subjects and participated in diagnostic evaluations. K.S. and T.K. wrote the manuscript. M.K., Y.D., T.Y. and Y.N. contributed to the overall GWAS design.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study design and subjects. We performed a three-stage GWAS of lung adenocarcinoma in the Japanese population using independent samples. The characteristics of each case-control group are shown in **Supplementary Table 1**. The discovery GWAS samples consisted of 1,722 cases from the National Cancer Center Hospital (NCCH) and 5,846 controls from the BioBank Japan project[22], Osaka-Midosuji Rotary Club (MRC) and the Pharma SNP consortium (PSC). The BioBank Japan project (see URLs) was begun in 2003 for the collection of genomic DNA, serum and clinical information from 300,000 individuals diagnosed with any of 47 diseases by a collaboration network of 66 hospitals in all areas of Japan[22]. The subjects from MRC were 1,018 healthy volunteers, and the subjects from PSC were 906 Japanese healthy volunteers from whom immortalized B lymphoblast cell lines were established by the PSC. The cell lines were obtained from the Japan Health Sciences Foundation (JHSF)/Health Science Research Resources Bank (HSRRB). Individuals with any cancer were excluded from the control group.

The validation study consisted of two independent cohorts. The first validation cohort included 2,955 cases with lung adenocarcinoma and 7,036 controls. The cases included 1,747 subjects from the BioBank Japan project and 1,208 subjects from NCCH. All control subjects were from the BioBank Japan project. Individuals with any cancer were excluded from the control group. The second validation cohort included 1,379 cases with lung adenocarcinoma from the NCCH and 1,166 controls (cancer-free volunteers) from the NCCH and the Keio University in Tokyo[23]. Individuals with any cancer were excluded from the control group.

All of the cases with lung adenocarcinoma were diagnosed by cytological and/or histological examination according to the World Health Organization classification[24]. The cases from NCCH in the GWAS and first validation sets consisted of cases for which enough DNA for large-scale SNP analyses were available, and the cases in thee second validation set consisted of cases for which less DNA were available, for example, because only small noncancerous tissues were available for DNA extraction. Controls from the BioBank Japan project were genotyped previously. Genome-wide genotyping data obtained before and after 2010 were used as the controls for the GWAS and the first validation study described below, respectively, and thus there was no specified rationale for the selection of control subjects other than time of genotyping. Eight controls and 1,529 cases with lung adenocarcinoma from BioBank Japan and 906 control subjects from the Osaka-MRC were used in the previous GWAS[10]. All the participants provided written informed consent. This project was approved by the ethical committees of each participating institution.

Sample preparation and genotyping. Genomic DNA was extracted from peripheral blood leukocytes or noncancerous lung tissues using standard methods.

In the GWAS, we genotyped 1,722 cases with lung adenocarcinoma from the NCCH using the Illumina HumanOmni1-Quad Chip. For the controls in the GWAS, we used genome-wide data from 5,846 individuals with cerebral aneurysms, chronic obstructive pulmonary disease or glaucoma, which were genotyped using the Illumina Human OmniExpress Genotyping BeadChip.

In the study of the first validation cohort, we genotyped 2,955 cases with lung adenocarcinoma using the multiplex PCR-based Invader assay (Third Wave Technologies), as previously described[10]. The control group consisted of genome-wide data from 7,036 individuals with epilepsy, nephrosis syndrome, atopic dermatitis, urinary tract stone disease or Basedow's (Graves') disease, which were genotyped using the Illumina Human OmniExpress Genotyping BeadChip. The same quality control criteria were applied as in the GWAS (see below) to confirm that no unexpected duplicates or probable relatives were present between the control subjects of the GWAS and those of the first validation study.

In the study of the second validation cohort, we genotyped 1,379 cases with lung adenocarcinoma and 1,166 controls using the TaqMan method, according to the protocol for the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Quality control. Systemic quality control was performed on the raw genotyping data from 709,857 SNPs in DNA samples obtained from 7,568 subjects, consisting of 1,722 cases and 5,846 controls, using PLINK (v 1.06)[25]. Forty-four subjects were excluded because they showed gender discrepancies based on their X chromosome genotypes (7,524 subjects remained). Next, SNPs were excluded according to the following criteria: (i) 19,993 SNPs were not mapped on autosomal chromosomes; (ii) 102,929 SNPs had a minor allele frequency <0.01; and (iii) a total of 48,769 SNPs had a call rate <0.99 and genotype distributions that clearly deviated from those expected by Hardy-Weinberg equilibrium ($P < 1.0 \times 10^{-6}$). Together, 538,166 SNPs in autosomal chromosomes passed the quality-control filters and were used for the GWAS.

