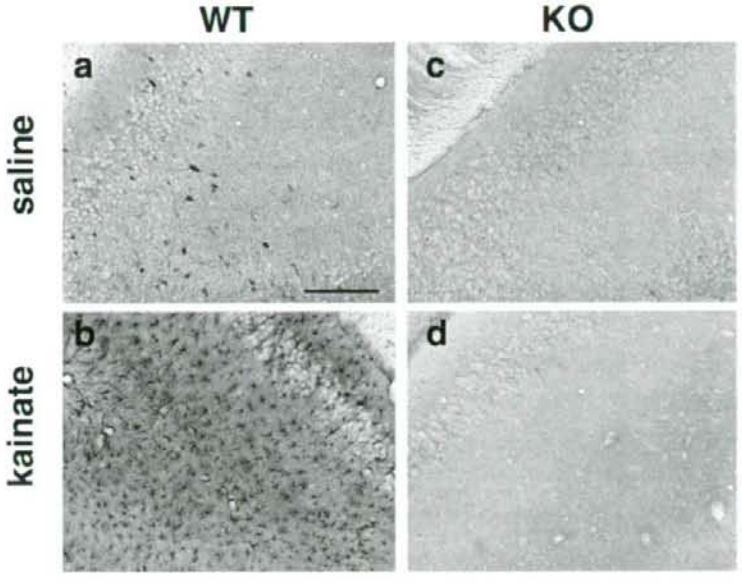

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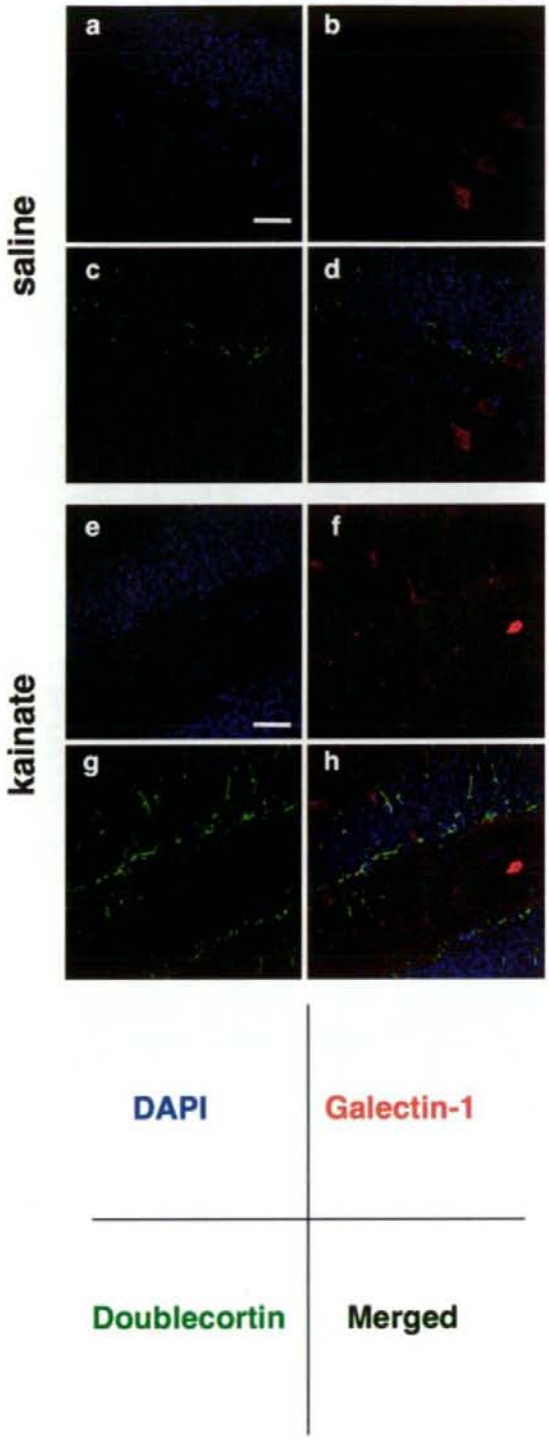

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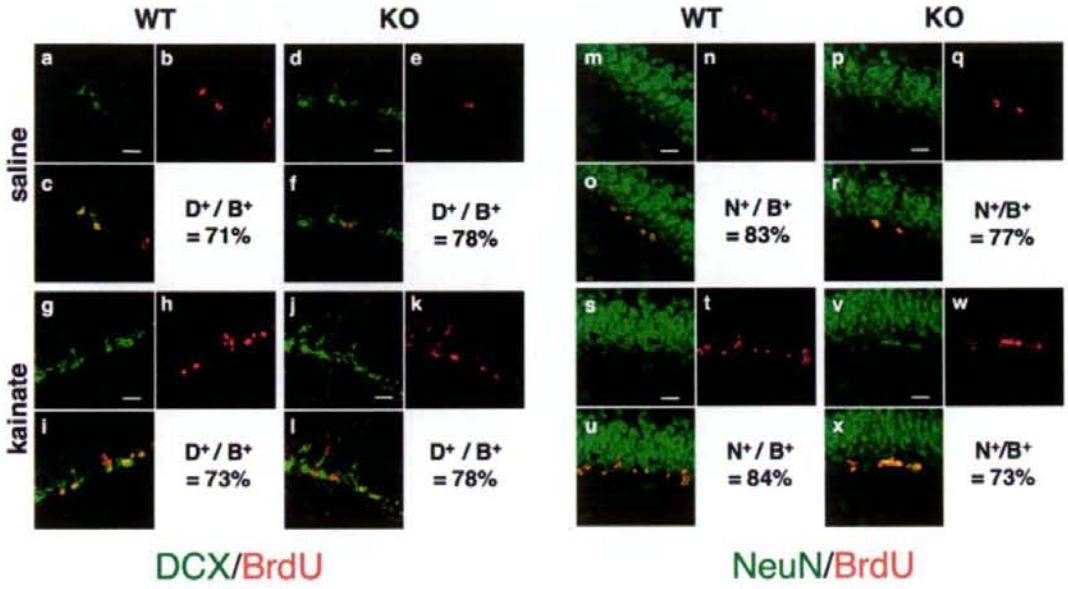

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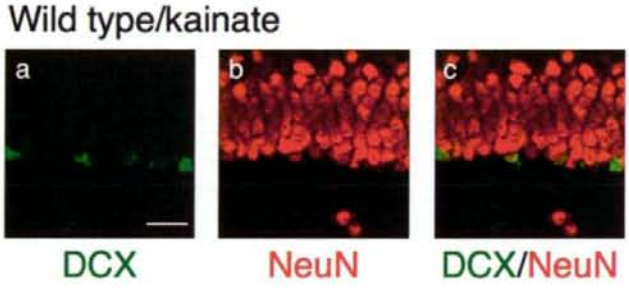

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HUMAN MUTATION 29(1), 182–189, 2008

METHODS

Multiplex PCR-Based Real-Time Invader Assay (mPCR-RETINA): A Novel SNP-Based Method for Detecting Allelic Asymmetries Within Copy Number Variation Regions

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We report the development of a real-time Invader assay combined with multiplex PCR (mPCR-RETINA), an SNP-based approach that can measure the allelic ratio in copy number variation (CNV) regions of a genome. RETINA monitors the real-time fluorescence intensity of each allele during the Invader assay and detects allelic asymmetries caused by genomic duplication/multiplication in heterozygous individuals. By combining mPCR-RETINA and real-time quantitative PCR that detects total copy number, we can estimate the copy number of each allele in CNV regions, which should be useful for investigating the functional significance of allele copy number with disease susceptibilities and drug responses. Also, mPCR-RETINA can efficiently refine the detailed structures of CNV regions. Due to the combination of RETINA with multiplex PCR, mPCR-RETINA requires a very small amount of genomic DNA for analysis (0.1–0.38 ng/locus). Additionally, mPCR-RETINA has clear advantages in its simple protocol and target-specific reaction, even in nonunique regions. We believe mPCR-RETINA will provide a significant contribution to identifying functional alleles in CNV regions. Hum Mutat 29(1), 182–189, 2008. © 2007 Wiley-Liss, Inc.

KEY WORDS: CNV; duplication; multiplication; SNP; multiplex PCR; real-time Invader assay

INTRODUCTION

Copy number variations (CNVs) are relatively common in the human genome, estimated to be approximately 12% of the all genomic regions [Redon et al., 2006]. Since copy number differences in functional genes may affect the quantity of gene products, some of them are likely to be associated with susceptibility to various diseases as well as the efficacy or adverse reaction to certain drugs [Gasche et al., 2004; Gonzalez et al., 2005; Aitman et al., 2006; Padiath et al., 2006]. For example, the multiplication of functional alleles in the *CYP2D6* gene was shown to be correlated with ultrarapid metabolization of codeine, which results in life-threatening opioid intoxication [Gasche et al., 2004]. Since qualitative (allele copy number) and quantitative (gene copy number) alterations affect critical phenotypes, it is essential to develop new methods that can measure copy number values of both genes and alleles to investigate the functional significance of CNVs [Freeman et al., 2006].

So far, more than 2,000 CNV regions have been identified by various methods, including BAC-array comparative genomic hybridization (CGH) [Redon et al., 2006; Iafrate et al., 2004; Sharp et al., 2005; Locke et al., 2006], an oligonucleotide array called ROMA [Sebat et al., 2004], fosmid paired-end sequence mapping [Tuzun et al., 2005], and the SNP mapping array [Redon et al., 2006], and are summarized in the Database of Genomic Variants (http://projects.tcag.ca/variation). However, the genomic regions that are deleted or duplicated/multiplied are not well defined due to the technical limitations in these methods: for example, BAC-array CGH detects relatively large CNV regions (>50 kb); the resolution in the ROMA method is low due to its low coverage of the genome; the sample size used for fosmid paired-end sequence is very small; the marker density in the SNP mapping array is low in some parts of the genomic regions [Sharp et al., 2006; Freeman et al., 2006]. Thus, many of the reported CNV regions must have their detailed structures defined to investigate the presence or absence of a copy number difference in the functional gene unit. Recently, high-density oligonucleotide tiling array CGH has been frequently used to precisely define CNV breakpoints [Locke et al., 2006; Urban et al., 2006]. This method

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has the advantage of high resolution, but its capability in the target-specific recognition of probes is not sufficient, especially in nonunique regions that are known as CNV hotspots [Locke et al., 2006; Sharp et al., 2006].

