TABLE 2 Concordance between the OSNA assay and the histology examination in study 1

OSNA	Histopathologically negative lymph nodes
Positive	0
Negative	121
Total	121

Concordance rate for negative samples: 1.0 (95% CI: 0.976–1.0)

TABLE 3 Correlation between OSNA and the 2-mm-interval histopathological examination in study 2

OSNA	Pathology		Total
	Positive	Negative	
Positive	79	7	86
Negative	4	295	299
Total	83	302	385

Concordance rate: 0.971 (95% CI: 0.950–0.984)

Sensitivity: 0.952 (95% CI: 0.881–0.987)

Specificity: 0.977 (95% CI: 0.953–0.991)

Kappa value: 0.916 (95% CI: 0.868–0.965)

for the 95% CI (0.976–1.0). This result was above the lower limit of 0.89 for the expected negative concordance rate of 0.99 (Table 2).

Study 2

Diagnostic concordance was obtained between the OSNA assay and the 2-mm-interval histopathological examination with H&E staining in 79 positive LNs and 295 negative LNs, of 385 LNs analyzed (Table 3). Hence, the concordance rate was 0.971 with a lower limit of 0.950 (95% CI: 0.950–0.984), which was not below the 0.85 lower limit of the 95% CI, as the target concordance rate was 0.95. Therefore, the judgment produced by the OSNA assay was equivalent to that produced by the 2-mm-interval histopathological examination. A sensitivity of 0.952 (95% CI: 0.881–0.987) and a specificity of 0.977 (95% CI: 0.953–0.991) for the OSNA assay were also calculated from study 2 (Table 3).

CK19 Protein Levels in Primary Tumors

IHC with the CK19 antibody indicated that all 85 primary CRC tumors tested for study 2 exclusively expressed the CK19 protein. The majority of the tumors (80 of 85: 94.1%) was stained over ≥10% of the entire area, while 5 tumors (5.9%) were stained over less than 10% (Table 4). Of these 5 "slight expression" tumors, 2 cases were found to be LN metastasis-positive by the histopathological examination method. When the 6 metastasis-positive LNs were dissected from the 2 patients and subjected to the OSNA assay, the average CK19 mRNA copy number was 20,000 copies/μl, with a range of 3,800–50,000 copies/μl.

TABLE 4 CK19 protein expression status in primary tumors (study 2)

	Number of patients (%)		
	Expression[a]	Slight expression[a]	No expression
Adenocarcinoma			
Well	31	0	0
Moderately	42	3	0
Poor	5	1	0
Mucinous	1	1	0
Adenosquamous carcinoma	1	0	0
Total	80 (94.1%)	5[b] (5.9%)	0 (0%)

[a] Immunohistochemical staining was performed with the anti-CK19 antibody. *Expression* the area stained is ≥10%, *Slight Expression* the area stained is <10%, *No expression* no stained area

[b] CK19 mRNA in 6 positive lymph nodes from 2 of 5 patients who had slight CK19 protein expression in the primary tumor. Average: 20,000 copies/μl ($N = 6$), range: 3800 copies/μl–50,000 copies/μl

Further Analysis of Discordant LN Samples

Discordance was observed in the analysis of 11 LNs in study 2. There were 4 LNs judged negative by the OSNA assay and positive by the 2-mm-interval histopathological examination, while 7 LNs were judged positive by the OSNA assay and negative by the histopathological examination (Table 3). Additional analyses were performed to explore possible sources for this discordance.

All 4 LNs judged negative by the OSNA assay and positive by histopathological examination displayed CK19 protein expression in the additional prepared sections, with the largest diameter ranging from 0.7 to 4.0 mm (Table 5A). In the 7 LNs judged positive by the OSNA assay and negative by histopathological examination, the copy number of CK19 mRNA ranged from 270 to 10,000 copies/μl (Table 5B). Additional examination of the pathological blocks by IHC and H&E staining did not provide a new metastatic focus for LNs No. 6–11; however, a CK19-positive metastasis (0.5 mm) was detected in LN No. 5 (Table 5B).

Moreover, in study 2, discordant LN samples between 2-mm-interval pathological examination and common pathological examination using 1 section with the largest cutting surface, were confirmed by observation of each section in pathological blocks. Among 83 positive LNs

TABLE 5 Further analysis of discordant cases

No.	pStage	Histology (additional)		OSNA copies/μl
		CK19 expression (largest metastasis)	Section number metastasis positive/tested	
(A) OSNA negative, histopathological examination positive				
1	IIIB	Yes (4 mm)	9/9	ND
2	IIIB	Yes (2 mm)	2/14	ND
3	IIIA	Yes (1 mm)	2/6	ND
4	IIIA	Yes (0.7 mm)	5/7	83
(B) OSNA positive, histopathological examination negative				
5	IIIC	Yes (0.5 mm)	1/4	630
6	IIIC	No	0/7	690
7	IIIB	No	0/6	7,700
8	IIIC	No	0/16	10,000
9	IIA	No	0/20	270
10	IIA	No	0/18	400
11	IIIC	No	0/11	8,800

No.	pStage	2-mm-interval histopathological examination		Common pathological examination[a] (1 section)
		Largest metastasis	Section number metastasis positive/tested	
(C) Negative LNs with common pathological examination among 83 positive LNs[b]				
i	IIIB	2.0 mm	2/3	Negative
ii	IIIC	1.5 mm	2/3	Negative
iii	IIIB	2 mm	1/3	Negative
iv	IIIA	0.35 mm	1/3	Negative
v	IIIC	4.0 mm	1/3	Negative
vi	IIIC	6.0 mm	2/3	Negative

ND not determined

[a] Common pathological examination: 1 section with the largest cutting surface was used

[b] Positive LN was examined at 2-mm-interval pathological examination (study 2)

judged by 2-mm-interval pathological examination, 6 LNs (7.2%) were judged negative by 1 central section with the largest cutting surface (Table 5C). On the other hand, all 302 negative LNs judged by 2-mm-interval pathological examination were concordantly negative by 1-level pathology. The ability in detection of LN metastases by 2-mm-interval pathology test was significantly superior to that by 1-level pathology test (McNemar's test: $P = .041$).

DISCUSSION

Previous studies reported diagnosis of LN metastasis of CRC by the OSNA method.[15,16] These works are fundamental research studies that examined LN metastasis by both the OSNA method and the pathological examination. On the other hand, the present multicenter (8 hospitals) study is the first translational one to acquire OSNA-based molecular testing as a clinical practice, in which a total of 506 LNs were tested. For this purpose we strictly conducted a 2-phase study with the distinct aims. Thus, study 1 was designed for the purpose of denial of false-positive reaction by OSNA for metastasis-negative LNs. Study 2 was intended to validate OSNA performance in clinical judgment including metastasis-positive LNs.

False-positive results are a major concern when transferring new molecular biology-based testing methods to clinical practice.[17] Study 1 confirmed that the OSNA assay did not yield false-positive results for histopathologically negative LNs dissected from patients with benign colorectal disease and CRC (pStage 0/I). The negative concordance rate in study 1 was 1.0 (95%CI: 0.976–1.0), and CK19 mRNA did not amplify in 115 of 121 histologically negative LNs (95%) (Fig. 2). Therefore, the OSNA assay is unlikely to yield false-positives for histopathologically negative LNs, when the cutoff copy number is set at 250 copies/μl (see the section Materials and Methods for this value).

Study 2 confirmed that the OSNA assay provides a judgment performance equivalent to that of 2-mm-interval histopathological examinations. The OSNA assay exhibited a concordance rate of 0.971 (95% CI: 0.950–0.984) against the 2-mm-interval histopathological examination, with a κ value of 0.916 (95% CI: 0.868–0.965), confirming the high equivalence between the 2 methods (Table 3). However, as long as half of the LNs were provided for OSNA assay and the other half were used for histological analysis, discordance cannot be avoided because of allocation bias, that is, the localization of metastasis in LN pieces. In Table 5B, 6 of 7 samples (No. 6–11) again provided negative results by additional histological examination even with OSNA-positive results. Of the 6 discordant samples, 4 LNs (No. 6, 7, 8, 11) were collected from stage III node-positive cases. It is therefore likely that small cancer clusters might be present in LN pieces provided for OSNA. Indeed, RT-PCR assay revealed that CK19 mRNA was expressed in LN (No. 6, 7, 8) using LN pieces provided for OSNA (data not shown).