Next, an additional 75 unexpected duplicates or probable relatives in the GWAS were excluded based on pairwise identity by state according to their PI_HAT values in PLINK (all PI_HAT > 0.25) (7,449 subjects remained). Heterozygosity rates were calculated using PLINK, and more than 6 s.d. from the mean was used as the exclusion criterion. A principal components analysis (PCA) was performed on the genotype data from the samples, along with European (CEU), African (YRI) and east Asian (Japanese (JPT)) and Han Chinese (CHB)) individuals obtained from the phase II HapMap database using smartpca[26]. The PCA revealed no evident population substructure (**Supplementary Fig. 1a**) and identified seven outliers for exclusion (7,442 subjects remained). Most subjects fell into a known main cluster (Hondo) of the Japanese population (**Supplementary Fig. 1b**), and 414 subjects that fell far from the Hondo cluster[27] were excluded. The remaining 7,028 subjects, consisting of 1,695 cases and 5,333 control subjects, were used for the GWAS. The lack of population substructure between the cases and controls was validated by PCA of the subjects in the Hondo cluster (**Supplementary Fig. 1c**).

Statistical analyses. In the GWAS and the validation study, the statistical significance of the association with each SNP was assessed using a logistic regression trend test in the R program. Age and gender were included as covariates. Heterogeneity across the two stages was examined using the Breslow-Day test[28].

Imputation. We performed SNP imputation for each individual in the GWAS using the IMPUTE7 program[29]. The 1000 Genomes Project database (June 2010 release) was used as a reference panel. After excluding imputed SNPs with a low genotype information content (<0.5), posterior probability score (<0.90), call rate (<0.90), minor allele frequency (<0.01) or Hardy-Weinberg equilibrium ($P < 1.0 \times 10^{-7}$), imputed SNPs with $r^2 > 0.3$, residing 200–500 kb upstream or downstream of the two newly identified marker SNPs, were subjected to association analyses.

Software. For general statistical analyses, we used the R statistical environment version 2.6.1 or PLINK1.06 (ref. 25). We used LocusZoom to plot regional association plots[30].

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Prostate stem cell antigen gene is expressed in islets of pancreas

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Abstract: Prostate stem cell antigen (PSCA) is a glycosylphosphatidylinositol-anchored cell surface antigen with an organ-dependent expression pattern in cancers; e.g., up-regulated in prostate cancer and down-regulated in gastric cancer. Previously it was reported that PSCA is not expressed in the normal pancreas but aberrantly expressed in pancreatic cancer. In this present study, we identified PSCA expression in islets of the pancreas by immunohistochemistry, which was co-localized with four islet-cell markers: insulin, glucagon, somatostatin and pancreatic polypeptide. In our investigation of the transcription start site of *PSCA*, we found a non-coding splicing variant of *PSCA* as well as authentic *PSCA* transcripts in mRNA samples from a normal pancreas. Both the transcripts were also identified in several pancreatic cancer cell lines. We previously reported that PSCA expression is correlated to the methylation status of the enhancer region in gastric and gallbladder cancer cell lines but not in pancreatic cancer cell lines, suggesting that PSCA expression is regulated in a different mode in pancreatic cancer from that in gastric and gallbladder cancers.

Key words: GPI-anchored protein, Islet cells, Pancreatic cancer, Splicing variant, Immunohistochemistry

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Introduction

The prostate stem cell antigen (PSCA) gene encodes a glycosylphosphatidylinositol (GPI)-anchored membrane protein with an unknown biological function [1]. As with other GPI-anchored proteins, it is thought that PSCA locates in a lipid raft on the outer surface of the cell membrane, a special microdomain enriched in glycosphingolipids, cholesterol and other lipidated proteins, and has some functional involvement in subcellular signal transduction [2]. *PSCA* was originally identified as a gene up-regulated in prostate cancer [3]. It is also up-regulated in other tumors including urinary bladder cancer, renal cell carcinoma, hydatidiform mole and ovarian mucinous tumor, where PSCA is thought to abet tumor progression [1]. In contrast, down-regulation of the gene was reported in gastric and gallbladder cancers, where it may act as a tumor suppressor [4, 5]. Moreover, there is another type of expression pattern: no expression in normal tissues but expressed in malignant counterparts, which is seen in pancreatic cancer [6], non-small cell lung cancer [7] and glioma [8]. It was reported that PSCA expression was not observed in the normal pancreas by northern blotting or immunohistochemistry and that its transcript could be a practical biomarker in detecting pancreatic cancer [6]. In this study, we identified PSCA expression in islets of the normal pancreas, which harbor endocrine cells, by immunohistochemistry using a mouse monoclonal anti-PSCA antibody.

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Materials and Methods

Immunohistochemistry

Embedded normal pancreas specimens on glass were purchased from BioChain Institute (Newark, CA, USA). Immunohistochemistry (Fig. 1A) was performed by the same procedure as described in the previous studies [4, 5] with mouse monoclonal anti-PSCA antibody whose specificity had been proved previously [4], rabbit anti-proliferating cell nuclear antigen (PCNA) antibody (sc-7907, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were incubated at 4°C overnight with both antibodies simultaneously, and then with alkaline phosphatase conjugated anti-mouse IgG antibody and peroxidase conjugated anti-rabbit IgG antibody (NICHIREI Biosciences Inc., Tokyo, Japan) for an hour at room temperature. PSCA protein was visualized by Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA, USA) and PCNA by Vector NovaRED Substrate Kit for Peroxidase (Vector Laboratories). The fluorescent immunohistochemistry (Fig. 1B) was conducted using anti-PSCA, rabbit anti-insulin (sc-9186, Santa Cruz Biotechnology), rabbit anti-glucagon (ab18461, Abcam Japan, Tokyo, Japan), rabbit anti-somatostatin (A0566, Dako Japan, Tokyo, Japan) and goat anti-pancreatic polypeptide (NB100-1793, Novus Biologicals, Littleton, CO, USA) antibodies for primary antibodies, and anti-mouse IgG antibody-Rhodamin, anti-rabbit IgG-FITC and anti-goat IgG-FITC conjugates (sc-2300, sc-2090, sc-2024, respectively, Santa Cruz Biotechnology) for secondary antibodies. The fluorescent signals were obtained by Zeiss LSM510 META Confocal Imaging System (Carl Zeiss MicroImaging, LLC, Thornwood, NY, USA).