The Invader assay, coupled with multiplex PCR, is one of the SNP genotyping methods with the highest accuracy [Ohnishi et al., 2001; International HapMap Consortium, 2005]. The basic principle of the PCR Invader assay is shown in Fig. 1a. In our routine SNP genotyping, we simultaneously amplified 96 fragments of SNP sites by multiplex PCR. After that, the PCR products were put into 384-well plates, and the Invader assay was performed for each SNP with unique probes corresponding to each allele [Ohnishi et al., 2001]. This method was originally developed as an endpoint assay, and fluorescence intensities were only measured after a 15-minute to 60-minute incubation of the Invader reaction [Ohnishi et al., 2001; Nevilie et al., 2002]. Although this method generates clear and accurate genotyping results, it was not investigated as to whether this assay could be applied to the detection of CNVs. In this study, we modified the Invader assay to detect real-time fluorescence during the enzymatic reaction after multiplex PCR (multiplex PCR real-time Invader assay [mPCR-RETINA]). We show that mPCR-RETINA can measure the allelic ratio utilizing allelic asymmetries caused by genomic duplication/multiplication in heterozygous individuals, and determine the allele copy number in CNV regions by combining the total copy number data obtained from real-time quantitative PCR. We also show that mPCR-RETINA can be useful for refining breakpoints within duplicated/multiplied regions.

MATERIALS AND METHODS

Genomic DNA

We used 180 genomic DNA samples consisting of 30 trios of European ancestry (CEU) and 30 trios of Yoruba (YRI) that were for the International HapMap project [International HapMap Consortium, 2005]. These DNA samples were purchased from the Coriell Cell Repositories (Camden, NJ).

PCR-RETINA

We used the reported PCR primers, invader probes, and allele probes for the *CYP2D6* assays [Nevilie et al., 2002]. The remaining PCR primers were designed by Primer Express 1.5 (Applied Biosystems, Foster City, CA), and the other invader probes and allele probes were designed and synthesized under the reported criteria [Mast and de Arruda, 2006]. The sequences of all primers and probes are listed in Supplementary Table S1 (available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). Fluorescence resonance energy transfer (FRET) probes labeled with FAM or Yakima Yellow were purchased from Third Wave Technologies (Madison, WI). Rox dye (Sigma, St. Louis, MO) was used for the normalization of reporter signals. We used Takara Ex Taq HS (Takara, Shiga, Japan) for all PCR amplification according to the manufacturer's instructions with a primer concentration of 100 nM in all assays. PCR was performed on GeneAmp 9700 (Applied Biosystems) with a reaction volume of 5 μl in a single PCR. The PCR condition of the *CYP2D6* assays was initiated at 95°C for 2 minutes followed by 35 cycles at 95°C for 15 sec and 68°C for 4 minutes. The PCR condition of the *MRGPRX1* assays was initiated at 95°C for 2 minutes followed by 35 cycles at 95°C for 15 sec, 58°C for 30 sec, and 72°C for 1 minute. For the multiplex PCR, the PCR condition was initiated at 95°C for 2 minutes followed by 37 cycles at 95°C for 15 sec, 58°C for 30 sec, and 72°C for 4 minutes with a reaction volume of 10 μl [Ohnishi et al., 2001]. After PCR, the products were diluted up to 10-fold and used as templates for RETINA. We carried out RETINA for each SNP with the reaction volume of 4 μl on ABI prism 7900 (Applied Biosystems) following the protocol recommended by Third Wave Technologies. Data analysis was performed with Excel (Microsoft, Redmond, WA).

Estimating Total Copy Number by Taqman Assays

We carried out Taqman assays to estimate the total copy number of CNV regions. We first used the reported Taqman (Applied Biosystems) assay for *CYP2D6* [Bodin et al., 2005]. However, since we found a three-base insertion in some YRI individuals at the reverse primer site of the reported assay, we designed a new reverse primer by Primer Express and then performed copy number analysis again to obtain accurate data in the analysis of YRI individuals. For *MRGPRX1*, we designed all assays using Primer Express 1.5. These Taqman probes were labeled with FAM at the 5′ end and linked by nonfluorescence quencher (NFQ) and minor groove binder (MGB) at the 3′ end. As the reference gene, we used the RNase P assay (Applied Biosystems) labeled with VIC. All Taqman assays were performed following the reported protocols and copy number calculation was conducted by the delta-delta threshold cycle (Ct) method [Bodin et al., 2005]. We assumed the samples with a median delta Ct value were two-copy and used them as a calibrator. All samples were examined in duplicate and the average copy number values were used in the scatter plot analysis. The primer and probe sequences of all assays are listed in Supplementary Table S1.

Direct Sequencing

Two YRI samples showing discordant results between PCR-RETINA and the Taqman assay were amplified by PCR under the reported reaction condition [Dorado et al., 2005]. The amplified DNAs were subjected to direct DNA sequencing on the ABI Prism 3700 sequencer (Applied Biosystems) and analyzed with Polyphred software (University of Washington, Seattle, WA; http://droog.gs.washington.edu/PolyPhred.html). The primer sequences for direct sequencing are listed in Supplementary Table S1.

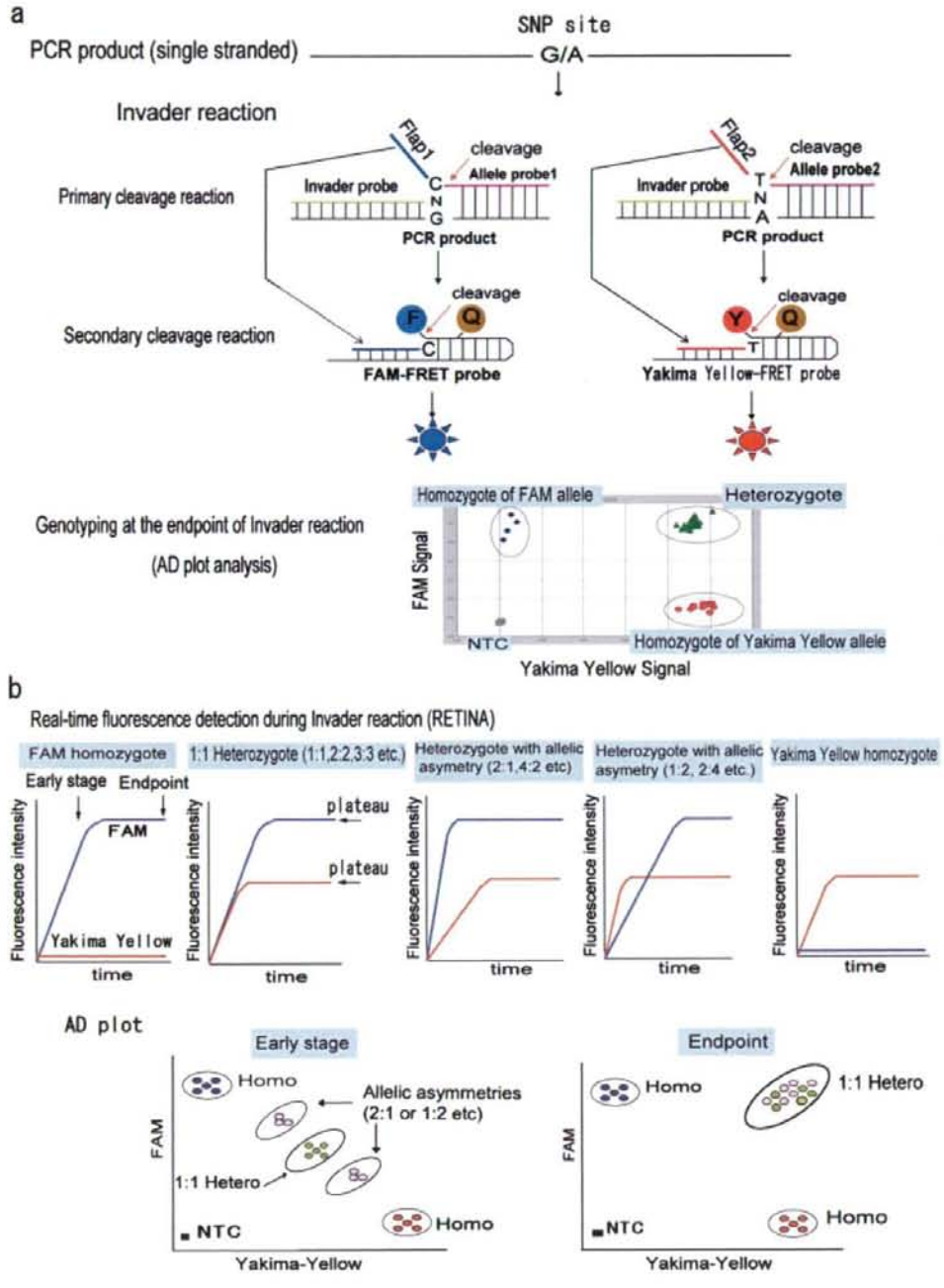
RESULTS

Detection of Allelic Asymmetries Using Artificial Templates

We first synthesized two artificial 80-base oligonucleotide templates corresponding to the genomic sequence of an rs2114912 SNP locus to investigate the detection capability of allelic asymmetries in the PCR Invader assay (the sequences are shown in Supplementary Table S1). We generated standard samples with a range of allelic ratios from 8:1 to 1:8 using these oligonucleotides. After the amplification of DNA fragments by PCR, we performed real-time fluorescence detection every 30 seconds during the 30-minute Invader reaction (RETINA). In allelic discrimination (AD) plot analysis, the samples with various allelic asymmetries in the concentration of each allele were clearly separated in proportion to the allelic ratio in the early stage of the reaction, but were merged as one heterozygote cluster in 20 minutes or more of the reaction (Fig. 2b–d). Among the AD plots at various time points during the Invader reaction, the time point just before the FAM or Yakima Yellow fluorescence signal became saturated could provide the best separation of the clusters with allelic asymmetries. The biochemical schema of PCR-RETINA is shown in Fig. 1b.