Because of the localization of metastases in the LN, it is entirely likely that the current method of histopathological examination, which examines only the section with the largest cutting surface, suffers from more overlooked metastases than 2-mm-interval histopathological examination . In fact, histopathological examination using the section with the largest cutting surface missed metastases in 6 of 83 positive LNs (7.2%) (study 2, Table 5C), and McNemar's test indicated statistical superiority in the 2-mm-interval pathology ($P = .041$). Despite the fact that the LNs were divided into halves to separate the analyses, the OSNA assay exhibited a high sensitivity of 95.2% (95% CI: 88.1%–98.7%) compared with the 2-mm-interval histopathological examination method (Table 3). Therefore, the OSNA assay may reasonably be expected to maximize the identification of localized metastases, resulting in more accurate metastasis detection in LNs, if a larger portion of the LN (or the entire LN) is analyzed in this manner.

The CK19 marker was also used to analyze primary CRC tumors, similar to a previous analysis of breast cancer samples. CK19 protein expression was observed in all 85 primary CRC tumors, regardless of their histological types (Table 4). Although 5 of 85 cases (5.9%) showed relatively low CK19 protein expression, the OSNA assay successfully detected CK19 mRNA (average: 20,000 copies/μl) in all 6 positive LNs from 2 of 5 cases (Table 4). Thus, the OSNA assay with CK19 mRNA as a metastasis marker was capable of detecting CK19 mRNA accurately, even in LNs from patients showing low CK19 protein expression in primary tumors.

Currently, various studies are attempting to stratify the group at high risk for recurrence among stage II colon cancer cases, and these studies have identified several specific risk factors.[18,19] It has also been reported that the potential occult metastases overlooked by the current histopathological examination (H&E staining) method are valuable for assessing recurrence among stage II patients.[6,7] In study 2, the OSNA assay identified metastasis-positive LNs (No. 9, 10; Table 5B) that were excised from 2 of 16 patients who were diagnosed as pStage II. In the cases of these 2 patients, if the OSNA assay had been used as a standard clinical practice, the metastases overlooked by histopathological examination may have been detected by the OSNA assay, suggesting the possibility of upstaging from stage II to stage III (2 of 16: 12.5%). Thus, the OSNA assay may enable more accurate LN staging and might contribute to reducing the recurrence rates of stage II colon cancer. It is also reported that RT-PCR assay is a useful tool to detect micrometastasis in LNs that is associated with poor prognosis in stage II CRC.[9] Considering OSNA starts from the lysate without RNA purification, OSNA should be more convenient than RT-PCR in clinical practice. In this regard, further large-scale clinical study is essential in stage II CRC.

In conclusion, the OSNA assay can facilitate the highly accurate diagnosis of LN metastases in CRC through the specific detection of CK19 mRNA. This method has great potential as a new, rapid, automated molecular-biology-based examination method, increasing the accuracy of LN metastases examination in CRC patients.

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Original Contribution

Association Between Plasma 25-Hydroxyvitamin D and Colorectal Adenoma According to Dietary Calcium Intake and Vitamin D Receptor Polymorphism

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The anticarcinogenic potential of vitamin D might be mediated by not only calcium metabolism but also other mechanisms initiated by vitamin D receptor (VDR). The authors measured plasma 25-hydroxyvitamin D in healthy volunteer examinees who underwent total colonoscopy in Tokyo, Japan, 2004–2005, and evaluated its influence on colorectal adenoma, both alone and in interaction with *VDR* polymorphisms, which correspond to the *Fok*I and *Taq*I restriction sites. The main analysis of plasma 25-hydroxyvitamin D included 737 cases and 703 controls. Compared with the lowest quintile of plasma 25-hydroxyvitamin D, only the highest was related to a significantly decreased odds ratio of colorectal adenoma (odds ratio = 0.64, 95% confidence interval: 0.45, 0.92). In contrast, all but the lowest quintile of dietary calcium intake presented similarly reduced odds ratios (odds ratio for the highest = 0.67, 95% confidence interval: 0.47, 0.95). Of note, the association between plasma 25-hydroxyvitamin D levels and colorectal adenoma was modified by the *Taq*I polymorphism of the *VDR* gene ($P_{interaction} = 0.03$) but not by dietary calcium intake ($P_{interaction} = 0.93$). These observations highlight the importance of vitamin D in colorectal tumorigenesis. Vitamin D might protect against colorectal neoplasia, mainly through mechanisms other than the indirect mechanism via calcium metabolism.

adenoma; calcium; case-control studies; intestine, large; Japan; polymorphism, single nucleotide; vitamin D

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism; VDR, vitamin D receptor.

Accumulating evidence has indicated that adequate levels of vitamin D may confer protection against the risk of colorectal cancer and adenoma, a well-established precursor lesion of colorectal cancer (1, 2). Recent meta-analyses of vitamin D intake and colorectal neoplasia have generally shown a weak inverse association (2, 3), while those of serum/plasma 25-hydroxyvitamin D, the predominant form of vitamin D in the circulation, have fairly consistently demonstrated a significant inverse association (3–5). This discrepancy in the magnitude of the association may reflect the fact that vitamin D in the body is derived from not only the diet but also the skin, where a substantial amount of pre-vitamin D can be synthesized from 7-dehydrocholesterol through stimulation by solar ultraviolet B radiation (6).

The primary role of vitamin D is the maintenance of calcium homeostasis, the disruption of which is also related to colorectal carcinogenesis (2, 7). Vitamin D exerts its effects on calcium metabolism through binding to vitamin D receptor (VDR), a member of the nuclear receptor superfamily, which regulates the transcription of genes involved in calcium absorption from the small intestine. The VDR gene (*VDR*) has a number of single nucleotide polymorphisms (SNPs), including rs2228570 (previously rs10735810) and rs731236. These 2 polymorphisms, which correspond to the *Fok*I and *Taq*I restriction sites, respectively, have been intensively explored over the last decade for their possible association with colorectal tumorigenesis (8, 9). The *Fok*I polymorphism exists at exon 2 of the *VDR* gene, and the *Taq*I polymorphism exists at exon 9. Given their distinctly separate locations, it is likely that the *Fok*I and *Taq*I polymorphisms are differently related to the development of colorectal neoplasia, if indeed they are related (9).

Although several epidemiologic studies have investigated the association between circulating vitamin D levels and colorectal neoplasia in conjunction with total/dietary calcium intake (10–15), few have done so in consideration of *VDR* polymorphisms (13, 14), despite the fact that the anticarcinogenic potential of vitamin D might be mediated by not only calcium metabolism but also other mechanisms initiated by VDR. Here, we measured plasma concentrations of 25-hydroxyvitamin D in 1,520 middle-aged and elderly Japanese and evaluated its influence on colorectal adenoma, both alone and in interaction with dietary calcium intake and the *Fok*I and *Taq*I polymorphisms of the *VDR* gene.

MATERIALS AND METHODS

Study population

The Colorectal Adenoma Study in Tokyo (16, 17), a case-control study conducted by the Research Center for Cancer Prevention and Screening, a branch of the National Cancer Center of Japan, was specifically designed to investigate environmental and genetic factors related to the early stage of colorectal carcinogenesis among healthy volunteer examinees of a colorectal cancer screening program. The Research Center conducts its cancer screening programs on a research basis and accordingly requires all examinees to provide written informed consent prior to admission to the use of data and materials collected through the screening programs to be used for medical research. This means that virtually no examinee refuses to participate in medical research initiated by the Research Center. Examinees who attend the Research Center are primarily self-referred, and more than 90% reside in Tokyo and its 6 neighboring prefectures, collectively called the Kanto region. The study protocol was approved by the institutional review board of the National Cancer Center.