Cell lines

The cell lines analysed in this study were described elsewhere; pancreatic cancer cell lines [6], gastric cancer cell lines [4], and gallbladder cancer cell lines [5].

RNA ligase-mediated rapid amplification of 5′ cDNA end (RLM-5′ RACE)

The RLM-5′ RACE was conducted on commercially available total RNA isolated from normal human pancreas (BioChain, Newark, CA, USA), and on total RNA isolated from the pancreatic cancer cell lines, using a GeneRacer kit (Invitrogen, Tokyo, Japan). A primer for the 1st strand synthesis (gene specific primer 1) was designed on the sequence in the 2nd exon, and nested PCR was performed with two sets of primers; a GeneRacer 5′ primer and the gene specific primer 1, and a GeneRacer 5′ nested primer and a primer (gene specific primer 2) containing a sequence partially overlapped with the gene specific primer 1 (Table 1). The PCR products were cloned into pCR4-TOPO vector (Invitrogen) and sequenced.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

The templates were synthesized from commercially available total RNA isolated from normal human pancreas, stomach and gallbladder (BioChain), or total RNA isolated from the pancreatic, gastric and bladder cancer cell lines, using the ThermoScript RT-PCR System (Invitrogen). Quantitative RT-PCR in Figs. 2A and 3A was performed by TaqMan Gene Expression Assay (Life Technologies Japan, Tokyo, Japan; Applied Biosystems assay ID: Hs00194665_m1 for PSCA, Applied Biosystems Part No. 4326317E for glyceraldehyde-3-phosphate dehydrogenase [GAPDH]), which was conducted for 40 cycles under a condition of 2 steps of temperature: 95°C for 15 seconds and 60°C for 60 seconds, by ABI PRISM 7900HT Sequence Detection System (Life Technologies Japan). Quantitative RT-PCR in Fig. 2C was performed with gene expression assay using SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan), conducted in 40 cycles under a condition of 2 steps of temperature: 95°C for 5 seconds and 60°C for 30 seconds, by the ABI PRISM 7900HT Sequence Detection System. The relative transcript level was calculated using the Ct value of *GAPDH* transcript as reference.

DNA methylation analysis

The DNA methylation status of the *PSCA* enhancer in the pancreatic cancer cell lines was analysed by bisulfite-Pyrosequencing procedure in the same manner as described in the previous report [5]. DNA samples were isolated from the pancreatic cancer cell lines with FlexiGene DNA Kit (Qiagen, Tokyo, Japan) and treated with EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. DNA methylation of the *PSCA* gene (GenBank accession No. NG_011722) was investigated by bisulfite-Pyrosequencing at *PSCA* enhancer region (position, -3,090 to -2,880) which is in the *PSCA* enhancer region located at -2.7 to -3 kb from the transcription initiation site [5]. PCR was performed in triplicate under a condition of 45 cycles of 3 steps of