Detection of Gene Duplications/Multiplications in the Human Genome

To examine whether PCR-RETINA could be applied to allelic-asymmetry detection within CNV regions, we investigated two representative CNVs corresponding to the *CYP2D6* and *MRGPRX1* loci [Lovlie et al., 1996; Tuzun et al., 2005; Redon et al., 2006]. We selected three SNPs for *CYP2D6* from a previous report [Nevilie et al., 2002] and three SNPs for *MRGPRX1* from the dbSNP database (the SNP locations are shown in Supplementary Figs. S1a and S2a, respectively). In the *CYP2D6* assay, we found that two CEU subjects and nine YRI subjects were placed outside of the three major clusters in the AD plots in at least one of the three loci (Supplementary Fig. S1b and c). We confirmed all of these 11 samples to have three copies of *CYP2D6* by means of the reported Taqman assay (Supplementary Fig. S1d and e) [Bodin et al., 2005]. On the other hand, PCR-RETINA was unable to identify some individuals who were indicated to have three copies



or one copy by the Taqman assay, because they contained three copies of the same allele or a deletion of one allele.

PCR-RETINA analysis at the *MRGPRX1* locus revealed several clusters in the AD plots of both populations. Individuals placed outside of the major clusters were confirmed to have three or more copies by the Taqman assay (Supplementary Fig. S2b–e). In this evaluation, PCR-RETINA was also unable to identify a few individuals having four copies, because they had two copies each of both alleles and were plotted in the heterozygote cluster. These experiments indicate that PCR-RETINA could detect individuals with allelic asymmetries, but could not detect individuals with multiplied allelic symmetries, homozygous individuals with duplications/multiplications, or individuals with deletions.

In the experiment of *CYP2D6* using YRI samples, we observed discordances in the results of two samples genotyped by PCR-RETINA and the reported Taqman assay. These samples were estimated to have one copy (a deletion in one chromosome) in the reported Taqman assay, but PCR-RETINA judged these two individuals to be heterozygous (Supplementary Fig. S3a and b). Subsequently, we performed direct sequencing of this region and found a 3-base insertion (4578-4579insCAT in M33388), that was not previously reported, in the region corresponding to the reverse primer site of the reported Taqman assay in these individuals (Supplementary Fig. S3c). Hence, we performed a copy number analysis by the Taqman assay using a new reverse primer, and confirmed the PCR-RETINA result to be correct (Supplementary Fig. S3d).

Estimation of Allele Copy Number

Through these experiments, we found that PCR-RETINA could estimate the copy number of each allele by combining the information of dot positions in the AD plot graph and the total copy number measured by the Taqman assay. To confirm the accuracy of this estimation, we conducted the standard curve analysis for the rs4756975 locus using CEU individuals. Standard samples were prepared by mixing the two-copy homozygote samples (NA07034 and NA12056) in the range of allelic ratios from 8:1 to 1:8. We performed PCR-RETINA in standard samples with various allelic ratios and analyzed them at 3 minutes of Invader reaction (Fig. 3a). We plotted the log of the fluorescence intensity ratio (FAM intensity/Yellow intensity) on the X-axis, the log of the allele mixing ratio on the Y-axis, and calculated a linear regression curve (Fig. 3b). This standard curve was adjusted using the fluorescence intensity ratio of two-copy heterozygous individuals. After the measurement of the total copy number by the Taqman assay, we performed PCR-RETINA and calculated the copy number of each allele by the linear regression curve. Individuals with various allelic ratios estimated by standard curve analysis were clearly separated by relative dot position in the AD plot (Fig. 3c and d).

Applying PCR-RETINA To Refine Breakpoints of Genomic Duplication/Multiplication

We then applied PCR-RETINA to refine the breakpoints of genomic duplication/multiplication, which is essential to judge whether the functional unit or a part of a certain gene was duplicated or multiplied. From the Database of Genomic Variants, four CNV regions were reported around the *MRGPRX1* gene: Variation_0415 (8 kb) and Variation_0416 (13.4 kb) were found from the fosmid paired-end sequence, Variation_2907 (52.8 kb) from the SNP mapping array, and Variation_3838 (263.3kb) from BAC-array CGH. We designed 26 SNP-based assays covering the largest candidate region (Variation_3838) and performed PCR-RETINA for 90 CEU individuals (Supplementary Fig. S4). Our data clearly indicated that the allelic asymmetries were limited only within the region between rs2220067 and rs7110426, and no additional cluster was found at any loci outside of this region (Fig. 4 and Supplementary Fig. S5). Therefore, we considered that the boundaries of the duplicated/multiplied region were likely to be located between rs12364167 and rs2220067 on one side, and between rs7110426 and rs11024893 on the other side. We confirmed this result by four Taqman assays at the adjacent region of the boundaries. Consequently, we defined only the *MRGPRX1* gene to be present in this CNV region (Fig. 4). Since the University of California Santa Cruz (UCSC) Genome Browser indicated that the boundaries of both sides of the duplicated/multiplied region were located within long interspersed repetitive element 1 (LINE-1) repeats (Fig. 4), we presume that this CNV of *MRGPRX1* occurred within these repeats possibly as a result of nonallelic homologous recombination [Burwinkel and Kilimann, 1998]. The same analysis using YRI individuals also supported the data obtained from CEU individuals (data not shown).

Evaluation of Multiplex PCR RETINA (mPCR-RETINA)

Last, we evaluated the feasibility of the combination of multiplex PCR and RETINA (mPCR-RETINA). We performed 26-plex PCR and RETINA corresponding to the 26 loci mentioned above and compared the AD plot patterns in each locus between 26-plex PCR and PCR with a single primer set. Although we used only 10 ng of genomic DNA for the 26 SNP loci (0.38 ng/site) in the multiplex PCR, the patterns of the AD plots of RETINA were almost identical to those of the single fragments (Supplementary Fig. S5). This suggests that multiplex PCR products can be sufficiently applied as a template of RETINA.

FIGURE 1. **a: A schema of SNP typing by the PCR Invader assay. The PCR Invader assay is a simultaneous detection method for two different alleles in an SNP. Two allele probes are designed for each target SNP, complementary to each allele. Each allele probe has a different universal flap sequence at the 5′ end. One invader probe is designed at the opposite site of allele probes. In the primary reaction, Cleavase VIII cuts the allele probes with a single-base invasive structure by an invader probe at the SNP site when the allele probes are hybridized to the complementary target DNA. The red arrows indicate the cleavage sites. The cleaved allele probes with a flap and a nucleotide of the SNP site from the primary reaction hybridize to the specific FRET probes and behave as invader probes in the secondary reaction. Cleavase VIII cuts the FRET probe with an invasive structure by the flap sequence, resulting in fluorescence signal generation. The primary and secondary Invader reactions occur simultaneously. If the probes do not hybridize perfectly at the site of interest and no overlapping structure is formed, no cleavage occurs and no fluorescence signal is generated. SNP genotyping is usually performed by clustering analysis in a two-dimensional allelic discrimination (AD) plot at the endpoint of the reaction. b: The principle of detecting allelic asymmetries by RETINA. In the PCR Invader assay, fluorescence signals are usually saturated and reach the plateau phase within 5 to 10 minutes (early stage) of the Invader reaction. This plateau effect induces all samples with various allelic asymmetries to a 1:1 fluorescence intensity ratio and allelic asymmetries cannot be discriminated in the AD plot at the end point of the reaction. Samples with allelic asymmetries show different patterns from a 1:1 heterozygote in real-time fluorescence signal curves and RETINA can discriminate these samples in the AD plot at an early stage of the reaction (before saturation). The time point just before the reaction is saturated usually provides the best separation of the clusters with allelic asymmetries.**