Eligible subjects were defined in advance as men aged 50–79 years and women aged 40–79 years who underwent total colonoscopy from the anus to the cecum and who were without a history of colorectal adenoma, any malignant neoplasia, ulcerative colitis, Crohn's disease, familial adenomatous polyposis, carcinoid tumor, or colectomy. Of a consecutive series of 3,212 examinees undergoing magnifying colonoscopy with indigo carmine dye spraying between February 2004 and February 2005, 2,234 met these conditions. On the basis of the pit pattern of colorectal lesions, namely, the characteristics of mucosal crypts, 526 men and 256 women were determined to have at least 1 adenoma and were thus included as adenoma cases. Pit-pattern classification based on magnifying chromoendoscopy has been detailed elsewhere (18). Of the remaining 1,452 examinees, we identified 482 men and 721 women as potential controls who were also free from other benign lesions (e.g., hyperplastic polyps, inflammatory polyps, and diverticula). Because there were fewer potential controls than cases in men, all potential male controls were included in the study, whereas female controls were randomly selected from potential controls and frequency matched to the female cases in 5 age categories (40–49, 50–54, 55–59, 60–64, and ≥65 years of age) and 2 screening periods (first and second halves). The screening period was matched because standard operating procedures were improved during the first half period after the establishment of the Research Center, which might have influenced, for example, the accuracy of diagnosis. Finally, the study enrolled 526 cases and 482 controls in men and 256 cases and 256 controls in women. A total of 242 male and 104 female cases had adenomas of ≥5 mm in diameter and were referred to clinical hospitals for definitive diagnosis and treatment. Of 1,362 adenomatous lesions referred to the National Cancer Center in 2004–2008, 1,221 (90%), 53 (4%), and 88 (6%) were histologically confirmed as adenoma, early cancer, and nonneoplastic lesions, respectively (unpublished data).

Blood collection and laboratory procedures

Blood is collected from all examinees of the Research Center for research purposes almost without exception. Examinees were scheduled for blood collection prior to any cancer screening procedures on the first day of screening. Fasting venous blood was drawn into a vacutainer tube with ethylenediaminetetraacetic acid (EDTA). The vacutainer tubes were centrifuged to obtain the plasma and buffy coat layer, and the blood samples were preserved at −80°C until analysis. Plasma and buffy coat samples were available for all subjects of this study.

Plasma 25-hydroxyvitamin D concentrations were measured by a radioimmunoassay method by using a commercially available reagent (Kyowa Medex, Tokyo, Japan) with a minimum detection level of 6 ng/mL at an external laboratory (SRL, Tokyo, Japan). The laboratory reported intra- and interassay coefficients of variation of 5.96% and 5.31% for plasma 25-hydroxyvitamin D concentrations of 25.0 and 20.1 ng/mL, respectively. All laboratory personnel were blinded with respect to case and control status.

Genomic DNA was extracted from white blood cells in the buffy coat layer by using a FlexiGene DNA kit (Qiagen, Hilden, Germany) in our laboratory. More than 90% of buffy coat samples provided a sufficient amount of genomic DNA to perform genotyping. The *Fok*I and *Taq*I polymorphisms of the *VDR* gene were analyzed by using the TaqMan SNP genotyping assays (Applied Biosystems, Foster City, California). These analyses were carried out with blinding to case and control status.

Self-administered questionnaire

Prior to cancer screening, all examinees were encouraged to complete a self-administered questionnaire concerning lifestyle and socioeconomic characteristics, as well as personal and family medical history. Details of the questionnaire have been described elsewhere (16, 17). Although some examinees left individual items blank, no examinee refused to answer any substantial portion of the questionnaire.

The questionnaire also included a food frequency questionnaire of 145 food and beverage items with standard portions/units and 9 frequency categories. The amount of each food consumed per day in the past year was first calculated from the responses, and then total energy and nutrient intakes, including calcium, were estimated by reference to the *Standard Tables of Food Composition in Japan*, Fifth Revised Edition (19). The food frequency questionnaire of the present study was

essentially the same as that used in a large prospective cohort study among a Japanese population (20, 21). A validation study conducted among subsamples of cancer screening examinees revealed that the dietary calcium intake estimated from this food frequency questionnaire was relatively well correlated with that from 4-day dietary records, with deattenuated Spearman's correlation coefficients for energy-adjusted calcium intake of 0.64 and 0.61 for men and women, respectively (unpublished data).

Statistical analysis

Odds ratios and 95% confidence intervals of colorectal adenoma for plasma 25-hydroxyvitamin D, dietary calcium intake, and the *Fok*I and *Taq*I polymorphisms of the *VDR* gene were estimated by using an unconditional logistic regression model. Dietary calcium intake was energy adjusted for each sex by using a linear regression model with natural logarithm-transformed intakes of total energy and calcium as independent and dependent variables, respectively (22). Plasma 25-hydroxyvitamin D concentrations and dietary calcium intake were divided into sex-specific quintiles by cutoff points derived from the distribution among controls. Statistical adjustment was made in a manner similar to that in our previous studies of colorectal adenoma (16, 17). Model 1 controlled for sex, matching variables (i.e., age categories and screening periods), and season of blood collection (spring, summer, fall, and winter). Model 2 adjusted for the same variables as model 1 and additionally for cigarette smoking (never, ≤20, 21–40, and >40 pack-years), alcohol drinking (never, past, <150, 150–299, and ≥300 g/week), body mass index (<21.0, 21.0–22.9, 23.0–24.9, and ≥25.0 kg/m^2), family history of colorectal cancer (yes or no), and nonsteroidal anti-inflammatory drug use (yes or no). Model 2 also adjusted for attained adult height, an indicator of gross energy intake in childhood and adolescence, and average daily energy intake in the past year. These variables were divided into quintiles, the cutoff points of which were based on the sex-specific distribution among controls. Linear trends in the odds ratios of colorectal adenoma were examined by assigning ordinal values to quintiles of plasma 25-hydroxyvitamin D and dietary calcium intake.

We then investigated the influence of plasma 25-hydroxyvitamin D on colorectal adenoma in interaction with dietary calcium intake and the *Fok*I and *Taq*I polymorphisms of the *VDR* gene. Three genotypes of each *VDR* polymorphism were dichotomized on the basis of the dominant model, with the first homozygous for the major allele and the second heterozygous and homozygous for the minor allele combined. Similarly, quintiles of plasma 25-hydroxyvitamin D and dietary calcium intake were reduced to 2 levels, namely, lower and higher, on the basis of their association with colorectal adenoma. The likelihood ratio test with 1 df was used to evaluate whether dietary calcium intake and the *VDR* polymorphisms modified the association between plasma 25-hydroxyvitamin D and colorectal adenoma.

Of 1,443 subjects without extreme energy intakes (<800 or >4,200 kcal/day) or calcium supplement use, 3 subjects had missing information, 1 with regard to height and 2 for cigarette smoking. These were then excluded, and the analyses of plasma 25-hydroxyvitamin D and dietary calcium intake were conducted in the remaining 737 cases and 703 controls. Of note, we excluded calcium supplement users, who accounted for <4% of study subjects, and focused our analysis on dietary calcium intake. In the analyses of the *Fok*I and *Taq*I polymorphisms of the *VDR* gene, 7 and 8 subjects with an undetermined genotype were excluded, respectively, from 1,332 subjects with a sufficient amount of genomic DNA to perform genotyping, leaving 1,325 (684 cases, 641 controls) and 1,324 (684 cases, 640 controls), respectively, for inclusion. Two-sided P values less than 0.05 were regarded as statistically significant. All statistical analyses were carried out using SAS, version 9.1, software (SAS Institute, Inc., Cary, North Carolina).

RESULTS

Table 1 shows selected characteristics of controls according to plasma 25-hydroxyvitamin D level. Increasing levels of plasma 25-hydroxyvitamin D were associated with older age and a higher intake of dietary vitamin D, while other selected characteristics were not related to plasma 25-hydroxyvitamin D levels.