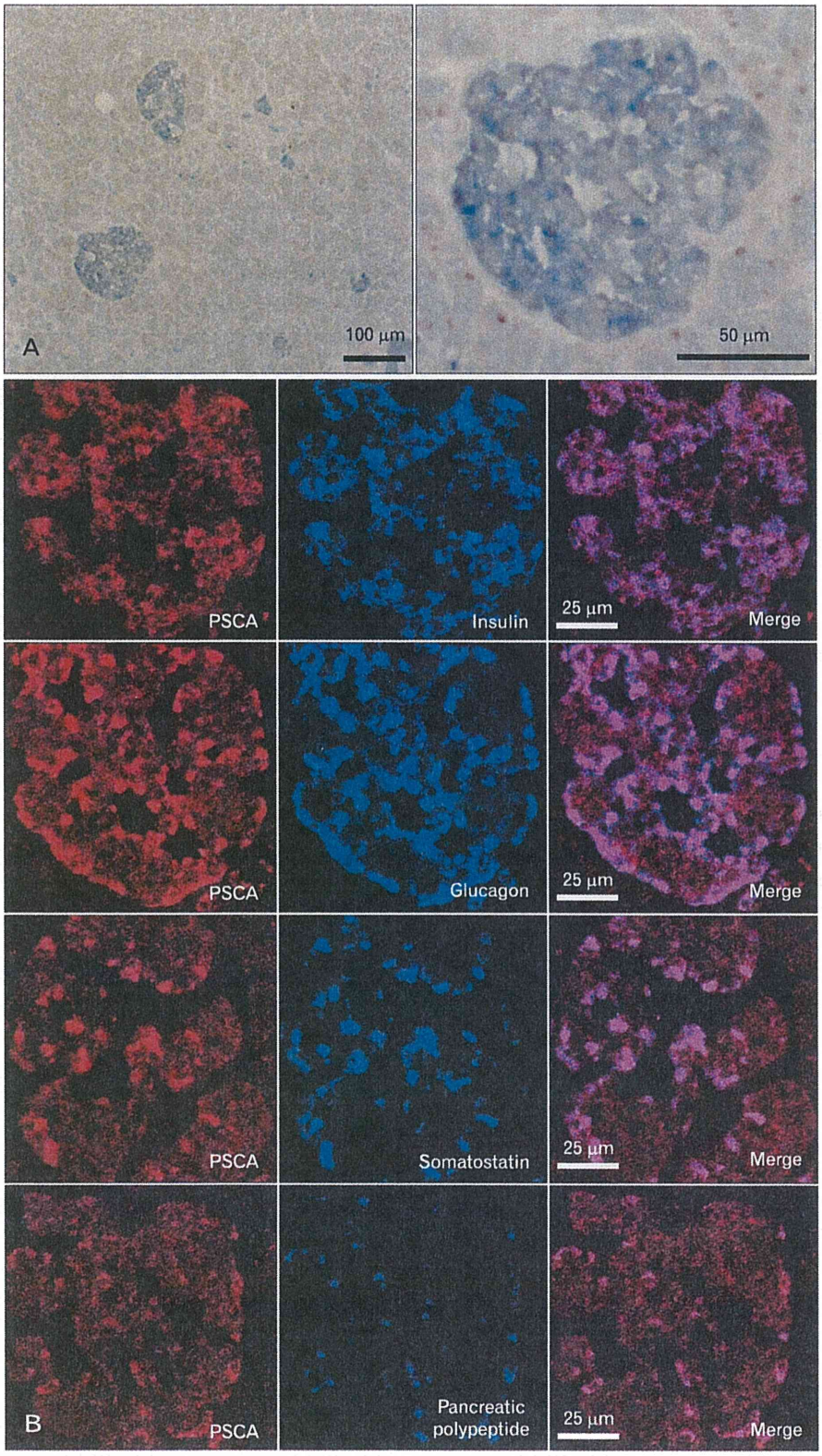

Fig. 1. Prostate stem cell antigen (PSCA) is expressed in the islet of human normal pancreas (immunohistochemistry). (A) Double staining with anti-PSCA (blue) and anti-proliferating cell nuclear (brown) antibodies. The right panel is magnification of the islet shown in the left column. (B) Fluorescent double staining of anti-PSCA antibody (red) and four islet-cell markers (blue), insulin (β cell), glucagon (α cell), somatostatin (δ cell) or pancreatic polypeptide (PP cell). Each series of the image was obtained by focusing on the islet-cell markers.

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Table 1. Primers

Primers for RLM-5' RACE	
Gene specific primer 1	5'-GCAGTTCAAGCTGCAGCCTTTGC-3'
Gene specific primer 2	5'-AGCTGCAGCCTTTGCTGATGACGGTC-3'
Primers for quantitative RT-PCR using SYBR Premix Ex Taq II	
PSCA variant 1	5'-CAGTGACCACGAAGGCTGTG-3'
	5'-CCTGGGCTTTGCAGGAGTAG-3'
PSCA variant 2	5'-AGCTGGGGTCCAATCCATAC-3'
	5'-CAGTCCTCGTTGCTCACCTG-3'
GAPDH	5'-ACAGTCAGCCGCATCTTCTT-3'
	5'-GTTAAAAGCAGCCCTGGTGA-3'
PCR primers for bisulfite-pyrosequencing methylation assay (position, -3090 to -2880)	
	5'-TAACAACCCCTATCCCATCC-3'
	5'-GCTGCTCCGGTTCATAGATTTAACAACCCC TATCCCATCC-3'
5'-biotinylated universal primer	5'-GCTGCTCCGGTTCATAGATT-3'
Sequence primers for bisulfite-pyrosequencing methylation assay (position, -3090 to -2880)	
	5'-TATTATTAAATATTTTTTGG-3'
	5'-ATTTATAAATATTTGAGG-3'
	5'-TATAGAGATAGATAAGAAAA-3'
	5'-GGGAGTGGTTAGAGT-3'

RLM-5' RACE, RNA ligase-mediated rapid amplification of 5' cDNA end; RT-PCR, reverse transcription polymerase chain reaction; *PSCA*, prostate stem cell antigen; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

temperature, 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute, after incubation at 95°C for 15 minutes with HotStarTaq (Qiagen). Biotinylated PCR product was purified and proceeded to the Pyrosequencing reaction, which was performed using the Pyrosequencing 96 Sample Prep Tool (Qiagen) and PSQ96MA (Biotage, Uppsala, Sweden). The data of the methylation status for the CpG site in bisulfite-treated sequences were obtained using the primers shown in Table 1, and the average frequency of methylated CpG sites was used as the methylation status index (%5mC).