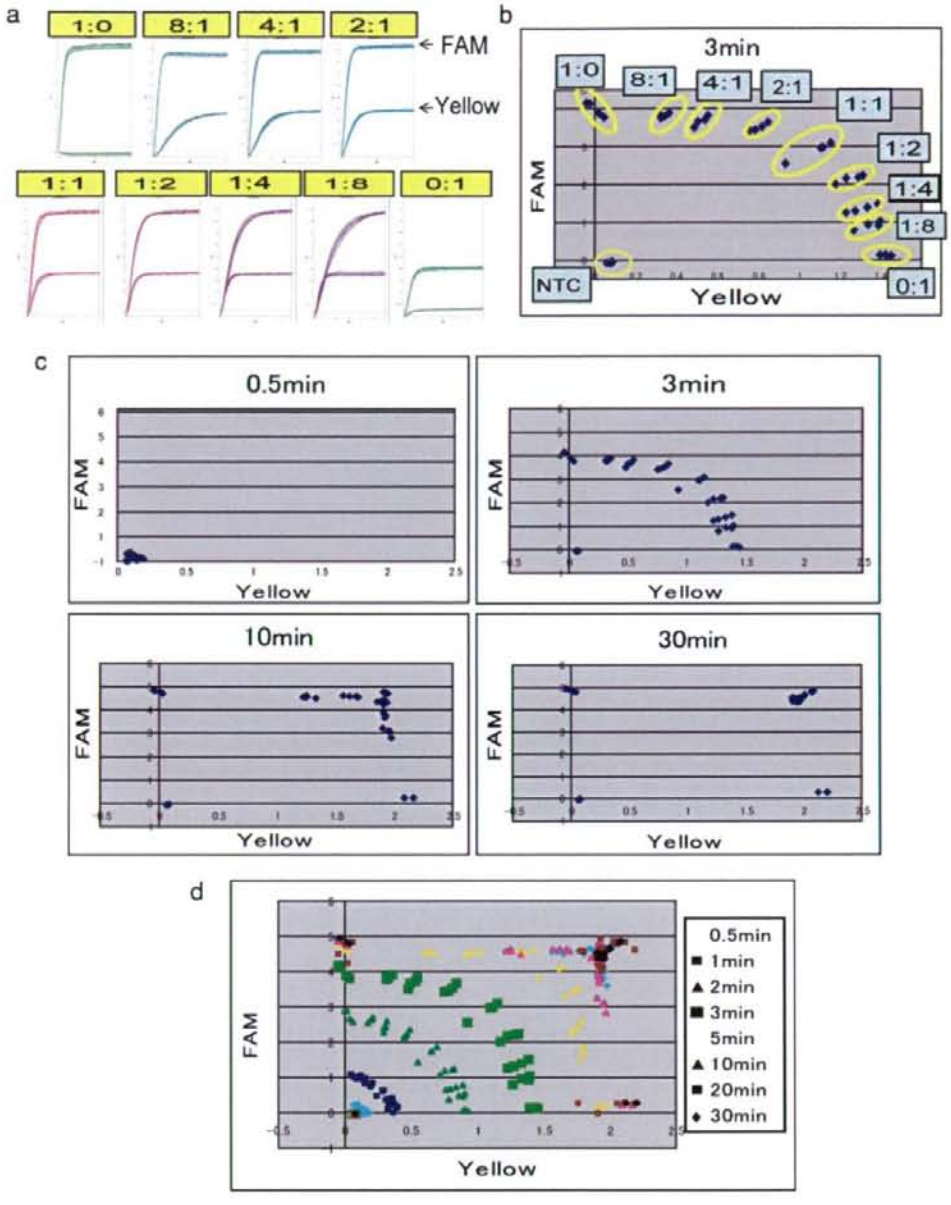


FIGURE 2. **Detection of allelic asymmetries by PCR-RETINA using artificial templates. All experiments were performed in quadruplicate. a:** Real-time detection of fluorescence signals during the Invader assay. The ratios in the boxes indicate the allelic ratio of each standard sample. Reporter fluorescence signal values (FAM and Yakima Yellow) after the normalization using passive reference (Rox) were plotted. The vertical axis is the normalized reporter signal (FAM or Yakima Yellow), and the horizontal axis is the reaction time every 30 seconds. **b:** The AD plot of PCR-RETINA at 3 minutes in the Invader reaction. The vertical axis is the normalized FAM allele signal, and the horizontal axis is the normalized Yakima Yellow allele signal. **c:** The AD plots at 0.5, 3, 10, and 30 minutes in the Invader reaction. **d:** Transition of cluster patterns showing allelic asymmetries during the 30-minute Invader reaction.

Since we have been successfully performing 96-plex PCR using 10 ng of genomic DNA for SNP genotyping, the amount of genomic DNA for mPCR-RETINA will be reduced to a minimum of 0.1 ng for one SNP locus.

DISCUSSION

It is crucial to refine the breakpoints of CNVs to judge whether or not CNVs represent the copy number differences in the

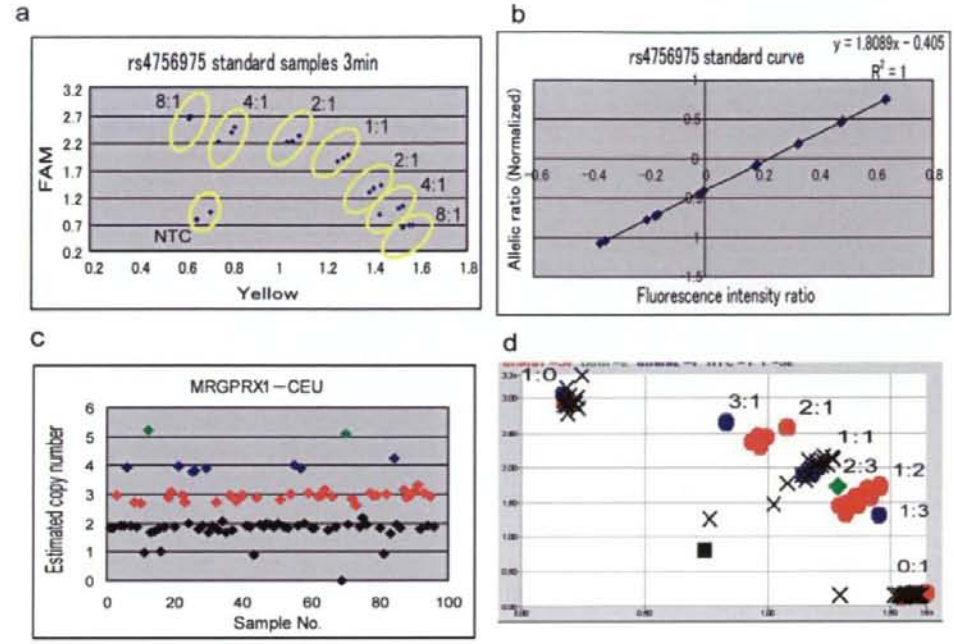


FIGURE 3. **Confirmation of the allele copy number by the standard curve method. a: AD plot of standard samples with various allelic ratios (8:1, 4:1, 2:1, 1:1, 1:2, 1:4, and 1:8) at a 3-minute time-point of the Invader reaction. b: Standard curve by standard samples with known allelic ratio. c: Total copy number obtained by Taqman assay. Closed red diamonds, blue diamonds, and green diamonds indicate three-copy, four-copy, and five-copy individuals, respectively. Closed black diamonds indicate two-copy, one-copy, and zero-copy individuals. d: The AD plot of CEU samples at the 3-minute RETINA reaction. Closed red circles, closed blue circles, and green diamonds indicate individuals with three copies, four copies, and five copies, respectively. X indicates two-copy, one-copy, or zero-copy individuals. Closed black square indicates no template control (NTC). The numbers in the AD plot indicate the estimated allele ratios calculated by the standard curve.**

functional gene unit. Also, it is essential to determine the allele copy number in the CNV regions that may be associated with various phenotypes in medical genetics and pharmacogenetics. Using mPCR-RETINA, we demonstrated here that allelic-asymmetry analysis of a particular locus could define the region of the genomic duplication/multiplication. Additionally, we showed that mPCR-RETINA could accurately determine the allelic ratio and could estimate the allele copy number in CNV regions by a combination with real-time quantitative PCR.

We first examined whether the PCR Invader assay, our standard SNP genotyping method, could be applicable to detect CNVs. Real-time fluorescence monitoring using artificial templates revealed that heterozygote samples with various allelic ratios were clearly separated in an early stage of the reaction, but were merged as one heterozygote cluster in a later phase. This disappearance of allelic asymmetries in the later phase of the Invader reaction is caused by a saturation effect of fluorescence signals originating from a depletion of FRET probes. Real-time fluorescence signal curves showed that both allele signals were saturated in the later phase of the Invader reaction (Fig. 2a). Hence, heterozygote samples with various allelic ratios eventually all come to have the same signal intensity and merge as one heterozygote cluster. Thus, real-time fluorescence monitoring (RETINA) is needed for an efficient detection of allelic asymmetries in the Invader assay.

When we applied PCR-RETINA to the human genome, we found that with PCR-RETINA it is possible to infer the allelic ratio with good precision, but impossible to infer the overall copy number within CNV regions. This characteristic of PCR-RETINA makes it difficult to detect individuals with multiplied allelic symmetries, homozygous individuals with duplications/multiplications, or individuals with deletions. The main reason why PCR-RETINA cannot detect the overall copy number is another saturation effect, namely, a plateau effect of PCR products. In general, a quantity of PCR products reflects the initial copy number of template DNA (genomic DNA) in an exponential phase of the PCR reaction, but does not reflect it in a later phase due to a plateau effect. In our present protocol, most of the PCR products are in the plateau phase and do not reflect the initial copy number of template genomic DNA. In contrast, the allelic ratio is constant through the PCR reaction because the plateau effect of PCR equally influences the amplification efficiencies of both alleles. Additionally, the difference in the input quantity of genomic DNA among the samples has no influence on the allelic ratio, though it usually affects the estimation of the total copy number. As a result, the plateau effect provides sufficient templates with accurate allelic ratios for RETINA and gives good signal intensities within a few minutes of the Invader reaction. For these reasons, PCR-RETINA enables us to estimate the allelic ratio with good precision. To estimate both the total copy number and allele copy number by mPCR-RETINA, further modification is needed.

To our knowledge, up to now melting curve analysis [Ruiz-Ponte et al., 2000; Timmann et al., 2005], the SNP-based real-time PCR-based method using the Taqman assay [Lo et al., 2003; Yu et al.,