Plasma 25-hydroxyvitamin D levels were inversely associated with the prevalence of colorectal adenoma (Table 2), albeit in a nonlinear manner. Compared with the lowest quintile of plasma 25-hydroxyvitamin D, only the highest showed a statistically significant decrease in the adjusted odds ratio of colorectal adenoma (odds ratio (OR) = 0.64, 95% confidence interval (CI): 0.45, 0.92). A similar pattern was noted when the analysis was replicated in men and women separately ($P_{\text{interaction}} = 0.30$) (Web Table 1, the first of 3 Web tables posted on the *Journal*'s Web site (http://aje.oupjournals.org/)). Given the well-known seasonal variation in circulating levels of 25-hydroxyvitamin D, we also conducted a stratified analysis by season of blood collection, which revealed that the association between plasma 25-hydroxyvitamin D levels and colorectal adenoma was not modified by season of blood collection ($P_{\text{interaction}} = 0.55$) (Web Table 2). A nonlinear inverse association was also observed for dietary calcium intake, although this differed from that for plasma 25-hydroxyvitamin D: Using the first quintile of dietary calcium intake as reference, we found that the second showed a significant decrease in the adjusted odds ratio of colorectal adenoma (OR = 0.64, 95% CI: 0.45, 0.90), while the third to fifth showed no further decline. Again, we saw no apparent difference in the association by sex ($P_{\text{interaction}} = 0.70$) (Web Table 1). When mutually adjusted for plasma 25-hydroxyvitamin D and dietary calcium intake, the odds ratio for the highest quintile of plasma 25-hydroxyvitamin D was 0.66 (95% CI: 0.46, 0.95), whereas those for the second and fifth quintiles of dietary calcium intake were 0.65 (95% CI: 0.46, 0.92) and 0.69 (95% CI: 0.48, 0.99), respectively. The *Fok*I and *Taq*I polymorphisms of the *VDR* gene were not associated with the prevalence of colorectal adenoma (Table 2). Genotype frequencies among controls were in agreement with Hardy-Weinberg equilibrium for both *VDR* polymorphisms ($P = 0.79$ and 0.82 for *Fok*I and *Taq*I, respectively).

Table 1. Selected Characteristics of Controls According to Plasma 25-Hydroxyvitamin D Level, the Colorectal Adenoma Study in Tokyo, Japan, 2004–2005[a]

Characteristic	Plasma 25-Hydroxyvitamin D Level[b]									$P_{difference}$[c]
	Quintile 1 (Lowest)			Quintile 3 (Middle)			Quintile 5 (Highest)			
	No.	%	Median (IQR)	No.	%	Median (IQR)	No.	%	Median (IQR)	
Continuous variables										
Plasma 25-hydroxyvitamin D, ng/mL			16 (14–19)			24 (24–26)			32 (31–34)	<0.001
Age, years			57 (54–63)			60 (56–65)			61 (57–65)	0.005
Height, cm			165 (158–169)			163 (156–169)			162 (155–168)	0.60
Energy intake, kcal/day			1,855 (1,540–2,212)			1,829 (1,594–2,182)			1,894 (1,599–2,227)	0.96
Dietary vitamin D intake, μg/day			6.0 (4.3–7.7)			6.6 (4.7–8.4)			7.2 (4.9–10.0)	0.02
Dietary calcium intake, mg/day			542 (383–685)			565 (422–784)			590 (459–781)	0.15
Categorical variables										
Men	86	66.6		95	65.5		100	63.6		0.73
Ever smoker	64	49.6		74	51.0		70	44.5		0.79
Ever drinker	93	72.0		111	76.5		121	77.0		0.89
Overweight or obesity	33	25.5		32	22.0		30	19.1		0.71
NSAID user	12	9.3		13	8.9		8	5.1		0.53
Family history of colorectal cancer[d]	19	14.7		17	11.7		20	12.7		0.91

Abbreviations: IQR, interquartile range; NSAID, nonsteroidal antiinflammatory drug.

[a] Presenting characteristics of controls in quintiles 1, 3, and 5.

[b] Respective median (range) of each plasma 25-hydroxyvitamin D quintile by sex—for men, quintile 1: 18 ng/mL (1–20); quintile 3: 25 ng/mL (24–26); quintile 5: 33 ng/mL (≥31); for women, quintile 1: 15 ng/mL (1–17); quintile 3: 23 ng/mL (22–24); quintile 5: 30 ng/mL (≥28).

[c] Based on the Wilcoxon rank-sum test for median difference and the Fisher exact test for percentage difference.

[d] History of colorectal cancer in parents and siblings.

Among 737 cases, 325 had a largest adenoma of ≥5 mm in diameter (44.1%). Excluding 12 cases with missing information, 388 had the largest adenoma at the proximal colon (53.5%), 259 at the distal colon (35.7%), and 78 at the rectum (10.8%). We then investigated the association of plasma 25-hydroxyvitamin D and dietary calcium intake with the size and location of the largest adenoma using a multinomial logistic regression model (Table 3). The inverse association of plasma 25-hydroxyvitamin D and dietary calcium intake was even more striking in cases with a largest adenoma of ≥5 mm in diameter. By location of the largest adenoma, the inverse association of plasma 25-hydroxyvitamin D was most pronounced in cases of proximal colon adenoma, whereas that of dietary calcium intake was most prominent in rectal adenoma cases.

We further evaluated the association of plasma 25-hydroxyvitamin D and dietary calcium intake with colorectal adenoma stratified by major risk factors of colorectal adenoma, namely, smoking and drinking habits and body fatness. Although no interaction of dietary calcium intake with body fatness was seen, such an interaction was suggested for plasma 25-hydroxyvitamin D ($P_{interaction} = 0.05$), in which the odds ratio of colorectal adenoma for the highest compared with lowest quintile was statistically significant in subjects with a body mass index of <23 kg/m^2 but not in those of ≥23 kg/m^2 (Web Table 3). With respect to smoking and drinking habits, we did not see any effect modification for either plasma 25-hydroxyvitamin D or dietary calcium intake (data not shown).

Table 4 shows the association of plasma 25-hydroxyvitamin D with colorectal adenoma according to dietary calcium intake and *VDR* polymorphism. Although we saw no multiplicative interaction, higher levels of plasma 25-hydroxyvitamin D and dietary calcium intake combined were related to the greatest decrease in odds ratio of colorectal adenoma (OR = 0.49, 95% CI: 0.33, 0.72). With regard to the *VDR* polymorphisms examined, we observed a significant interaction with the *Taq*I polymorphism ($P_{interaction} = 0.03$), for which an inverse association of plasma 25-hydroxyvitamin D was more evident in heterozygotes and homozygotes for the minor allele combined ($P_{trend} = 0.001$) than in homozygotes for the major allele ($P_{trend} = 0.25$). When examined in heterozygotes or homozygotes for the minor allele of *Taq*I, the adjusted odds ratio of colorectal adenoma for higher compared with lower levels of plasma 25-hydroxyvitamin D was 0.32 (95% CI: 0.16, 0.65).

DISCUSSION

In this study, we found a nonlinear inverse association of plasma 25-hydroxyvitamin D and dietary calcium intake with colorectal adenoma. Moreover, we noted a significant

Table 2. Association of Plasma 25-Hydroxyvitamin D, Dietary Calcium Intake, and Vitamin D Receptor Polymorphisms With Colorectal Adenoma, the Colorectal Adenoma Study in Tokyo, Japan, 2004–2005

Variable	No. of Subjects		Model 1[a]		Model 2[b]	
	Cases	Controls	OR	95% CI	OR	95% CI
Plasma 25-hydroxyvitamin D[c]						
Quintile 1 (lowest)	145	129	1	Referent	1	Referent
Quintile 2	132	128	0.89	0.63, 1.26	0.86	0.60, 1.24
Quintile 3 (middle)	157	145	0.90	0.64, 1.26	0.91	0.64, 1.29
Quintile 4	175	144	1.01	0.72, 1.41	1.03	0.73, 1.46
Quintile 5 (highest)	128	157	0.66	0.47, 0.94	0.64	0.45, 0.92
P_{trend}				0.08		0.09
Dietary calcium intake[d]						
Quintile 1 (lowest)	201	140	1	Referent	1	Referent
Quintile 2	124	140	0.58	0.42, 0.81	0.64	0.45, 0.90
Quintile 3 (middle)	141	141	0.64	0.46, 0.88	0.78	0.55, 1.10
Quintile 4	142	140	0.63	0.45, 0.87	0.80	0.56, 1.13
Quintile 5 (highest)	129	142	0.55	0.39, 0.77	0.67	0.47, 0.95
P_{trend}				0.002		0.13
FokI genotype[e,f]						
FF	274	260	1	Referent	1	Referent
Ff	324	294	1.06	0.83, 1.34	1.01	0.79, 1.29
ff	86	87	0.93	0.66, 1.32	0.91	0.63, 1.31
Ff/ff	410	381	1.03	0.82, 1.29	0.99	0.78, 1.25
TaqI genotype[e,f]						
TT	523	492	1	Referent	1	Referent
Tt	156	139	1.06	0.82, 1.39	1.06	0.81, 1.40
tt	5	9	0.56	0.18, 1.70	0.47	0.15, 1.51
Tt/tt	161	148	1.03	0.80, 1.34	1.03	0.79, 1.34

Abbreviations: CI, confidence interval; OR, odds ratio.