Results

It was previously reported that PSCA was not expressed in the normal pancreas, but we identified PSCA expression in the islets of the normal human pancreas by immunohistochemistry, and double-staining for 4 islet-cell markers revealed that PSCA is expressed in all of the 4 islet cells: α, β, δ, and PP cells (Fig. 1). Pancreatic expression was confirmed by quantitative RT-PCR (Fig. 2A). To identify the regulatory region for PSCA expression in the pancreas, we first performed RLM-5' RACE to identify the transcription start site (TSS) of the *PSCA* transcripts, which revealed two splicing variants in the pancreas: one is the authentic transcript (variant 1, NCBI accession No. NM_005672) generally detected in other tissues; the other is a non-coding type (variant 2, NR_033343) transcribed at the TSS about 1 kb upstream of that of the authentic one, suggesting that the regulatory region of variant 2 is different from that of the authentic (Fig. 2B). As the amount of variant 2 was small, it may be expressed in the islets. We also examined the expression of variant 2 in the normal stomach and gallbladder and in cell lines derived from their cancers, in addition to pancreatic cancer cell lines, by RT-PCR and found the transcript in all the normal tissues and the cell lines except MIA Paca2 (Fig. 2C). Variant 2 expression was dominant over variant 1 expression in the normal pancreas and the pancreatic cancer cell lines, although it codes no protein. We previously reported that variant 1 expression in gastric and gallbladder cancer cell lines is associated with the DNA methylation level in the enhancer region: the cell lines with relatively high PSCA expression tend to have a lower methylation level [5], but the relation was not observed in pancreatic cancer cell lines (Fig. 3).

Discussion

Although its function is unknown, in the normal prostate epithelium, PSCA is expressed in late intermediate cells in the differentiation process but not in mature secretary cells [9]. In the stomach, PSCA is mainly expressed in the epithelium of the middle portion of the gastric gland, which harbors a pre-pit cell, a transit amplifying precursor of a mucous-secreting pit cell [5]. In these organs, PSCA seems to have a role in the cells differentiating, rather than in differentiated mature cells. It was reported that pancreatic islets contain multipotent stem cells and that the islet cells continue to proliferate slowly for turning over [10, 11]. In this sense, the islet cells possess a property of immature cells, and PSCA may have a function related to the differentiation and/or proliferation of the cells.

In this study, we performed immunohistochemistry on the normal human pancreas, which revealed PSCA expression in islet cells. The expression was confirmed by RT-PCR and, in the process of identification of the TSS in the pancreas, we observed two types of *PSCA* transcript in the normal pancreas as well as in pancreatic cancer cell lines. It is important to note the fact that PSCA is expressed in the normal pancreas, because there are several reports suggesting that PSCA can be utilized as a biomarker of pancreatic cancer [12-14] and it is

