2–3.0/1.3–12.9/1.0–2.7	0.004/0.008/0.058	
					Dominant	CC	1.9/3.2/1.6	1.3–2.8/1.2–10.7/1.0–2.6	0.001/0.011/0.029	
					Recessive	CC + CT	1.2/1.7/1.2	0.9–1.7/0.9–3.2/0.8–1.7	0.184/0.089/0.431	
			309	211	CT	CC	0.8/0.5/0.8/0.7	0.5–1.5/0.3–0.9/0.4–1.7/0.3–1.7	0.521/0.008/0.493/0.419	Non-cardia/cardia/non-cardia diffuse/non-cardia intestinal
					TT	CC	1.7/0.6/1.7/1.2	0.9–3.0/0.3–1.2/0.8–3.6/0.5–2.9	0.069/0.105/0.155/0.651	
					Dominant	CC	1.1/0.5/1.1/0.9	0.7–1.8/0.3–0.9/0.6–2.1/0.4–1.9	0.689/0.010/0.849/0.717	
					Recessive	CC + CT	1.9/0.9/1.9/1.5	1.2–3.0/0.5–1.6/1.1–3.5/0.7–2.9	0.005/0.766/0.018/0.246	
Zeng *et al.*(19)	rs2294008C>T	Chinese	460	549	CT	CC	1.38	1.06–1.78	0.018	Gastric cancer
					TT	CC	1.66	1.03–2.69	0.038	
					Dominant	CC	1.42	1.10–1.82	0.006	
Song *et al.*(20)	rs2294008 C>T	Korean	3245	1700	Per-allele	C	1.29	1.18–1.41	<0.01	Gastric cancer
					CT	CC	1.50	1.28–1.76	<0.01	
					TT	CC	1.71	1.43–2.04	<0.01	
Sala *et al.*(21)	rs2294008 C>T	Caucasian	411	1530	Log-additive		1.42/1.47/1.54/1.52	1.23–1.66/1.19–1.81/1.20–1.96/1.20–1.93	6.5×10^{-6}/0.0003/0.0005/0.0005	Gastric cancer/non-cardia/diffuse/intestinal
					CT	CC	1.46/1.43/1.32/1.68	1.23–1.66/0.98–2.10/0.84–2.07/1.08–2.62	3.7×10^{-5}/0.0015/0.0018/0.0022	
					TT	CC	2.02/2.15/2.31/2.34	1.49–2.76/1.41–3.26/1.43–3.73/1.43–3.83		

†Major, major allele; minor, minor allele. ‡Genetic model for the biological effect of risk alleles (rs2294008:T, rs2976392:A). §Genetic model as reference (odds ratio = 1).

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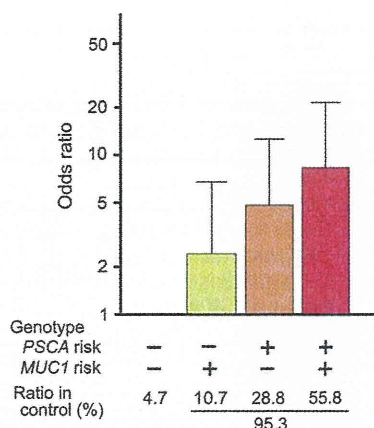

Fig. 1. Prostate stem cell antigen (*PSCA*) and Mucin 1 (*MUC1*) genotypes are associated with risk for diffuse-type gastric cancer (DGC). Association studies were performed with a distinct model for each risk allele's effect, dominant for rs2294008 (risk genotype: TT and TC; protective genotype: CC) and recessive for rs4072037 (risk genotype: AA; protective genotype: GG and GA), using genotype data of rs2294008 in *PSCA* and rs4072037 in *MUC1* (Japanese 605 DGC cases and 1264 controls). Bar, upper bound of 95% confidence interval.