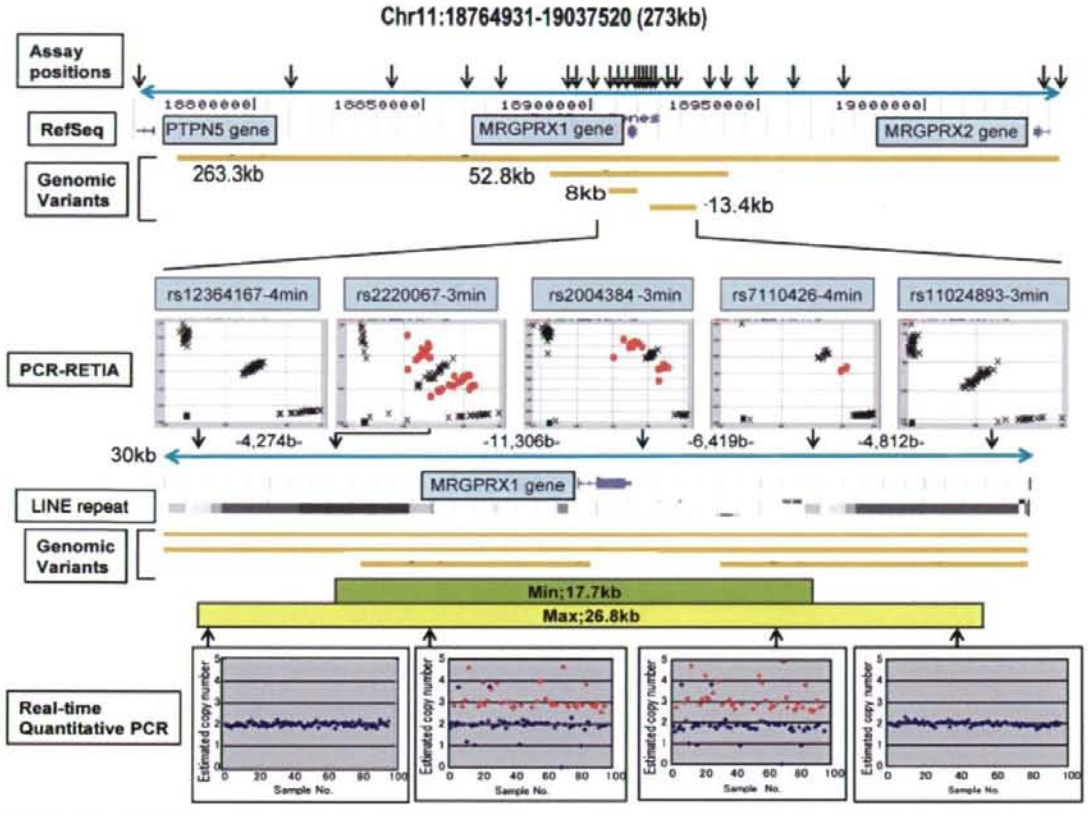


FIGURE 4. **The refinement of the breakpoints of the duplicated/multiplied region including the *MRGPRX1* gene. The region covering the largest genomic variation (Variation_3838) is shown in the upper side of the figure. The map of the *MRGPRX1* gene and its flanking regions are extracted from the UCSC Genome Browser and Database of Genomic Variants. Arrows indicate assay positions of PCR-RETINA and Taqman assays. Representative AD plots especially related to the determination of boundaries of the duplicated/multiplied region are shown in the center of the figure. Closed red circles in the AD plots indicate the individuals with allelic asymmetries. Copy number measurements by the Taqman assays are shown in the lower side of the figure. Red closed diamonds indicate the individuals with allelic asymmetries in at least one of the 26 assays. The boxes marked Min and Max indicate the minimum and maximum size of the duplicated/multiplied regions refined by PCR-RETINA.**

2006], and the single-base extension on matrix-assisted laser-desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry [Tsui et al., 2005] have been used to detect allelic asymmetries or measure the allelic ratio. However, melting curve analysis and the Taqman assay require expensive target-specific fluorescence probes and a large amount of genomic DNA, and are not suitable for multiple target sites. The single-base extension on MALDI-TOF mass spectrometry involves multiple steps and is labor-intensive. In contrast, PCR-RETINA needs only PCR and the Invader assay, and is very simple and rapid. The adoption of multiplex PCR and no need to synthesize target-specific fluorescence probes can minimize assay costs and save genomic DNA [Lyamichev et al., 1999; Ohnishi et al., 2001]. Additionally, mPCR-RETINA has an advantage in target-specific reactions even in regions of repetitive sequences. The design flexibility of amplicon size (0.1 kb–1 kb or longer) in multiplex PCR facilitates finding unique sequences for PCR primers. Also, the characteristic of the Cleavase VIII enzyme (Third Wave Technologies) that recognizes the specific triplet structure at a target SNP site provides a much higher target-specific reaction compared to the methods by hybridization alone [Lyamichev et al., 1999]. As a proof of that, PCR-RETINA successfully performed three SNP assays (rs2220067, rs11517776 and rs11024893) within Line-1 repeats (Fig. 4; Supplementary Fig. S5).

As PCR-RETINA is a post-PCR detection method, there is a possible risk of cross-contamination. To prevent or minimize this risk, we physically segregate the sample setup and the postreaction workup in the laboratory. In addition, most of the liquid handling processes are conducted by credible robotics in the cleanest environment possible and filtered tips are used in manual mixing or dispensing. We always have "no template control" wells in every plate to monitor cross-contamination and great care is taken to prevent such contamination.

In conclusion, we developed mPCR-RETINA as a new method for detecting allelic asymmetries in CNV regions. mPCR-RETINA can be used for a refinement of duplicated/multiplied regions and the measurement of the copy number of each allele in CNV regions by combining it with real-time quantitative PCR. mPCR-RETINA has clear advantages in saving genomic DNA, target-specific reactions, ease of use, and cost effectiveness. Although

many CNV regions have been reported so far, the functional significance in most of these regions has not been clarified yet. By using mPCR-RETINA and real-time quantitative PCR, we can determine the copy number of each allele in multiple SNP loci of CNV regions efficiently, and examine the relationship of CNV regions with the pathogenesis of common diseases and drug responses. We believe mPCR-RETINA will be a powerful tool in elucidating the functional significance of CNVs for various critical phenotypes.

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Impact of serum total cholesterol on the incidence of gastric cancer in a population-based prospective study: The Hisayama study

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The results of prospective studies that have examined the association between serum cholesterol levels and the incidence of gastric cancer remain controversial. To examine this issue in a general population, a total of 2,604 subjects aged 40 years or older were followed up prospectively for 14 years. During the follow-up period, gastric cancer developed in 97 subjects. The age- and sex-adjusted incidence of gastric cancer by quartiles of serum cholesterol level, namely, <4.06, 4.06–5.32, 5.33–6.04 and ≥6.05 mmol/L, were 3.9, 3.3, 3.1 and 2.1 per 1,000 person–years, respectively. The risk of gastric cancer increased with decreasing cholesterol level (age- and sex-adjusted hazard ratio [HR], 1.22; 95% confidence interval [CI], 1.01–1.49; $p = 0.04$ for a decrease of 1 mmol/L in serum cholesterol level). This inverse association remained unchanged even after adjustment for other confounding factors, namely, *Helicobacter pylori* infection, atrophic gastritis, family history of malignant neoplasm, smoking habits, body mass index, hemoglobin A1c, white blood cell count and dietary factors (adjusted HR, 1.28; 95% CI, 1.03–1.58; $p = 0.02$). This association was significant for intestinal-type gastric cancers, but not for diffuse-type. As regards cancer stage, the inverse cholesterol-cancer association was marginally significant for early gastric cancer after multivariate-adjustment (adjusted HR, 1.25; 95% CI, 0.97–1.61; $p = 0.09$), but was not for advanced gastric cancer probably due to the small number of cases. In conclusion, our findings suggest that low serum cholesterol levels are an independent risk factor for developing gastric cancer, especially intestinal-type gastric cancer.

Key words: cohort study; serum cholesterol; gastric cancer; incidence; *Helicobacter pylori*

Introduction

It is well known that a high level of serum cholesterol is one of the major risk factors for coronary heart disease.[1–3] On the other hand, some cross-sectional and prospective studies have indicated that serum cholesterol levels at the lower end of the distribution are associated with higher non-coronary mortality,[4–7] especially cancer mortality.[7–11] With respect to gastric cancer, a limited number of studies suggest this inverse association,[4,7,12] while others do not.[6,8,9] In most of these studies, the end points were cancer death,[4,6,7,9] in which there would be possible preclinical effect of cancer and effects of competing risk of cardiovascular disease. Sherwin *et al.* suggested that serum cholesterol level decreased in 2–3 years before death.[7] The strong positive association between serum cholesterol level and cardiovascular mortality is generally recognized[2]: subjects with high cholesterol levels are more likely to suffer from cardiovascular disease, and therefore tend to be censored by cardiovascular death before developing gastric cancer. These phenomena might result in an apparent inverse association between serum cholesterol levels and mortality from gastric cancer. To the best of our knowledge, only 2 prospective studies to date have examined the association between serum cholesterol level and the development of gastric cancer,[8,12] but the results of these studies were inconsistent: a Swedish cohort study[12] demonstrated a significant inverse association between serum cholesterol level and the incidence of gastric cancer, but no such association was found in a Finnish cohort study.[8] In addition, in these studies, not all of the well known risk factors for gastric cancer, such as the status of *Helicobacter* (*H.*) *pylori* infection, smoking habits and dietary factors, were taken into consideration. Thus, in the present study, we assessed the association between serum cholesterol level and the incidence of gastric cancer in a population-based cohort study, taking these risk factors into account.

Material and methods

Study population

The town of Hisayama is located in a suburban area adjacent to Fukuoka City, a large urban center on Kyushu Island in the southern part of Japan. The population of the town is ~7,500 and has been stable for the past 40 years. According to the 1985 census, the age and occupational distributions of the Hisayama population are almost identical to those of Japan as a whole.[13] The dietary patterns of the residents are also similar to those of the participants in the National Nutrition Survey, which selected its subjects from 300 areas throughout Japan.[14]

In 1988, a total of 2,742 Hisayama residents aged 40 years or older (80.9% of the total population in that age group) underwent a health check-up. After the exclusion of 132 persons with a history of gastrectomy or gastric cancer, 5 who died during the examination period, and 1 who missed serum total cholesterol measurement, a total 2,604 subjects (1,071 men and 1,533 women) were enrolled in the study.

Follow-up survey

The precise method of follow-up used in this study has been described previously.[15] Briefly, the study population was followed up for 14 years, from December 1988 through November 2002 by repeated health check-ups that were conducted every 1–2 years. For all those who did not undergo regular check-ups or who moved out of town, health status was checked every year by mail or telephone. In addition, a daily monitoring system was established by the study team and local physicians or members of the Division of Health and Welfare of the town. To identify new occurrences of gastric cancer in the cohort, at least once a week, members of our study group surveyed not only local clinics in the town but also major hospitals around the town, to which Hisayama residents were usually admitted if need be and monitored records of barium meal examinations, upper endoscopic examinations and biopsy diagnosis. We also checked all records from annual mass screenings for gastric cancer that used upper gastro-intestinal series. Furthermore, to find any concealed gastric cancer, autopsies were performed on 361 (75.4%) of the 479 subjects who died during the follow-up period.

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The diagnosis of gastric cancer was confirmed by histologic examination of specimens obtained by gastrectomy, endoscopic mucosal resection or autopsy in all cases. The tumors were categorized as either intestinal-type or diffuse-type, according to the classification described by Lauren.[16] Subjects were also classified into 2 categories by cancer stage, namely, early gastric cancers which were limited to the mucosa or submucosal layer of the stomach and advanced cancers which extended into the muscularis propria of the stomach.

During the follow-up period, only 1 subject was lost to follow-up, and gastric cancer developed in 97 subjects (68 men and 29 women), including 3 concealed cancers (3.4%) first diagnosed at autopsy. Among these cases, there were 8 subjects (8.2%) who had 2 synchronous gastric cancers (double cancers), resulting in 105 cancer lesions. Among the 8 subjects with double cancers, 3 had 2 cancers of different histologic subtypes and remaining 5 had 2 cancers of the same histologic subtype. As regards cancer stage, 3 subjects had both early and advanced gastric cancers, and remaining 5 had 2 cancers of the same cancer stage. We could not determine cancer stage in another 2 cases.