[a] Model 1 was adjusted for sex, age, screening period, and season of blood collection.

[b] Model 2 was adjusted for the same variables as model 1 and additionally for cigarette smoking, alcohol drinking, body mass index, family history of colorectal cancer, nonsteroidal antiinflammatory drug use, daily energy intake, and height.

[c] Respective median (range) of each plasma 25-hydroxyvitamin D quintile by sex—for men, quintile 1: 18 ng/mL (1–20); quintile 3: 25 ng/mL (24–26); quintile 5: 33 ng/mL (≥31); for women, quintile 1: 15 ng/mL (1–17); quintile 3: 23 ng/mL (22–24); quintile 5: 30 ng/mL (≥28).

[d] Respective median (range) of each dietary calcium intake quintile by sex—for men, quintile 1: 288 mg/day (1–366); quintile 3: 514 mg/day (463–567); quintile 5: 867 mg/day (≥717); for women, quintile 1: 419 mg/day (1–498); quintile 3: 676 mg/day (613–742); quintile 5: 1,069 mg/day (≥881).

[e] The number of subjects providing sufficient genomic DNA to perform genotyping was 1,332.

[f] For *FokI* and *TaqI*, 7 and 8 subjects with undetermined genotype were excluded, respectively.

interaction between plasma 25-hydroxyvitamin D and the *TaqI* polymorphism of the *VDR* gene. These findings underline the importance of vitamin D in colorectal carcinogenesis, at least in its early stage.

Circulating levels of 25-hydroxyvitamin D have been evaluated in at least 7 prospective studies of colorectal cancer and 6 observational studies of colorectal adenoma (best summarized by Gandini et al. (23)). However, only 2 of these were conducted in an Asian or, more specifically, Japanese population (6, 24). Although neither reported a straightforward overall association, the investigation of colorectal adenoma showed a nonlinear inverse association, similar to ours, but only in subjects who provided blood during the winter season (24). With respect to total/dietary calcium intake, we are aware of at least 4 observational studies of colorectal cancer in Asian populations (21, 25–27) but no study of colorectal adenoma in a similar population. Even when the lower consumption levels in Asian than Western populations were considered, all studies consistently reported an inverse association (21, 25–27).

A recent comprehensive review that estimated optimal concentrations of 25-hydroxyvitamin D for multiple health

Table 3. Association of Plasma 25-Hydroxyvitamin D and Dietary Calcium Intake With the Size and Location of the Largest Adenoma, the Colorectal Adenoma Study in Tokyo, Japan, 2004–2005

Variable	Size of Largest Adenoma						Location of Largest Adenoma[a]								
	≥5 mm in Diameter			<5 mm in Diameter			Proximal Colon			Distal Colon			Rectum		
	No. of Cases	OR[b]	95% CI	No. of Cases	OR[b]	95% CI	No. of Cases	OR[b]	95% CI	No. of Cases	OR[b]	95% CI	No. of Cases	OR[b]	95% CI
Plasma 25-hydroxyvitamin D[c]															
Quintile 1 (lowest)	70	1	Referent	75	1	Referent	75	1	Referent	53	1	Referent	17	1	Referent
Quintile 2	56	0.75	0.48, 1.17	76	0.97	0.64, 1.47	80	1.00	0.66, 1.51	40	0.74	0.45, 1.21	9	0.49	0.20, 1.16
Quintile 3 (middle)	67	0.81	0.52, 1.25	90	1.03	0.69, 1.55	74	0.82	0.54, 1.24	65	1.10	0.70, 1.74	18	0.87	0.41, 1.82
Quintile 4	79	0.94	0.61, 1.43	96	1.12	0.74, 1.67	93	1.03	0.68, 1.55	58	0.96	0.60, 1.54	18	0.88	0.42, 1.85
Quintile 5 (highest)	53	0.54	0.34, 0.86	75	0.74	0.49, 1.13	66	0.63	0.41, 0.96	43	0.62	0.38, 1.02	16	0.68	0.31, 1.46
P_{trend}			0.06			0.35			0.07			0.21			0.72
Dietary calcium intake[d]															
Quintile 1 (lowest)	101	1	Referent	100	1	Referent	96	1	Referent	75	1	Referent	29	1	Referent
Quintile 2	53	0.55	0.36, 0.84	71	0.76	0.51, 1.14	60	0.63	0.41, 0.95	48	0.72	0.46, 1.13	16	0.59	0.29, 1.17
Quintile 3 (middle)	67	0.74	0.49, 1.13	74	0.84	0.56, 1.26	73	0.78	0.51, 1.17	52	0.87	0.55, 1.37	12	0.47	0.22, 1.00
Quintile 4	54	0.60	0.39, 0.94	88	1.04	0.70, 1.56	83	0.90	0.60, 1.36	44	0.76	0.47, 1.22	13	0.55	0.26, 1.16
Quintile 5 (highest)	50	0.50	0.32, 0.79	79	0.88	0.58, 1.33	76	0.74	0.49, 1.13	40	0.66	0.41, 1.09	8	0.29	0.12, 0.70
P_{trend}			0.009			0.91			0.57			0.17			0.007

Abbreviations: CI, confidence interval; OR, odds ratio.

[a] Twelve cases had missing information on the location of the largest adenoma.

[b] Adjusted for sex, age, screening period, season of blood collection, cigarette smoking, alcohol drinking, body mass index, family history of colorectal cancer, nonsteroidal antiinflammatory drug use, daily energy intake, and height.

[c] Respective median (range) of each plasma 25-hydroxyvitamin D quintile by sex—for men, quintile 1: 18 ng/mL (1–20); quintile 3: 25 ng/mL (24–26); quintile 5: 33 ng/mL (≥31); for women, quintile 1: 15 ng/mL (1–17); quintile 3: 23 ng/mL (22–24); quintile 5: 30 ng/mL (≥28).

[d] Respective median (range) of each dietary calcium intake quintile by sex—for men, quintile 1: 288 mg/day (1–366); quintile 3: 514 mg/day (463–567); quintile 5: 867 mg/day (≥717); for women, quintile 1: 419 mg/day (1–498); quintile 3: 676 mg/day (613–742); quintile 5: 1,069 mg/day (≥881).

Table 4. Association of Plasma 25-Hydroxyvitamin D With Colorectal Adenoma According to Dietary Calcium Intake and Vitamin D Receptor Polymorphism, the Colorectal Adenoma Study in Tokyo, Japan, 2004–2005

Variable	Plasma 25-Hydroxyvitamin D								$P_{interaction}$
	Quintiles 1–4 (Lower)				Quintile 5 (Higher)				
	No. of Cases	No. of Controls	OR[a]	95% CI	No. of Cases	No. of Controls	OR[a]	95% CI	
Dietary calcium intake									0.93
Quintile 1 (lower)	169	113	1	Referent	32	27	0.69	0.38, 1.26	
Quintiles 2–5 (higher)	440	433	0.73	0.54, 0.98	96	130	0.49	0.33, 0.72	
FokI genotype[b,c]									0.27
FF	228	212	1	Referent	46	48	0.85	0.53, 1.36	
Ff/ff	338	291	1.06	0.82, 1.38	72	90	0.65	0.44, 0.96	
TaqI genotype[b,c]									0.03
TT	423	388	1	Referent	100	104	0.80	0.57, 1.11	
Tt/tt	143	113	1.17	0.87, 1.57	18	35	0.43	0.23, 0.79	

Abbreviations: CI, confidence interval; OR, odds ratio.

[a] Adjusted for sex, age, screening period, season of blood collection, cigarette smoking, alcohol drinking, body mass index, family history of colorectal cancer, nonsteroidal antiinflammatory drug use, daily energy intake, and height.

[b] The number of subjects providing sufficient genomic DNA to perform genotyping was 1,332.

[c] For *FokI* and *TaqI*, 7 and 8 subjects with undetermined genotype were excluded, respectively.

outcomes, including colorectal cancer, concluded that the most advantageous concentrations of 25-hydroxyvitamin D began at around 30 ng/mL for all endpoints assessed (28), with which our observations essentially agree. With regard to dietary calcium intake, a pooled analysis of 10 cohort studies reported a threshold effect of dietary calcium intake in which all quintiles above the lowest showed a similar decrease in the risk of colorectal cancer (7), which strongly supports our present results.