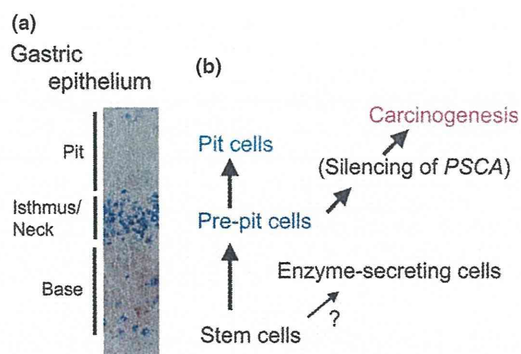

Fig. 2. Prostate stem cell antigen (*PSCA*) regulates proliferation of pre-pit cells in gastric epithelium? (a) PSCA is mainly expressed in the epithelium in the middle portion, the isthmus and neck regions, of the gastric gland (immunohistochemical double stain; blue for PSCA and brown for proliferating cell nuclear antigen). Together the two regions are called the isthmus/neck region as the boundary between the two regions is often ambiguous. Weak PSCA expression is also observed in the epithelium of the pit region. (b) The isthmus/neck region harbors pre-pit cells, a precursor of pit cells, which are rapidly proliferating to compensate for rapid turnover of pit cells. It is hypothesized that PSCA regulates proliferation of pre-pit cells, which also contributes to prevention of carcinogenesis in the epithelium.

Table 3. Cancer-type dependent expression status of the prostate stem cell antigen (*PSCA*) gene

Upregulation	Downregulation
Prostate cancer[25]	Esophageal cancer[34]
Urinary bladder cancer[26]	Gastric cancer[12,34]
Renal cell carcinoma[27]	Gallbladder cancer[35]
Hydatidiform mole[28]	
Ovarian mucinous tumor[29]	
(Aberrant expression)†	
Pancreatic cancer[30]	
Non-small-cell lung cancer[31]	
Glioma[32]	

†Upregulation in cancer but no expression in normal tissue.

histopathological investigation in Japan, which revealed that the smallest lesions of dysplasia and carcinoma seem to be confined to the region.[36]

It was demonstrated that the rs2294008 in the gene is the functional SNP affecting transcriptional activity of the *PSCA* promoter. However, the biological effect of the T allele still seems to be considerably controversial. The rs2294008 determines the position of the translation initiation codon; the T allele makes itself part of the codon encoding as the first methionin (ATG); in contrast, the C allele replaces the encoded amino acid from methionine to threonin (ACG), resulting in a change of the first methionine position. The T allele associated with the risk for GC has a negative effect on the promoter activity in gastric, urinary bladder and gallbladder cancer cell lines.[12,22,35] Therefore, it is anticipated that people possessing the T allele have a lower amount of PSCA protein in the organs than those possessing the C allele. However, recent microarray transcriptome analyses showed that normal and malignant urinary bladder tissues from people with the T allele contained more *PSCA* transcripts than those from people with the C allele.[37] The discrepancy between the *in vitro* reporter assay and the *in vivo* expression data further suggests a complexity of the PSCA regulation, which might be influenced by tissue-specific transcriptional factors and DNA methylation, as shown in gastric and gallbladder cancer cell lines.[35] In contrast, a recent study reported that the C allele of rs2294008 in *PSCA* was associated with an increased risk of duodenal ulcer (DU; odds ratio = 1.84; $P = 3.92 \times 10^{-33}$ in a recessive model).[38] Moreover, the results of functional analyses showed that the C allele changes the subcellular localization of PSCA protein from the cell surface to the cytoplasm and also reduced the protein's stability.[38] As PSCA might have several functions, some of which could be contradictory, in the context of tissues and pathological states, it is likely that the functional effect of rs2294008 might also differ. In particular, the reciprocal association between the rs2294008 alleles and two major HP-related gastrointestinal diseases is notable; the T allele predisposes to GC, while the C allele confers an increased risk for DU. It is known that patients with DU have a decreased risk for GC, but it depends on the location of gastritis; DU patients with chronic corpus gastritis have an increased risk of GC.[39] This could be explained by the relation between the location of gastritis and the amount of acid secretion; corpus-predominant gastritis is accompanied by hypochlorhydria and results in the highest risk for GC, whereas antrum-predominant gastritis is associated with hyperchlorhydria and predisposes to DU disease.[40,41] As PSCA is not expressed in the duodenum, its function in the stomach might affect DU development, possibly through such effects as those related to acid secretion, location and extent of HP infection or gastritis. It was also reported that the T allele was especially associated with non-cardia GC in Chinese and Caucasian patients but with cardia GC in Korean patients. [14,18–21]