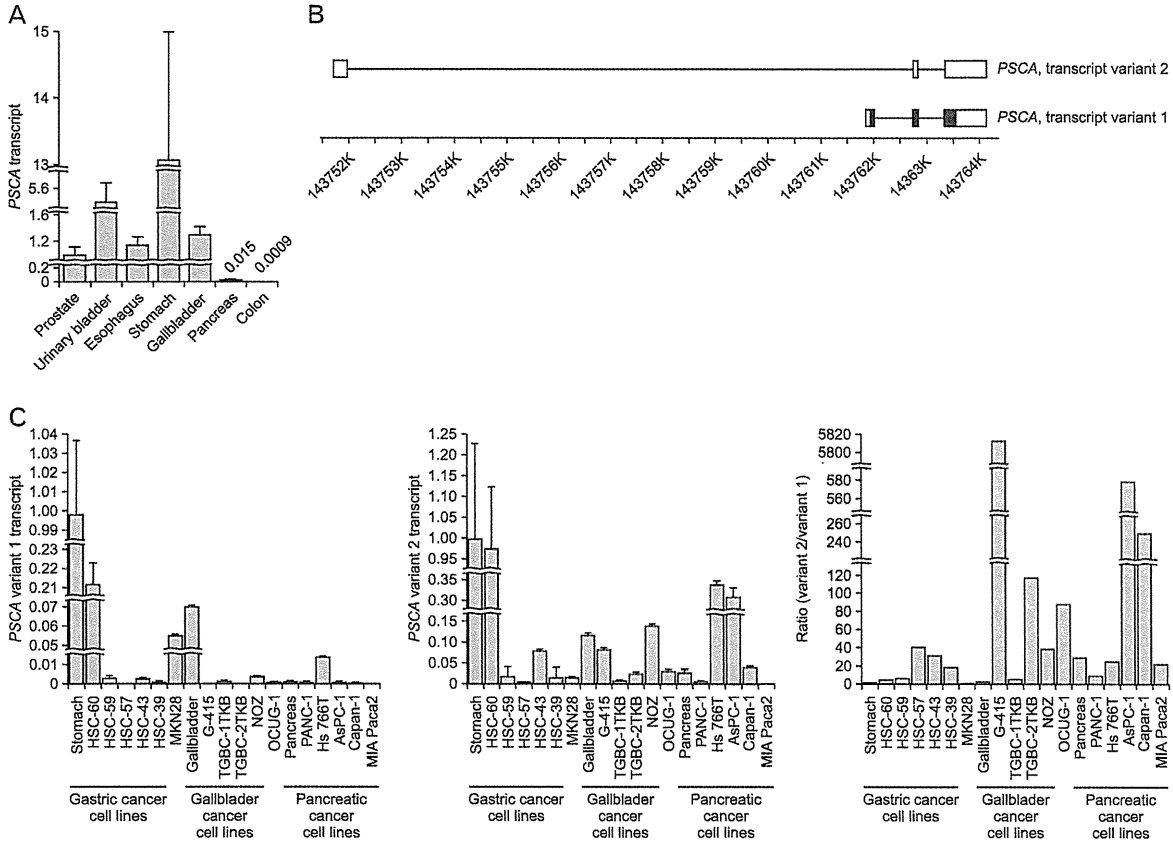

Fig. 2. Two splicing variants of the prostate stem cell antigen (*PSCA*) transcript are expressed in normal pancreatic, gastric and gallbladder tissues and in cancer cell lines derived from their cancers. (A) Quantitative reverse transcription polymerase chain reaction (RT-PCR) revealed *PSCA* transcript (variant 1) in human normal pancreas. Colon is negative control as PSCA is not expressed in that organ. (B) Schematic representation of the structure of the two variants. Position in chromosome 8q24 is based on NCBI Build 37.3. (C) Quantitative RT-PCR revealed expression of variants 1 and 2 in the normal tissues and the cancer cell lines, and variant 2 is dominant in the pancreas and pancreatic cancer cell lines.

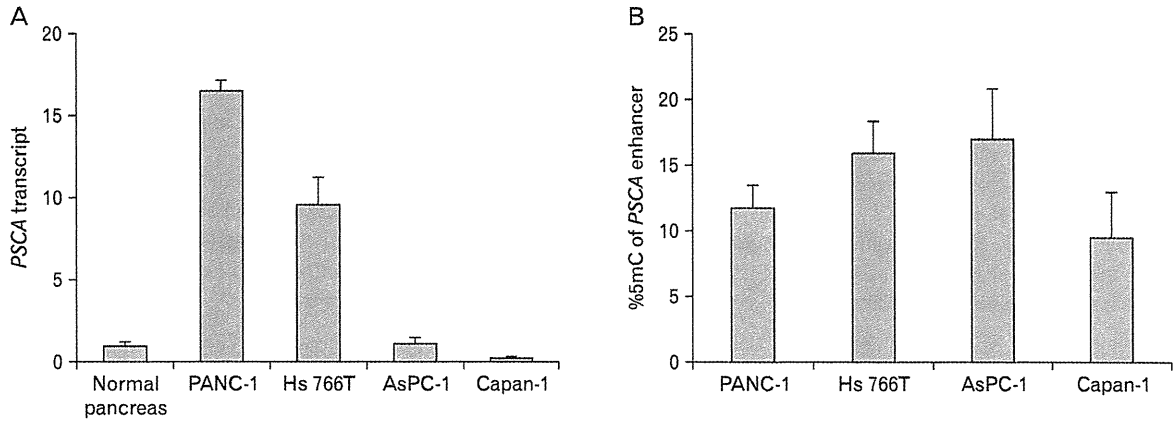

Fig. 3. Bisulfite-pyrosequencing revealed that the DNA methylation status of the prostate stem cell antigen (*PSCA*) enhancer (B) is not correlated to expression level (A) in the pancreatic cancer cell lines.

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considered as a target molecule in the treatment of pancreatic and prostate cancers [15, 16]. Clinical applications targeting PSCA have a potential risk of leading to misdiagnosis and adverse events in medical practice.

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Association of dietary and genetic factors related to one-carbon metabolism with global methylation level of leukocyte DNA

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Global hypomethylation of leukocyte DNA has been associated with an increased risk of cancer. As dietary and genetic factors related to one-carbon metabolism may influence both the methylation and synthesis of DNA, we investigated associations between these factors and the global methylation level of peripheral blood leukocyte DNA based on a cross-sectional study of 384 Japanese women. Dietary intake of folate and vitamins B2, B6, and B12 was assessed with a validated semiquantitative food frequency questionnaire. Five polymorphisms in methylenetetrahydrofolate reductase (*MTHFR*) (rs1801133 and rs1801131), methionine synthase (*MTR*) (rs1805087), and methionine synthase reductase (*MTRR*) (rs10380 and rs162049) were genotyped. Global DNA methylation of leukocyte DNA was quantified using Luminometric Methylation Assay. A linear trend of association between methylation and dietary and genetic factors was evaluated by regression coefficients in a multivariable linear regression model. Mean global methylation level (standard deviation) was 70.2% (3.4) and range was from 59.0% to 81.2%. Global methylation level significantly decreased by 0.36% (95% confidence interval, 0.03–0.69) per quartile category for folate level. Subgroup analysis suggested that alcohol drinking modified the association between folate intake and global methylation level ($P_{interaction}$ = 0.01). However, no statistically significant association was observed for intake of vitamins B2, B6, and B12, alcohol consumption, or five single nucleotide polymorphisms of *MTHFR*, *MTR*, and *MTRR*. We found that higher folate intake was significantly associated with a lower level of global methylation of leukocyte DNA in a group of healthy Japanese females. (*Cancer Sci* 2012; 103: 2159–2164)