We saw no multiplicative interaction between plasma 25-hydroxyvitamin D and dietary calcium intake. Previous observational studies of primary colorectal cancer and adenoma have also failed to identify such interaction (10–15). Although these findings do not rule out the existence of biologic interaction, they may suggest that vitamin D exerts an anticarcinogenic effect on the large intestine itself, and that its influence on calcium homeostasis plays only a minor role in colorectal tumorigenesis.

Although not nonsynonymous, the *TaqI* polymorphism of the *VDR* gene appears to be in linkage disequilibrium with a series of polymorphisms in the 3′ end of the *VDR* gene (29), for example, the polyadenylated microsatellite in the 3′ untranslated region, the length of which likely determines messenger RNA stability and hence likely affects intracellular levels of VDR (30). To date, the 2 studies of colorectal neoplasia that have examined the *TaqI* polymorphism in conjunction with vitamin D, as measured by dietary intake (31) or circulating levels ((14); the results were shown in the text only), indicated the absence of any obvious interaction.

We investigated effect modification by the *VDR* gene using 2 traditional SNPs, although the gene spans approximately 100 kilobases and has numerous genetic polymorphisms. In fact, sequencing of the *VDR* gene in a Japanese population identified >20 SNPs with a minor allele frequency of >0.05, including *FokI* and *TaqI* polymorphisms, at least some of which would serve as tag SNPs to capture the common variation in the gene (32). Further, recent genome-wide scans revealed several genes associated with circulating 25-hydroxyvitamin D concentrations (33, 34). Our findings, based on a limited number of SNPs in a single gene, provide at most an intriguing insight into the gene-environmental interaction in the vitamin D pathway.

The strengths of the present study include its measurement of plasma 25-hydroxyvitamin D concentrations, which may provide a relatively accurate classification of study subjects by vitamin D status. In addition, the provision of total colonoscopy to all study subjects likely decreased the possibility of misclassification between cases and controls. Conversely, a major limitation is its cross-sectional nature, and the observed associations might have been due to reverse causality. In contrast to colorectal cancer, however, colorectal adenoma likely does not affect circulating levels of vitamin D, because colorectal adenoma is an asymptomatic benign tumor. A second limitation is that adenoma cases were not histologically confirmed and necessarily included those with an early cancer or nonneoplastic lesion. However, our preliminary survey reported an accuracy of diagnosis based on magnifying chromoendoscopy of 90%, a result similar to those previously reported (35, 36), and the influence of any misclassification caused by the technique is therefore likely to have been minimal. Third, we were unable to analyze groups of cases and their frequency-matched controls in single batches, because single groups contained too many subjects to allow placement in the same batch. Although the impact of variability in assay performance was not reduced by simultaneously analyzing all subjects in a matching category, blood samples were at least analyzed irrespective of case and control status, reducing differential misclassification between cases and controls. Fourth, blinded control samples from the study population were not available and were therefore not

included in the measurement of plasma 25-hydroxyvitamin D; quality control for this measurement was performed by an external laboratory by using nonblinded controls. Accordingly, the reported intra- and interassay coefficients of variation would likely have underestimated the true underlying variations. Finally, we did not match cases and controls by season of examination or blood collection. If such matching had been conducted, we could have taken better account of the seasonal variation in plasma 25-hydroxyvitamin D concentrations.

In summary, we found that both plasma 25-hydroxyvitamin D and dietary calcium intake were inversely associated with the prevalence of colorectal adenoma, albeit in a nonlinear manner. We further noted that plasma 25-hydroxyvitamin D levels interacted with the *Taq*I polymorphism of the *VDR* gene but not with dietary calcium intake. These observations highlight the importance of vitamin D in colorectal carcinogenesis, at least in its early stage. Vitamin D might protect against colorectal cancer and adenoma, mainly through mechanisms other than the indirect mechanism via calcium metabolism.

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Original Article

Red meat intake may increase the risk of colon cancer in Japanese, a population with relatively low red meat consumption

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Asian populations have changed from traditional to Westernized diets, with increased red meat intake. They are suggested to be particularly susceptible to the adverse effects of red meat on the development of colorectal cancers, however, few prospective studies of this putative link have been conducted. We examined associations between the consumption of red and processed meat and the risk of subsite-specific colorectal cancer by gender in a large Japanese cohort. During 1995-1998, a validated food frequency questionnaire was administered to 80,658 men and women aged 45-74 years. During 758,116 person-years of follow-up until the end of 2006, 1,145 cases of colorectal cancer were identified. Higher consumption of red meat was significantly associated with a higher risk of colon cancer among women [multivariate hazard ratios (95%CIs) for the highest versus lowest quintiles (HR): 1.48 (1.01, 2.17; trend p=0.03)], as was higher consumption of total meat among men [HR=1.44 (1.06, 1.98; trend p=0.07)]. By site, these positive associations were found for the risk of proximal colon cancer among women and for distal colon cancer among men. No association was found between the consumption of processed meat and risk of either colon or rectal cancer. In conclusion, red meat intake may modestly increase the risk of colon cancer in middle-aged Japanese, although the highest quintile of red meat consumption could be considered moderate by Western standards.

Key Words: meat, colon cancer, rectal cancer, prospective studies, Japan

INTRODUCTION

The linear increase in the incidence and mortality of colon cancer between 1970 and the mid-1990s among Japanese of both sexes occurred in parallel with an increase in the intake of meat, such as beef and pork products.[1-4] Despite this increase, however, intake is still lower in Japanese than Western populations (approx 78, 130, 160, 185, and 200 g per capita per day in Japan, UK, Italy, France, and US, respectively, according to the FAO food supply database, 1995).[3] Given findings that descendents of Japanese migrants to the US have a higher incidence of colorectal cancer than US-born Caucasians,[5,6] individuals of Asian ethnicity may be particularly susceptible to the adverse effects of the Westernized diet, including red meat intake, owing to exposure to other lifestyle risk factors, the modifying influence of genetic biological susceptibility factors, or both.

A recent joint report by the World Cancer Research Fund/American Institute for Cancer Research concluded that the evidence that red and processed meats are a cause of colorectal cancer is convincing.[7] Most prospective studies to date have been conducted in Western countries,[8-10] however, and we are aware of only five in Asian populations, including the Japanese,[11-15] most of which failed to demonstrate a clear positive association between red or processed meat intake and colorectal cancer risk.

Asian populations tend to differ from Western populations in colonic anatomy and pattern of intracolonic bacteria,[16,17] the latter of which relates to the production of secondary bile acids from primary bile acids (which are required to digest animal fat) and of endogenous *N*-nitroso compounds (NOC).[7,18,19] A number of potential differences in the distribution of possible confounders is also likely, with Asians having a higher distribution of smok-

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ers (among men) and higher consumption of salt-preserved fish, the major sources of exogenous NOC,[7,20] as well as a lower prevalence of obesity. Moreover, few prospective studies have evaluated the effect of red meat consumption on the risk of subsite-specific colon cancers, separately by gender,[21-23] although risk factors for and biological pathways of proximal and distal colon carcinogenesis have been suggested to differ. In Japan, incidence rates for colorectal cancer have reached those in Western countries (GLOBOCAN 2002). These findings highlight the importance of studies aimed at characterizing the influence of red meat consumption on the risk of colorectal cancer by sub-site in Asian populations.

In this study, we used a validated comprehensive food frequency questionnaire to examine associations between red meat and the risk of colorectal cancer in a population-based prospective cohort study in Japan. Particular focus was placed on the risk of colorectal cancer according to sub-site in relation to red meat intake.

MATERIALS AND METHODS

Study population

The Japan Public Health Center-based Prospective (JPHC) Study was conducted in two cohorts (Cohort I and II), initiated in 1990-1994. The study population was defined as all registered Japanese inhabitants aged 40-69 years in 11 public health center areas, as identified from the population registries maintained by the local municipalities. The study design has been described in detail previously.[24] The study protocol was approved by the institutional review board of the National Cancer Center, Tokyo, Japan.