The cell growth inhibition activity of PSCA was also demonstrated on gallbladder cancer (GBC) cells.[35] The GBC cells introduced with *PSCA* cDNA showed lower *in vitro* and *in vivo* growth than controls, and their invasion ability assayed with a Matrigel chamber was also attenuated.[35] PSCA is expressed homogenously in normal gallbladder epithelium, which is characterized by a mono-layer of columner cells and functions by absorbing water and electrolytes. It is possible that PSCA has a role in cell-division control and/or other activities such as active transport of molecules in gallbladder epithelium.

The product of the *PSCA* gene is glycosylphosphatidylinositol (GPI)-anchored membrane protein with unknown biological

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function.[42] It is believed that, as with other GPI-anchored proteins, PSCA might be located in a special microdomain called lipid raft, enriched in glycosphingolipids, cholesterol and other lapidated proteins, on the outer surface of the cell membrane. The lipid raft is known as the domain where molecular interaction for subcellular signaling is processed.[43] However, there have been no reports on the elucidation of the PSCA ligand or the molecule on which PSCA makes some modification. Our attempt to co-immunoprecipitate PSCA-associating molecules has not been successful, even with cross-linking of proteins using DSP (dithiobis[succinimidyl-proprionate]) or DTSSP (3,3′-dithiobis[sulfosuccinimidylpro-prionate]). The function of PSCA might be restricted to a preparation of the microenvironment on the cell membrane by changing the local composition of lipid and other molecules, which supports some molecular interaction required for subcellular signal transduction. If that is the case, its apparently organ-dependent opposing function, either tumor promotion or suppression, could be highly comprehensible.

MUC1 gene

The Mucin family (MUC1 to MUC21) consists of secretary and membrane-bound types, and MUC1 belongs to the latter.[44] After being translated, a single MUC1 peptide was cleaved to N-terminal and C-terminal subunits, designated as MUC1-N and MUC1-C, respectively, by autoproteolysis, but both the subunits remain associated by non-covalent binding and are localized to the cell membrane in the apical side of the epithelial cells. MUC1-N present on the cell surface has multiple glycosylation sites and is thought to be the second line of protection for cells against many types of insults, after the front layer of defense by the secretary mucins in mucus.[45] However, MUC1-C has a transmembrane domain and a cytoplasmic tail (CT), which is involved in subcellular signal transduction. The CT contains several phosphorylation sites and a β-catenin binding site. Phosphorylation of Thr in the TDRSPYEKV sequence within the CT stimulates interactions between the CT and β-catenin, which leads to nuclear localization of the complex for regulating genes including *p53*.[46,47] Previously, and maybe at present, MUC1 has been considered as an oncoprotein, because there are several studies demonstrating the correlation of MUC1 expression and the poor prognosis of cancer patients. It was also reported that MUC1 acts as a growth factor receptor on undifferentiated human embryonic stem cells.[48–50]

In addition to the GWAS conducted in Japan, the recently conducted GWAS on the Chinese population also listed 1q22 as a candidate for GC-related locus (rs4072037; odds ratio = 0.75, $P = 4.22 \times 10^{-7}$).[12,24] The association between non-cardia GC and the GC-related locus was also demonstrated in imputation analyses on large-scale Chinese case-control samples (rs4072037; odds ratio = 0.73, $P = 1.0 \times 10^{-4}$).[23] Moreover, an association between *MUC1* gene polymorphisms and GC has also been reported by other groups previously.[51,52] The *MUC1* gene contains a polymorphic number of tandem repeats, a variable number of tandem repeats (VNTR), which are shown by an electrophoresis pattern after restriction enzyme digestion. When the polymorphic allele is divided into large (L) and small (S) alleles, the latter was shown to associate with GC in Caucasians.[51,52] The association between the A allele of rs4072037 and DGC identified in Japanese patients was also found in Chinese and Caucasian patients.[53,54] The A allele of rs4072037 identified using the GWAS is in a linkage disequilibrium with the S allele in Japanese and Caucasian patients.[13,55] The results of these association studies on different ethnic populations strongly support the result of the Japanese GWAS; *MUC1* is a GC susceptibility gene.