DNA methylation plays an important role in the epigenetic mechanism of gene regulation[1,2] and cellular differentiation.[3] Aberrant genomic DNA methylation, both in specific genes and in the genome overall, is widely recognized to be associated with cancer.[4] For example, hypermethylation at promoter CpG islands in tumor suppressor genes is an important means of silencing transcription in carcinogenesis.[4] Global DNA hypomethylation in normally methylated regions is thought to contribute to carcinogenesis through the induction of genomic instability.[4] In addition, some previous evidence suggests that DNA hypomethylation could lead to the activation of oncogenes, and global DNA hypomethylation has been linked to hypomethylation in multiple promoter CpG islands.[4,5] Although many studies have investigated aberrant DNA methylation at the tissue level, there is great interest in epigenetic markers in peripheral blood and several epidemiological studies have found that hypomethylation of global peripheral blood cell DNA is associated with an increased cancer risk.[6–9] However, determinants of global methylation level among healthy individuals remain largely unexplored.

Folate and vitamin Bs in one-carbon metabolism are cofactors and cosubstrates for methylation and nucleic acid synthesis and also function as regulatory molecules of these processes.[10] Accumulating epidemiological evidence has suggested that folate intake is associated with a decreased risk of some sites of cancer such as esophagus, colorectum, and pancreas,[11] which implies that folate is associated with cancer risk through the mechanisms of DNA methylation and DNA synthesis. As folate is a universal methyl donor, which is necessary in DNA methylation, it is considered to be a potential determinant of the global methylation level of leukocyte DNA.[12] Intervention studies have suggested that folate might alter DNA methylation levels, but findings have been inconsistent.[12–16] Only a few of the previous observational studies examined associations of dietary and genetic factors related to one-carbon metabolism with global methylation level of leukocyte DNA, and their overall findings showed no association.[6–8] These inconsistent findings might be explained by differences in exposure level of nutrients related to one-carbon metabolism, differences in assay methods of global methylation level, and difference in the distribution of genetic factors related to one-carbon metabolism, either alone or in combination. In particular, no study has investigated the interaction of genetic factors such as SNPs and nutrient intake related to one-carbon metabolism with DNA methylation level.

Here, we used the well-characterized control group of a breast cancer case–control study in Nagano, Japan, to carry out a cross-sectional study to evaluate the associations of dietary and genetic factors related to one-carbon metabolism with the global methylation level of peripheral blood leukocyte DNA among Japanese women.

Materials and Methods

Study subjects. Subjects were the control group in a multicenter, hospital-based case–control study of breast cancer carried out from May 2001 to September 2005 at four hospitals in Nagano Prefecture, Japan. Details of this study have been described previously.[17,18] The study protocol was

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approved by the institutional review board of the National Cancer Center, Tokyo, Japan.

Briefly, healthy female individuals were selected from medical check-up examinees in two of the hospitals and confirmed to not have any cancer. Each subject was recruited as a control and matched for each case by age (within 3 years) and residential area during the study period; the cases were a consecutive series of 405 women aged 20–74 years with newly diagnosed, histologically confirmed invasive breast cancer who were admitted to one of the four hospitals during the survey period. Among potential control subjects, one declined to participate and two refused to provide a blood sample. Consequently, written informed consent was obtained from 405 matched pairs.

Data collection. Participants were asked to complete a self-administered questionnaire that included questions on demographic characteristics, anthropometric factors, smoking habit, family history of cancer, physical activity, medical history, and menstrual and reproductive history. Dietary habits were investigated using a 136-item semiquantitative FFQ that was developed and validated in a Japanese population.[19,20] In the FFQ, participants were questioned on how often they consumed the individual food items (frequency of consumption), as well as relative sizes compared to standard portions. Daily food intake was calculated by multiplying the frequency of each food item in the FFQ by its standard portion and relative size. Daily intakes of nutrients were calculated using the 5th revised and enlarged edition of the Standard Tables of Food Composition in Japan.[21] The validity of nutrient intakes estimated from the FFQ was evaluated in a subsample of the Japan Public Health Center-based Prospective Study, which includes Nagano as one its study areas. Estimated intake according to the FFQ was compared to that in four 7-day dietary records, one carried out in each of the four seasons. Spearman's correlation coefficients between energy-adjusted intakes estimated from the FFQ and from dietary records were 0.35–0.50 for folate, 0.34–0.45 for vitamin B2, 0.36–0.47 for vitamin B6, and 0.27–0.35 for vitamin B12.[18,19]

Participants provided blood samples at the time they returned their self-administered questionnaire. Whole blood in a 7-mL EDTA-2Na Vacutainer (Terumo, Tokyo, Japan) and serum samples were stored at −80°C until analyzed.