Participants in the present study were subjects in the JPHC study who responded to a self-administered 5-year follow-up questionnaire, which included comprehensive information on food intake and lifestyle factors, in 1995-1999, at age 45-74 years. This follow-up survey was used as the starting point in the present study. One public health center area (Tokyo) was excluded from the present analysis because cancer incidence data were not available.

After exclusion of 11,943 persons who had died, moved out of a study area, or were lost to follow-up before the starting point of the present study (1995-1999), the remaining 121,134 subjects were eligible for participation. Of these, 98,514 subjects responded to the questionnaire survey (46,029 men, 52,485 women; response rate: 81.3%) and were included in the present study.

Follow-up

Subjects were followed from the starting point (time that the FFQ for 5-year follow-up survey was completed) until December 31, 2006. Changes in residence status, including survival, were obtained annually from the residential registry in each area; or for those who had moved out of the study area, through the municipal office in the area to which they had moved. Mortality data for persons in the residential registry are forwarded to the Ministry of Health, Labour and Welfare, and are coded for inclusion in the national Vital Statistics. Residency registration and death registration are required by the Basic Residential Register Law and Family Registry Law, respectively, and the registries are thought to be complete. During the follow-up period in the present study, 7,658 (7.8%) subjects died, 3,970 (4.0%) moved out of the study area, and 318 (0.3%) were lost to follow-up.

The occurrence of cancer was identified by active patient notification from major local hospitals in the study area and from data linkage with population-based cancer registries, with permission from the local governments responsible for the cancer registries. Colorectal cancer cases were coded according to the International Classification of Diseases for Oncology, Third Edition (C18-C20), with colon cancer as C18 (C18.0-C18.5 for proximal colon cancer and C18.6-C18.7 for distal colon cancer) and rectal cancer as C19 and C20.[25] In our cancer registry system, the proportion of cases for which information was available from death certificates only was 2.6% of colorectal cancers. We confirmed 1,435 cases of newly diagnosed colorectal cancer among the 98,514 subjects by December 31, 2006.

Of the 98,514 respondents, we excluded subjects with a history of cancer (n=4,008), those who did not complete the diet component of the questionnaire (n=1,030), and those with extreme self-reported height or weight (≥200 cm, <20 kg; n=2,456). A history of cancer was defined as a diagnosis of cancer before the starting point or a self-report of cancer in the questionnaires. Of the remaining 91,020 subjects, 4,550 who reported extreme total energy intake (lower and upper 2.5 percentiles: 913 and 3,954 kcal/day, respectively), and subjects for whom values for any of the potential confounders were missing (n=5,812) were excluded, leaving 80,658 subjects (38,462 men, 42,196 women) for final analysis, including 1,145 with colorectal cancer (481 colon and 233 rectal cancer cases in men, and 307 colon and 124 rectal cancer cases in women). By sub-site, proximal and distal colon cancer accounted for 200 and 257 cases in men and 179 and 110 in women, respectively.

Food frequency questionnaire (FFQ)

The FFQ asked about the usual consumption of 138 foods and beverages during the previous year in standard portions/units and nine frequency categories.[26] The FFQ enquired about 16 meat items. The red meat items included 3 beef dishes (steak, grilled beef, and stewed beef), 6 pork dishes (stir-fried pork, deep-fried pork, stewed pork in Western style, stewed pork in Japanese style, pork in soup, and pork liver), 4 processed meat products (ham, sausage or Weiner sausage, bacon, and luncheon meat), and chicken liver. Poultry items included two chicken meals (grilled chicken and deep-fried chicken). Standard portion sizes were specified for each food item in three amount choices: small (50% smaller than standard), medium (same as standard) and large (50% larger). The amount of each food consumed (grams/day) was calculated from the responses. Energy and nutrient intake, excluding heme iron, were calculated using the Standardized Tables of Food Composition, 5th revised edition.[27] Heme iron intake was computed using the following proportions of iron for each type of meat: 69% for beef; 39% for pork, ham, bacon, and luncheon meats; 26% for chicken and fish (19 items); and 21% for liver.

The validity of the FFQ for the assessment of meat intake has been confirmed.[28,29] Spearman's correlation coefficients between energy-adjusted meat intake based on

the FFQ and those based on 28-day (or 14-day for one public health center area) dietary records among subsamples of men and women were 0.50 and 0.45 for Cohort I and 0.48 and 0.44 for Cohort II, respectively. Correlation coefficients for the reproducibility of the FFQ administered 1 year apart for men and women were 0.52 and 0.52 for Cohort I and 0.52 and 0.41 for Cohort II, respectively.[29,30] Correlation coefficients for the validity of the FFQ for assessment of specific meats for men were as follows: beef; 0.43, pork; 0.42, processed meat; 0.45, chicken; 0.20. For women as compared with men, the validity of the FFQ was comparable (unpublished data, Nanri, et al).

Statistical analysis

Person-years of follow-up were calculated for each subject from the starting point to the date of diagnosis, date of emigration from the study area, date of death, or end of the follow-up period (December 31, 2006), whichever occurred first. Subjects lost to follow-up were censored on the last confirmed date of presence in the study area. A total of 354,987 and 403,129 person-years for men and women, respectively, were accrued for the present analysis.

Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated for energy-adjusted meat consumption categories in quintiles based on the sex-specific distributions for men and women separately, with the lowest consumption category as the reference, using Cox proportional hazards models with adjustment for potential confounding variables according to the SAS PHREG procedure (SAS software, version 9.1, SAS Institute, Inc., Cary, North Carolina). The assumption of proportional hazards was established graphically; no deviation from proportionality was found. Person-years (follow-up time) was used for the underlying time metric in the Cox regressions. A residual model was used for energy adjustment.[31]

We conducted initial analyses by adjusting for age at the starting point (continuous) and study area (10 PHC districts). In the second multivariate model, we further adjusted for body mass index in kg/m^2 (<19, 19-22.9, 23-24.9, 25-26.9, and ≥27), smoking status (never, past, and current), alcohol consumption (none, occasional, 1-149, 150-299, 300-449, and ≥450 g of ethanol/week), physical activity in metabolic equivalent task-hours/day (<30, 30-34.9, 35-39.9, and ≥40), diabetes who either report of medication use for diabetes or a history of diabetes, screening examinations (yes/no) for fecal occult blood test, barium enema, and colonoscopy and quintiles of total energy, calcium, vitamins D and B-6, folate, and dietary fiber. This multivariate model was further adjusted for dried and salted fish intake in quintiles as a potential proxy for the intake of *N*-nitroso compounds.[20,32,33] Subjects for whom values for any of the potential confounders were missing were excluded from the final analysis, because findings did not materially differ when subjects with missing values were retained in the analyses (n=86,470) by assigning dummy variables for missing responses. Further, we conducted an additional analysis with the sub-site of colon cancer (proximal and distal) as endpoints. We also assessed linear associations (trend p-values) using the median values of meat intake for each quintile in the hazard models.

We additionally performed sub-group analyses according to age (<60 or ≥60 years), smoking status ("never" for nonsmokers or "past" and "current smoker" for ever smokers), body mass index (<25 or ≥25 kg/m^2), alcohol intake (<150 or ≥150 g ethanol/week, for men only), and Cohort (I or II). We sought to confirm whether extremely high meat affects the risk of colorectal cancer compared with very low meat intake. HRs were calculated for meat consumption categories in deciles. Throughout this paper, all p-values are two-sided, and statistical significance was determined at the p <0.05 level.

RESULTS

Red meat intake for men and women ranged from a median value of 15.4 and 13.6 g/day, respectively, in the lowest quintile to 102 and 93.0 g/day, respectively, in the highest. Subjects with higher red meat consumption were slightly younger.

Table 1 shows age-adjusted values for subject characteristics according to quintile of red meat consumption. For both men and women, subjects with higher red meat consumption were more likely to be overweight, and less likely to be heavy drinkers or participate in fecal occult blood test screening. They were also more likely to consume lower levels of calcium, dietary fiber, as well as dried and salted fish. Higher red meat intake was not associated with the prevalence of ever smoking, history of diabetes, or level of physical activity.