Risk factor measurement

Fasting and non-fasting blood samples were taken and transferred to the Japanese Medical Laboratory, Kyushu Branch, where serum total cholesterol levels were determined enzymatically using an analyzer (TBA-80S; Toshiba, Tokyo, Japan). The subjects were divided into 4 groups according to quartiles of serum total cholesterol level: <4.06, 4.06–5.32, 5.33–6.04 and 6.05 ≤ mmol/L. To isolate the effect of serum cholesterol levels on the occurrence of gastric cancer, several baseline factors in addition to age and sex were examined as potential confounders. Serum immunoglobulin G antibodies to *H. pylori* were assayed by quantitative enzyme immunoassay using a commercial kit (HM-CAP; Enteric Products, Westbury, NY). Assay values were interpreted as negative or positive according to the manufacturer's instructions. Serum pepsinogen test was assessed as a marker of gastric atrophy as well as a strong risk factor for gastric cancer.[17] The measurement of serum pepsinogen concentrations was carried out by immunoradiometric assay (PG I/II RIA BEAD; Dainabot, Tokyo, Japan). On the basis of the serum pepsinogen test proposed by Miki *et al.*,[18] the study subjects were divided into 2 groups, a positive pepsinogen test (serum pepsinogen I levels of ≤70 ng/mL and pepsinogen I/II ratios of ≤3.0) and a negative test (the rest). The positive test group was considered as presence of atrophic gastritis. Hemoglobin A1c (HbA1c) was measured by high pressure liquid chromatography and was used as an indicator of glucose tolerance status. White blood cell count was measured by the impedance method.[19] Body mass index (weight [kg]/height [m]^2) was used as an indicator of obesity. Information about smoking habits, alcohol intake and medical history, including a history of peptic ulcer disease and family history of malignant neoplasm, was obtained by means of a questionnaire administered to each subject. The subjects were divided into 2 groups by smoking status: smokers and non-smokers including former smokers. Alcohol intake was converted into average daily ethanol intake (g/day), and classified into 3 categories: none, 1–20 and ≥20 g/day. Those subjects engaging in sports more than 3 times per week during their leisure time were classified as belonging to the physically active group. Data on dietary factors were obtained by a semi-quantitative food frequency method.[20] A self-administered questionnaire concerning food intake over the last year, which consisted of 70 food items. Questionnaire was completed by each participant prior to the initiation of the study, and was checked by experienced dieticians and nutritionists, who presented the subjects with food models of actual size in the survey. The average food intake per day was estimated based on detailed descriptions of the frequency of eating and the quantity of each food. Nutritional intake was calculated using the 4th revision of the Standard Tables of Food Composition in Japan. The nutritional elements were adjusted for energy intake using the method described by Willett and Stampfer[21] in order to assess the independent contributions.

Statistical analysis

Mean values of the possible risk factors were adjusted for age and sex using the covariance method and were tested for trends across serum total cholesterol levels by multiple regression analysis. The frequencies of risk factors were adjusted for age and sex by the direct method and were tested for trends using the Cochran-Mantel-Haenszel χ^2 test.[22,23] The incidence of first occurrence gastric cancer was estimated with the person–year method adjusted for age and sex and compared by the Cox proportional hazards model.[24] All the study subjects were used as a standard population for age- and sex-adjustment. To estimate the risk factor adjusted hazard ratio (HR) and 95% confidence interval (CI), we first carried out a stepwise version of the Cox proportional hazards model, with $p < 0.2$ being required for entry into the model and for remaining there, using the possible confounding factors indicated in Table I. As a result, age, male sex, *H. pylori* infection, atrophic gastritis, family history of malignant neoplasm, smoking status, body mass index, HbA1c level, white blood cell count, and intake of total energy, salt, vitamin A and vitamin B_1 remained significant risk factors for gastric cancer. These selected variables were included in the Cox model as confounding factors. In the analysis according to histologic type and cancer stage, each of double cancers was stratified into its appropriate category. All tests were 2-sided, and a p value of <0.05 was considered to be statistically significant. We used the SAS statistical package for all statistical analyses (SAS, version 8; SAS Institute, Bremerton, Washington).

Ethical considerations

The study protocol was approved by the Human Ethics Review Committee of the Kyushu University Graduate School of Medical Sciences, and written informed consent was obtained from all subjects.

Results

Table I shows age- and sex-adjusted mean values or frequencies of potential risk factors for gastric cancer by quartiles of serum cholesterol level at baseline. The means of age, body mass index, HbA1c, white blood cell count and intake of total fat, vitamin B_2 and dietary fiber were significantly positively correlated with serum cholesterol levels (p for trend <0.05), while the frequencies of male sex, atrophic gastritis and smoking habits showed a significant negative correlation with serum cholesterol levels (p for trend <0.05). Those with lower serum cholesterol level tend to drink much more than those with higher serum cholesterol level. The frequencies of *H. pylori* infection, family history of malignant neoplasm, history of peptic ulcer, physical activity and the mean values for the intake of total energy, salt, vitamin A, vitamin B_1 and vitamin C were not found to be associated with serum cholesterol levels.

Figure 1 demonstrates the age- and sex-adjusted incidence rates of gastric cancer by quartiles of serum cholesterol level. The incidence rates significantly increased with descending quartiles of serum cholesterol level (p for trend = 0.04). Table II shows the age-(and sex-) adjusted and multivariate-adjusted HRs for gastric cancer by quartiles of serum cholesterol level. The significant inverse association was observed between cholesterol level and the risk of gastric cancer after age- or age- and sex-adjustment in men and both men and women together ($p = 0.04$), but was not in women. In both men and women together, this significant association remained unchanged even after adjustment for potential risk factors, namely, age, sex, *H. pylori* infection, atrophic gastritis, family history of malignant neoplasm, smoking habits, body mass index, HbA1c level, white blood cell count and intake of total energy, salt, vitamin A and vitamin B_1 (adjusted HR, 1.28; 95% CI, 1.03–1.58; $p = 0.02$, for a decrease of 1 mmol/L in serum

TABLE I – AGE- AND SEX-ADJUSTED MEAN VALUES OR FREQUENCIES OF POTENTIAL RISK FACTORS FOR GASTRIC CANCER ACCORDING TO QUARTILES OF SERUM CHOLESTEROL LEVEL IN 1988

Baseline selected variables	Serum cholesterol level (mmol/L)				
	<4.60 (n = 644)	4.60–5.32 (n = 664)	5.33–6.04 (n = 650)	6.05≤ (n = 646)	p for trend
Age, yrs	58.1 ± 0.3	59.6 ± 0.3	59.0 ± 0.3	60.1 ± 0.2	<0.01
Male sex, %	53.9	44.0	37.4	29.3	<0.01
H. pylori infection, %	73.0	72.3	72.1	69.8	0.20
Atrophic gastritis, %	39.3	31.5	34.1	28.9	<0.01
Family history of malignant neoplasm, %	9.4	9.2	10.0	9.1	0.56
History of peptic ulcer, %	14.5	13.5	14.1	16.4	0.58
Body mass index, kg/m^2	22.5 ± 0.1	22.6 ± 0.1	23.1 ± 0.1	23.5 ± 0.1	<0.01
Hemoglobin A1c, %	5.5 ± 0.03	5.5 ± 0.03	5.6 ± 0.03	5.7 ± 0.03	<0.01
White blood cell count, × 10^3/μl	5.4 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	5.7 ± 0.1	<0.01
Smoking habits, %	27.8	24.0	23.4	22.7	0.01
Alcohol intake, %					<0.01
None	65.2	69.4	70.7	72.7	
<20 g/day	11.1	9.5	11.0	10.3	
20≤ g/day	23.8	21.1	18.3	16.9	
Physical activity, %	10.4	10.4	9.8	11.4	0.99
Total energy intake, kcal/day	1701 ± 15	1711 ± 15	1716 ± 15	1693 ± 20	0.62
Salt intake, g/day	13.5 ± 0.2	12.9 ± 0.2	13.0 ± 0.2	12.7 ± 0.2	0.10
Total fat intake, g/day	47.0 ± 0.4	48.1 ± 0.4	48.5 ± 0.4	49.2 ± 0.4	<0.01
Vitamin A intake, IU/day	2875 ± 45	2892 ± 44	2919 ± 44	2873 ± 44	0.74
Vitamin B1 intake, mg/day	0.79 ± 0.02	0.80 ± 0.02	0.84 ± 0.02	0.80 ± 0.02	0.08
Vitamin B2 intake, mg/day	1.11 ± 0.01	1.14 ± 0.01	1.16 ± 0.01	1.18 ± 0.01	<0.01
Vitamin C intake, mg/day	75.3 ± 1.3	77.9 ± 1.3	77.9 ± 1.3	77.2 ± 1.3	0.64
Dietary fiber intake, g/day	10.4 ± 0.1	10.9 ± 0.1	10.9 ± 0.1	10.8 ± 0.1	0.03

Values are mean ± standard error or percentages.
Age is not adjusted for sex and male sex is not adjusted for age.

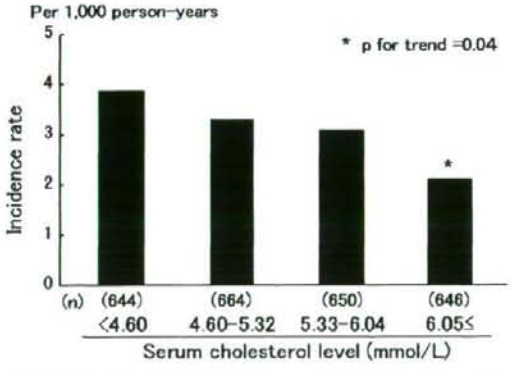

FIGURE 1 – Age- and sex-adjusted incidence rates of gastric cancer by quartiles of serum cholesterol level. The incidence rates of gastric cancer by the designated quartiles of serum cholesterol level, <4.06, 4.06–5.32, 5.33–6.04, and ≥6.05 mmol/L, were 3.9, 3.3, 3.1 and 2.1 per 1,000 person-years, respectively.

cholesterol level). These associations were substantially unchanged when analyzing the data after excluding those subjects who developed gastric cancer in the first 3 years of the follow-up period (Table III).