Laboratory analysis. Genomic DNA was extracted from the whole blood using a Qiagen FlexiGene DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Global DNA methylation was quantified by LUMA,[22,23] consequent to the findings of our in-house testing of several methods which found LUMA to be relatively reliable and unbiased in assessing small differences in global methylation levels of peripheral blood leukocytes. Three hundred nanograms of genomic DNA was cleaved with *Hap*II + *Eco*RI or *Msp*I + *Eco*RI in two separate 20-μL reaction tubes containing 2 μL of 10× T buffer (330 mM Tris-acetate, 100 mM Mg-acetate, 660 mM K-acetate, 5 mM DTT), 2 μL of 0.1% BSA, and 5 units of each of the restriction enzymes. The reactions were set up in a PSQ96 Plate Low (Qiagen) and incubated at 37°C for 1 h. Then 20 μL annealing buffer containing 200 mM Tris-acetate and 50 mM Mg-acetate (pH 7.6) was added to the cleavage reactions, and samples were assayed using a PSQ96 MA system (Biotage, Uppsala, Sweden). The instrument was programmed to add dNTPs in six steps, consisting of: step 1, dATPαS; step 2, mixture of dGTP + dCTP; step 3, dTTP; step 4, mixture of dGTP + dCTP; step 5, water; and step 6, dATP. Peak heights were calculated using the PSQ96 software. The *Hap*II/*Eco*RI and *Msp*I/*Eco*RI ratios were calculated as (dGTP + dCTP)/dATP for each reaction. The *Hap*II/*Msp*I ratio was then calculated as (*Hap*II/*Eco*RI)/(*Msp*I/*Eco*RI), which corresponds to the proportion of unmethylated CCGG. Restriction enzymes (*Hap*II, *Msp*I, and *Eco*RI) were purchased from Takara Bio (1053A, 1150A and 1040A, respectively; Shiga, Japan). PyroMark Gold Q96 Reagents for pyrosequencing were purchased from Qiagen (972804). DNA quantification was carried out using a Quan-iT PicoGreen dsDNA Reagent and kit (P7581; Invitrogen, Carlsbad, CA, USA). Intra-assay CV was 6.4% at a mean methylation level of 74% ($n = 20$).

In the present study, we focused on three genes, *MTHFR*, *MTR*, and *MTRR*, which are closely related to DNA methylation in one-carbon metabolism, and selected SNPs in consideration of the availability of functional information. Five polymorphisms in *MTHFR* (rs1801133 and rs1801131), *MTR* (rs1805087), and *MTRR* (rs10380 and rs162049) genes were genotyped by TaqMan SNP Genotyping Assays developed by Applied Biosystems (Foster City, CA, USA). Confirmation that the genotype frequencies were in Hardy–Weinberg equilibrium was done using a χ^2-test as quality control (all P values >0.05).

Statistical analysis. Nutrient intake (folate, vitamin B2, B6, and B12 intake) was adjusted for total energy intake using the residual method[24,25] and divided into quartile categories. Adjusted mean global methylation levels of leukocyte DNA were calculated according to nutrient intake and SNPs related to one-carbon metabolism using a multivariable linear regression model. To test linear trends for mean folate intake levels, regression coefficients (β) were calculated in the multivariable linear regression model using categories of each folate intake level as ordinal variables. The following variables were used for adjustment: age (continuous); BMI (continuous); smoking (never smokers, past smokers, current smokers); alcohol drinking (non-drinkers, occasional drinkers, regular drinkers of <150 g ethanol/week, regular drinkers of ≥150 g ethanol/week); and physical activity in the past 5 years (no, ≤2 days/week, ≥3 days/week). To investigate potential effect modification, subgroup analyses were carried out by nutrient intake and SNPs related to one-carbon metabolism, and tests for interaction were carried out. All reported P-values are two-sided, and significance level was set at $P < 0.05$. All statistical analyses were done with SAS software version 9.1 (SAS Institute, Cary, NC, USA).

Results

After exclusion of subjects who reported extremely low or high total energy intake (<500 or ≥4000 kcal, respectively) or had no DNA sample, 384 healthy Japanese women were included in the present analyses. Mean age and total calorie intake of women in the present study was 54.1 years and 1947.5 kcal, respectively. Mean global methylation level (SD) was 70.2% (3.4) and range was from 59.0% to 81.2%. Table 1 shows global methylation level according to age, BMI, smoking status, and physical activity, which were used for adjustment in Tables 2 and 3. None of these factors was associated with the level of global methylation.

Global methylation levels according to five dietary factors and five SNPs of genes for folate metabolic enzymes are shown in Table 2. We found a statistically significant association between folate intake level and the global methylation level of leukocyte DNA ($P = 0.030$). Global methylation level decreased by 0.36% (95% CI, 0.03–0.69) per quartile category for folate intake. No associations were found for vitamin B2, B6, or B12 intake, alcohol drinking, or five SNPs of *MTHFR*, *MTR*, and *MTRR*.

Association between mean global methylation level of leukocyte DNA and folate intake by factors related to one-carbon metabolism are shown in Table 3. Subgroup analyses revealed that alcohol drinking modified the association between folate intake and global methylation level ($P_{interaction} = 0.01$). The

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