It was demonstrated that the rs4072037 in *MUC1* has a biological function. In the gastric epithelium, variants 2 and 3 are the major *MUC1* transcript.[13] The rs4072037 is located in the 5′ side of the second exon of *MUC1* and determines the splicing acceptor site in the second exon, which in turn defines the type of variants; the G and A alleles result in the expression of variants 2 and 3, respectively (Fig. 3).[13,55] Consequently, the nine amino acid deletion in the second exon changes the supposed cleavage site of the N-terminal signal peptide, which might lead to a difference in the function of the encoded protein between the two splicing variants. It is understood that, in GC and other cancer cells that have lost cell polarity, the MUC1 protein interacts freely with other molecules including membrane receptors involved in cell growth and, consequently, it acts as an oncoprotein; in contrast, in normal epithelial cells, MUC1 is restricted to the apical surface of the cells where the interaction with other molecules is limited and it acts as a barrier against exogenous insults to the cells.[56] It is speculated that the rs4072037 affects the barrier function in the stomach of individuals through the determination of a major variant expressed in the stomach, which results in the difference in GC susceptibility. From a different viewpoint, *MUC1* was identified as associating with the serum magnesium level using GWAS.[57] Because a correlation between a low serum magnesium level and GC was suggested, it is possible that MUC1 affects GC susceptibility by playing a role in magnesium homeostasis.[58] However, it was demonstrated using the GWAS that hypomagnesaemia was correlated with the G allele of rs4072037, the protective allele for GC development.[57] As no correlation was observed between *MUC1* and IGC in the present study ([13] and Hiromi Sakamoto, Teruhiko Yoshida

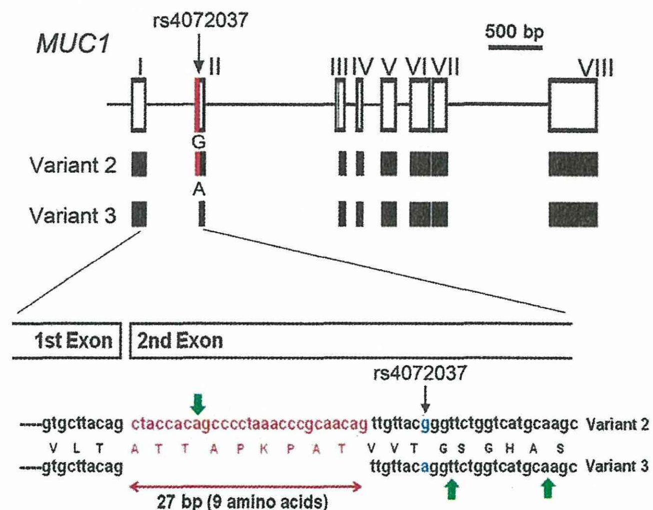

Fig. 3. Single nucleotide polymorphism (SNP) rs4072037 determines the major splicing variants expressed in the gastric mucosa. In the gastric mucosa, major splicing forms were variant 2 (NM_001018016) and variant 3 (NM_001018017). The allele of SNP rs4072037 is related to the splicing acceptor site selection in the second exon (upper panel). Nucleotide sequences of the first/second exon boundary of Mucin 1 (*MUC1*) variants 2 and 3 revealed using RNA ligase-mediated rapid amplification of the 5′ cDNA end on RNA samples from normal stomach and gastric cancer cell lines (lower panel).[13] In the present study, all variant 2 transcripts containing the first 27 bp of the second exon (double-headed red arrow) had a G allele at rs4072037, while all variant 3 lacking the 27 bp had A allele. This result is concordant with a previous report.[55] It is anticipated that deletion of the 27 bp, corresponding to nine amino acids, changes cleavage sites (green arrows) of the signal peptide among the variants. One-letter amino acid abbreviation is shown just below or above the second nucleotide of each codon.

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