Table IV shows the age- and sex-adjusted and multivariate-adjusted risk of gastric cancer for a decrease of 1 mmol/L in baseline serum cholesterol level by cancer histology and stage. The significant negative association was observed between serum cholesterol level and the risk of intestinal-type of gastric cancer (age- and sex-adjusted HR, 1.33; 95% CI, 1.06–1.67; p = 0.01). This significant association remained unchanged even after adjustment for potential risk factors (multivariate-adjusted HR, 1.39; 95% CI, 1.09–1.77; p < 0.01). But no such association was seen for diffuse-type gastric cancer. As regards cancer stage, the inverse cholesterol-cancer association was marginally significant for early gastric cancer after multivariate-adjustment (adjusted HR, 1.25; 95% CI, 0.97–1.61; p = 0.09), but was not for advanced gastric cancer probably due to the small number of cases.

Discussion

In a Japanese cohort study, we found an inverse association between serum cholesterol levels and the incidence of gastric cancer, especially intestinal-type gastric cancer. This significant association remained unchanged even after adjustment for potential risk factors, specifically, age, sex, *H. pylori* infection, atrophic gastritis, family history of malignant neoplasm, smoking habits, body mass index, HbA1c level, white blood count and dietary intake of total energy, salt, vitamin A and vitamin B_1. The strengths of our study include its longitudinal population-based study design, long duration of follow-up, almost perfect follow-up of subjects, and our examination of the data taking comprehensive risk factors into consideration, including *H. pylori* infection and smoking habits.

To date, several explanations of the inverse association between serum cholesterol levels and cancer risk have been proposed. In some epidemiological studies, the association between low serum cholesterol levels and cancer risk has been attributed to the influence of existing undiagnosed cancer on cholesterol level at the time of testing.[4,7,25] In the present study, however, the significant inverse association between serum cholesterol level and gastric cancer incidence was also observed after excluding cancers which developed in the first 3 years of the follow-up period. Thus, it can be said that the effect of pre-existing disease is negligible in our subjects. With respect to competing risk, cardiovascular death might induce a potential bias in the cholesterol–cancer association and affect it. In the present study, however, the mortality from all causes and cardiovascular deaths were not higher in our subjects with high serum cholesterol levels than in those with normal to low serum cholesterol levels (data not shown), suggesting that the effect of censor cases by cardiovascular disease might be negligible.

In our subjects, there was a clear inverse association between serum cholesterol and gastric cancer for intestinal-type cancers, but not for diffuse-type cancers. It is generally accepted that

TABLE II – HRS AND 95% CIS FOR GASTRIC CANCER ACCORDING TO QUARTILES OF SERUM CHOLESTEROL LEVEL AT BASELINE, 1988–2002

Cholesterol level (mmol/L)	No. of population at risk	No. of cases	Age- (and sex-)adjusted		Multivariate-adjusted[1]	
			HR (95% CI)	*p* value	HR (95% CI)	*p* value
Men						
<4.42	270	21	2.09 (0.99–4.45)	0.05	1.78 (0.82–3.89)	0.15
4.42–5.06	263	20	1.85 (0.87–3.97)	0.11	1.83 (0.84–3.99)	0.13
5.07–5.76	275	17	1.58 (0.72–3.45)	0.25	1.36 (0.60–3.05)	0.46
5.77≤	263	10	1.00		1.00	
Risk for a decrease of 1 mmol/L	1071	68	1.28 (1.01–1.63)	0.04	1.29 (0.99–1.68)	0.06
Women						
<4.78	383	7	1.42 (0.48–4.24)	0.53	1.68 (0.55–5.18)	0.36
4.78–5.47	391	6	1.13 (0.36–3.49)	0.84	1.33 (0.39–4.52)	0.21
5.48–6.25	382	10	1.79 (0.65–4.93)	0.43	1.88 (0.64–5.53)	0.25
6.26≤	377	6	1.00		1.00	
Risk for a decrease of 1 mmol/L	1,533	29	1.12 (0.80–1.58)	0.52	1.20 (0.83–0.33)	0.33
Men and women combined						
<4.60	644	32	1.65 (0.89–3.03)	0.11	1.62 (0.86–3.06)	0.13
4.60–5.32	664	27	1.37 (0.73–2.56)	0.32	1.49 (0.78–2.85)	0.23
5.33–6.04	650	22	1.29 (0.68–2.47)	0.43	1.10 (0.56–2.17)	0.78
6.05≤	646	16	1.00		1.00	
Risk for a decrease of 1 mmol/L	2,604	97	1.22 (1.01–1.49)	0.04	1.28 (1.03–1.58)	0.02

HR, hazard ratio; CI, confidence interval. In the stratified analyses by sex, HRs were not adjusted for sex.
[1]Adjusted for age, sex, *H. pylori* infection, atrophic gastritis, family history of malignant neoplasm, smoking status, body mass index, hemoglobin A1c, white blood cell count, and intake of total energy, salt, vitamin A and vitamin B1, using Cox's proportional hazards model.

TABLE III – HRS AND 95% CIS FOR GASTRIC CANCER AFTER EXCLUDING THOSE CASES WHICH DEVELOPED IN THE FIRST 3 YEARS OF FOLLOW-UP PERIOD ACCORDING TO QUARTILES OF SERUM CHOLESTEROL LEVEL AT BASELINE, 1988–2002

Cholesterol level (mmol/L)	No. of population at risk	No. of cases	Age- (and sex-)adjusted		Multivariate-adjusted[1]	
			HR (95% CI)	*p* value	HR (95% CI)	*p* value
Men						
<4.42	263	19	1.95 (0.91–4.20)	0.11	1.69 (0.76–3.76)	0.20
4.42–5.06	275	15	1.42 (0.64–3.18)	0.32	1.51 (0.67–3.43)	0.32
5.07–5.76	263	13	1.22 (0.54–2.79)	0.43	1.08 (0.45–2.56)	0.87
5.77≤	270	10	1.00		1.00	
Risk for a decrease of 1 mmol/L	1,071	57	1.26 (1.00–1.66)	0.06	1.42 (0.93–2.27)	0.07
Women						
<4.78	383	6	2.52 (0.63–10.12)	0.19	2.99 (0.71–12.62)	0.14
4.78–5.47	391	4	1.52 (0.34–6.79)	0.59	2.32 (0.49–11.04)	0.29
5.48–6.25	382	5	1.78 (0.43–7.46)	0.43	2.04 (0.44–9.44)	0.36
6.26≤	377	3	1.00		1.00	
Risk for a decrease of 1 mmol/L	1533	18	1.42 (0.89–2.18)	0.15	1.31 (0.96–1.73)	0.11
Men and women combined						
<4.60	644	29	2.07 (1.02–4.18)	0.04	2.06 (0.99–4.27)	0.05
4.60–5.32	664	18	1.30 (0.61–2.76)	0.50	1.55 (0.72–3.34)	0.26
5.33–6.04	650	17	1.42 (0.66–3.03)	0.37	1.23 (0.56–2.70)	0.62
6.05≤	646	11	1.00		1.00	
Risk for a decrease of 1 mmol/L	2,604	75	1.31 (1.04–1.66)	0.02	1.36 (1.08–1.72)	0.01

HR, hazard ratio; CI, confidence interval. In the stratified analyses by sex, HRs were not adjusted for sex.
[1]Adjusted for age, sex, *H. pylori* infection, atrophic gastritis, family history of malignant neoplasm, smoking status, body mass index, hemoglobin A1c, white blood cell count, and intake of total energy, salt, vitamin A and vitamin B1, using Cox's proportional hazards model.

intestinal-type cancer develops through a multistep process from normal gastric mucosa to chronic active gastritis, to gastric atrophy and intestinal metaplasia, and finally to dysplasia and cancer.[26] These steps include an inflammatory process through the activation of the proinflammatory transcription factor, nuclear factor κB (NFκB).[27] On the other hand, cholesterol is an important structural lipid and is required in order to maintain cell function.[28] An experimental study reported that long-term depletion of serum cholesterol induces NFκB activation, which could promote tumorigenesis.[29] Considering the circumstances mentioned above, it is postulated that low cholesterol levels have a causal effect on one of the processes of carcinogenesis. Further studies are warranted to elucidate this postulation.

H. pylori is known to be a major risk factor for the development of gastric cancer.[15,30] Some epidemiological studies indicate that *H. pylori* infection is associated with lipid metabolism.[31,32] *H. pylori* infection might induce elevated triglyceride levels and decreased high density lipoprotein cholesterol levels. In the present study, however, no clear correlation was observed between serum cholesterol levels and the status of *H. pylori* infection at baseline examination. Moreover, in our subjects, the observed inverse association between serum cholesterol level and the risk of gastric cancer did not change even after adjustment for the status of *H. pylori* infection. These findings imply that the inverse cholesterol–cancer association is independent of the status of *H. pylori* infection.

Consistent with previous studies,[10,33] our subjects with low serum cholesterol levels showed a higher frequency of current smoking than those with high serum cholesterol levels. However, our multivariate analysis showed that the inverse association between serum total cholesterol levels and the risk of gastric cancer remained significant even after adjusting for other confounding factors, including smoking habits. Although these findings suggest that the association between low serum cholesterol and gastric cancer is not affected by smoking habits, we could not confirm the true interaction between serum cholesterol and smoking status on gastric cancer incidence because of a small sample size in which we could not conduct stratification analysis by smoking status.

TABLE IV – AGE- AND SEX-ADJUSTED AND MULTIVARIATE-ADJUSTED HRS AND 95% CIS FOR GASTRIC CANCER FOR A DECREASE OF 1 MMOL/L IN SERUM CHOLESTEROL LEVEL AT BASELINE BY CANCER HISTOLOGY AND STAGE, 1988–2002

	No. of population at risk	No. of cases	Age- and sex-adjusted		Multivariate-adjusted[1]	
			HR (95% CI)	*p* value	HR (95% CI)	*p* value
Cancer histology						
Intestinal type	2,604	76	1.33 (1.06–1.67)	0.01	1.39 (1.09–1.77)	<0.01
Diffuse type	2,604	24	0.90 (0.62–1.31)	0.58	0.97 (0.66–1.44)	0.89
Cancer stage						
Early	2,604	66	1.18 (0.93–1.50)	0.18	1.25 (0.97–1.61)	0.09
Advanced	2,604	32	1.24 (0.88–1.74)	0.22	1.27 (0.88–1.83)	0.20

HR, hazard ratio; CI, confidence interval.
[1]Adjusted for age, sex, *H. pylori* infection, atrophic gastritis, family history of malignant neoplasm, smoking status, body mass index, hemoglobin A1c, white blood cell count, and intake of total energy, salt, vitamin A and vitamin B1, using Cox's proportional hazards model.

It is presumed that dietary factors, especially intake of antioxidant vitamins, modify the association between serum cholesterol level and cancer.[34,35] Those with low serum cholesterol would tend to ingest lower dietary antioxidant vitamins, such as vitamins A, C and E,[33] which are thought to have anti-cancer effects.[36,37] Moreover, cholesterol plays an important role in the absorption of lipid-soluble vitamins and controls the flow of these vitamins in and out of cell membranes.[38] In the present study, there were significant correlations between serum cholesterol levels and intake of total fat, vitamin B_2 and dietary fiber at baseline examination. However, the effect of low serum cholesterol level on the development of gastric cancer did not differ after adjustment for dietary factors. This fact suggests that dietary factors do not affect the association between low cholesterol level and the gastric cancer risk. Information on dietary factors in the present study was obtained using a semi-quantitative food frequency method, which is thought to be susceptible to measurement error. Thus, further studies are needed to verify the effects of dietary factors on the association between serum cholesterol and gastric cancer.

Several limitations of the present study must be mentioned. First, serum total cholesterol level was determined by a single measurement at baseline. Because of intra-individual variation in serum cholesterol during the long-term follow-up period, a single measurement could lead to misclassification of a person's usual cholesterol level. However, such a misclassification would tend to dilute the observed association and the long-term predictive value of cholesterol measurement would be attenuated.[11,39] Thus, the association that we have observed is likely to be conservative.

Second, we did not take anti-cholesterol treatment into account in our study. In Japan, cholesterol-lowering drugs were not commonplace at the time of the baseline examination, and thus very few subjects seemed to be under cholesterol-lowering treatment at baseline. Nevertheless, it is reasonable to assume that some subjects would have started cholesterol-lowering treatment during the follow-up period. Recent clinical trials have demonstrated that no difference is found in cancer mortality or incidence between subjects under cholesterol-lowering treatment and those with no such treatment.[40–42] Thus, the association between serum cholesterol and gastric cancer incidence demonstrated in the present study would be expected to remain significant after adjustment for anti-cholesterol treatment.

In conclusion, in the present population-based prospective study, low serum cholesterol levels were found to be a significant risk factor for developing gastric cancer, especially intestinal-type cancer. This association was independent of *H. pylori* infection status, smoking habits and dietary factors. Our data suggest that patients with low serum cholesterol should consider regular gastro-intestinal examination for the prevention of gastric cancer.

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Intake of Dairy Products and Periodontal Disease: The Hisayama Study

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Background: A previous study showed the relationship between the intake of dairy products and periodontitis, but the types of dairy products that confer a benefit on periodontal health status have not been determined.

Methods: We analyzed 942 subjects aged 40 to 79 years as part of a comprehensive health examination of Hisayama Town, Fukuoka, Japan. Probing depth (PD) and clinical attachment loss (CAL) were used as the periodontal parameters. The dietary survey was conducted using a semiquantitative food frequency method. We examined the relationship between the intake of dairy products, such as milk, cheese, and lactic acid foods (yogurt and lactic acid drinks), and periodontal condition.

Results: The daily intake of lactic acid foods in subjects with generalized deep PD or severe CAL was significantly lower than that in subjects with localized deep PD or slight CAL. In multivariate linear regression analysis, the increased intake of lactic acid foods was associated significantly with lower mean PD ($P=0.002$) and lower mean CAL ($P=0.003$). In multivariate logistic regression analysis, the subjects eating ≥55 g lactic acid foods per day had a significantly lower prevalence of deep PD and severe CAL compared to those not eating these foods after adjusting for confounding variables; the odds ratios for generalized deep PD and severe CAL were 0.40 (95% confidence interval [CI]: 0.23 to 0.70) and 0.50 (95% CI: 0.29 to 0.87), respectively.

Conclusion: The routine intake of lactic acid foods may have a beneficial effect on periodontal disease. *J Periodontol 2008;79:131-137.*

KEY WORDS

Cheese; dairy products; epidemiology; milk; periodontal disease; yogurt.

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Periodontal disease is a chronic malady that is prevalent in the adult population. Anaerobes in the oral cavity have a causal influence on periodontal disease,[1,2] and many host and environmental factors also play important roles in its progression.[3-5] Eating habits and nutrient intake are believed to be important factors in various chronic diseases, and some studies[6,7] reported that a low dietary intake of calcium and vitamin C is associated with severe attachment loss. One recent study[8] reported that subjects with high intakes of dairy products had a lower prevalence of periodontal disease than those with low intakes. Although the nutrients and components differ in each type of dairy product, such as milk, cheese, and lactic acid foods and drinks, the study[8] did not distinguish between them. Therefore, it is still not known whether the intake of dairy products, in general, or the intake of specific dairy products has a positive effect on periodontal disease.

We conducted a nutrition survey in an annual health examination of a community-dwelling adult population. In this study, we analyzed the relationship between periodontal condition and the detailed intake of dairy products to identify the type of dairy product that may have a beneficial effect on periodontal disease.

MATERIALS AND METHODS

Study Population

The town of Hisayama is a suburb of Fukuoka, a metropolitan area in southern

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Japan. Its population is ~7,500 and has remained stable for >40 years. Between July and September 1998, 982 Hisayama residents aged 40 to 79 years participated in a periodontal examination and a nutrition survey as part of a comprehensive health examination.[5] We excluded 40 subjects who had <10 teeth or lacked data for the variables studied. Consequently, 942 subjects (369 males and 573 females) were analyzed. The ethics committee of the Faculty of Dental Science, Kyushu University, and the Department of General Affairs and Health and Welfare of Hisayama approved the study design, data collection methods, and procedure for obtaining informed consent.

Oral Examination

Following the method of the Third National Health and Nutrition Examination Survey,[9] a periodontal examination was performed on one maxillary and one mandibular quadrant, selected randomly. The periodontal examination was carried out by one of four dentists trained to perform a clinical examination of oral health status. The examiner reliability of the periodontal examination was verified by an interexaminer calibration of outpatients visiting Kyushu University Dental Hospital; the κ value for the periodontal examination exceeded 0.8, suggesting very good interexaminer agreement. Probing depth (PD) and clinical attachment loss (CAL) were measured as the periodontal parameters at mesio-buccal and mid-buccal sites for all of the teeth in the two quadrants.

Dietary Survey and Other Exposure Information

The dietary survey was conducted using a semiquantitative food frequency method.[10] Before starting the survey, each participant completed a self-administered questionnaire concerning food intake over the last year, which consisted of 70 food items; the answers were checked by experienced dieticians and nutritionists who showed the subjects actual-size food models in the survey. The average dairy food intake was estimated based on detailed descriptions of the frequency of eating and the quantity of each food. We examined the intake of each dairy product and classified them into four items: milk (ordinary liquid, high-fat, and low-fat milk), cheese, lactic acid foods (yogurt and lactic acid drinks), and other dairy products (skim milk and coffee whitener). In advance, each subject completed a self-administered questionnaire that included items on lifestyle habits and medication use, which was checked by trained nurses. Participants answered items concerning the frequency of their alcohol intake over the previous year and the types and amounts of alcoholic beverages consumed habitually. The alcohol intake per drink was converted into the weight of 100% ethanol in grams.[5] Smoking habit for all of the subjects, including never, past, and current smoking, was estimated from the number of packs smoked per day multiplied by the number of years smoked (pack-years). Trained nurses measured body weight and height. The body mass index (BMI) was defined as body weight in kilograms divided by the square of the height in meters. Blood pressure was measured three consecutive times, after resting ≥5 minutes, using a standard mercury sphygmomanometer with the subjects in the sitting position, and the average value was used for the analysis. Blood samples were collected from an antecubital vein after an overnight fast. The laboratory analyses of the blood samples followed described methods.[11]

Statistical Analysis

We used the mean value of PD and CAL as a continuously variable periodontal parameter. In addition, we used the proportions of teeth with PD ≥4 mm and CAL ≥5 mm as a categorical periodontal variable and divided PD and CAL into two categories each: localized deep PD (0 to 19.99%; N = 754, 80.0%) or generalized deep PD (≥20%; N = 188, 20.0%) and localized severe CAL (0 to 9.9%; N = 757, 80.4%) or generalized severe CAL (≥10%; N = 185, 19.6%). To analyze the intake of each kind of dairy product as a categorical variable, the intake of milk was divided into quartiles (0 to 27.9, 28.0 to 89.9, 90.0 to 199.9, and ≥200.0 g/day). Because the intake of cheese, lactic acid foods, and other dairy products was 0 g/day in more than a quarter of the subjects, the subjects eating were divided into tertiles: cheese (0, 0.1 to 3.4, 3.5 to 6.9, and ≥7.0 g/day); lactic acid foods (0, 0.1 to 27.9, 28.0 to 54.9, and ≥55.0 g/day); and other dairy products (0, 0.1 to 1.9, 2.0 to 4.9, and ≥5.0 g/day). The differences in the mean values were evaluated using the Student t test. Differences in proportions were evaluated using the Pearson χ^2 test. We calculated partial correlations with adjustment for age and gender between periodontal parameters and each study variable. We used multivariate linear regression models to examine the relationship between periodontal parameters and each independent variable. The variables for which the partial correlation coefficient was significant were entered into the multivariate models. We performed multivariate logistic regression analysis to determine the effect of the intake of dairy products on PD and CAL, calculating the odds ratio (OR) and 95% confidence interval (CI). The variables that showed statistical significance in the bivariate analysis with the periodontal parameters were entered into the model. The statistical analyses were performed using a software program.¶

¶ SPSS version 15.0, SPSS Japan, Tokyo, Japan.