As shown in Table 2, higher consumption of red meat was significantly associated with a higher risk of colon cancer among women. Although a statistically significant association was not found between red meat consumption and colon cancer among men (point estimates of multivariate HRs increased), a significant association was seen for higher consumption of total meat. A significant association was seen between higher consumption of beef and pork and the risk of colon cancer among women. No association between the consumption of processed meat and risk of colon cancer was seen among either men or women. Positive associations of red meat, beef, and pork with the risk of colon cancer were more clearly seen after adjustment for dried and salted fish as a potential confounding factor than without this adjustment among women, but not among men (data not shown). No association was found between total meat, red meat or specific meat consumption and the risk of rectal cancer in either gender (Table 2). These results were not different substantially from those using gender combined quintiles (data not shown).

HRs for colon cancer among men with higher total meat intake were attenuated by further adjustment for heme iron, but were not substantially changed by further adjustment for saturated fatty acid intake, with corresponding multivariate HRs for the highest versus lowest quintile of 1.30 (95% CI: 0.87, 1.93; trend p=0.38) and 1.43 (95% CI: 0.95, 2.16; trend p=0.19), respectively. HRs for colon cancer among women with higher red meat intake were not substantially changed by further adjustment for heme iron, but were attenuated by further adjustment for saturated fatty acid intake, with corresponding HRs (95% CI) of 1.62 (1.01, 2.61; trend p=0.02) and 1.38 (0.84, 2.27; trend p=0.18). We further adjusted for

Table 1. Characteristics of subjects according to quintile (Q) of red meat intake for men and women: the JPHC Study, 1995 and 1998 (n= 80,658)

	Men						Women					
	Q1	Q2	Q3	Q4	Q5		Q1	Q2	Q3	Q4	Q5	
Median intake (g)	15	31	46	65	102		14	29	43	60	93	
Range	<24.1	≥24.1, <38.8	≥38.8, <54.6	≥54.6, <78.7	≥78.7		<22.0	≥22.0, <35.8	≥35.8, <50.4	≥50.4, <71.8	≥71.8	
No. of subjects	7,692	7,693	7,692	7,693	7,692		8,439	8,439	8,440	8,439	8,439	
Age	58.1	56.8	56.2	56.0	56.0		58.1	56.9	56.3	56.0	56.0	
(SD†)	(7.7)	(7.7)	(7.7)	(7.8)	(7.7)		(7.6)	(7.6)	(7.6)	(7.8)	(7.8)	
Meat intake (g/day, mean§)												
Total meat	20	39	56	77	127		17	36	52	71	115	
Red meat												
Beef	4	9	13	19	31		3	7	10	15	24	
Pork	8	18	26	37	67		8	18	26	36	65	
Processed meat	2	4	6	8	13		2	4	6	8	12	
Chicken	5	8	10	11	14		5	7	9	10	12	
BMI ≥25kg/m^2 (%¶)	25.7	26.6	27.4	29.7	33.0	**	27.4	27.0	26.6	27.8	31.9	**
Past smoker (%¶)	19.0	18.3	19.1	18.6	17.0		0.8	1.2	1.1	1.3	1.2	
Current smoker (%¶)	47.0	48.5	48.3	47.0	45.1	**	5.8	5.6	5.1	5.7	6.4	
Moderate drinker (>0, <300 g alc/w, %¶)	32.6	36.5	39.0	38.8	37.6	**	11.4	12.7	13.3	12.7	10.2	**
Heavy drinker (≥300 g alc/w, %¶)	39.5	35.7	31.1	27.8	19.5	**	1.9	1.4	1.2	0.7	0.4	**
Physical activity (MET-h‡/day, mean§)	32.9	32.8	32.7	32.5	32.1	**	31.8	32.0	32.0	31.8	31.5	**
Screening examination (yes, %¶)												
Fecal occult blood test	29.8	29.9	30.0	28.2	23.7	**	28.5	29.2	29.9	28.4	23.2	**
Barium enema	6.9	7.5	7.2	7.3	7.2		6.3	5.9	6.0	5.9	7.1	
Colonoscopy	8.6	8.4	8.5	7.9	7.0	**	6.8	6.4	6.7	6.3	6.2	
History of diabetes (%¶)	7.6	7.3	7.0	7.2	6.9		3.8	3.6	3.4	3.2	3.7	
Dietary intake (mean§)												
Total energy, kcal/d	2,132	2,146	2,133	2,105	2,048	**	1,893	1,891	1,872	1,863	1,838	**
Calcium, mg/d	554	534	522	505	462	**	656	624	592	557	491	**
Vitamin D, μg/d	10.1	10.1	10.3	10.4	9.8		10.7	10.4	10.5	10.3	9.3	**
Vitamin B_6, mg/d	1.57	1.58	1.59	1.61	1.60	**	1.54	1.53	1.53	1.53	1.50	**
Folate, μg/day	386	391	393	393	379		452	445	435	425	394	**
Dietary fiber, g/d	12.8	12.6	12.4	12.0	11.0	**	16.0	15.1	14.4	13.6	11.9	**
Dried and salted fish, g/d	20.1	19.2	18.8	18.4	15.4	**	21.1	20.2	19.4	18.6	15.0	**
Saturated fatty acid, g/d	12.7	14.4	16.0	17.89	22.28	**	14.5	16.0	17.1	18.4	22.0	**
Heme iron, mg/d	0.31	0.40	0.48	0.58	0.78	**	0.30	0.38	0.45	0.53	0.70	**

† SD, standard deviation; ‡ MET-h, metabolic equivalent task hours.
§ Values are age-adjusted least square means. ¶ Values are age-standardized proportions. **p<0.01; Trend tests across categories of red meat consumption were calculated by analysis of covariance for age-adjusted means and the Cochran-Mantel-Haenszel test for age-adjusted proportions.

cholesterol-lowering medications (4% and 7% user for men and women, respectively) or hormone replacement therapy (2.5% current user for women) in the multivariate analysis, but results for colon and rectal cancer did not substantially changed (data not shown).

On further analysis using of colon cancer sub-sites (proximal and distal) as endpoints, as shown in Table 3, higher consumption of total meat, red meat, and beef was marginally associated with a higher risk of distal colon cancer but not with the risk of proximal colon cancer among men. In contrast, higher consumption of red meat and beef was associated with a higher risk of proximal colon cancer but not with the risk of distal colon cancer among women. Higher consumption of processed meat was not associated with either proximal or distal colon cancer for either gender.

Stratified analyses according to age (<60 or ≥60 years) showed a clearer association between red meat intake and the risk of colon cancer among the older age group than the younger group for both men and women. Corresponding HRs (95% CI) for the older and younger age groups were 1.46 (0.95, 2.23; trend p=0.07) and 1.05 (0.66, 1.68; trend p=0.87), respectively, among men (259 and 222 cases, respectively), and 1.64 (0.95, 2.82; trend p=0.06) and 1.34 (0.78, 2.30; trend p=0.20), respectively, among women (152 and 155 cases, respectively). Further, significant positive associations were found between the consumption of total or processed meat for men, and beef or pork for women, and the risk of colon cancer among the older age group only (data not shown), although tests of interaction were not statistically significant between age and red meat, or any meat intake for the risk of colon cancer (data not shown). Stratified analyses according to smoking status (never or ever smoker) showed a clearer positive association between processed meat intake and the risk of colon cancer among male nonsmokers (HR: 1.79; 95% CI: 1.04, 3.10; trend p=0.02) than male ever-smokers (HR: 1.10; 95% CI: 0.77, 1.58; trend p=0.62), although tests of interaction were not statistically significant. The main results [positive association between total meat (among men), and red meat including beef and pork (among women) and the risk of colon cancer; and no association between meats (combined or separated) and the risk of rectal cancer among either gender] did not sub-

stantially changed in analyses stratified by body mass index, alcohol intake, or cohort (data not shown). Also, the results did not substantially changed in the analyses that excluded cases diagnosed during the first two years of follow-up (data not shown). When colon cancer was limited to invasive cases (269 cases in men and 186 in women), point estimates of multivariate HRs increased with red meat intake but did not reach statistically significant levels, with multivariate HRs (95%CIs) for the highest versus lowest quintiles of intake of 1.19 (0.78, 1.82; trend p=0.37) for men, 1.39 (0.85, 2.28; trend p=0.18) for women, and 1.30 (0.94, 1.78; trend p=0.08) for the two genders combined.

Finally, in analyses by deciles of meat consumption, higher processed meat intake showed a marginally significant association with the risk of colon cancer for men but not for women, with multivariate HRs for the highest versus lowest decile of 1.37 (95% CI: 0.92, 2.03; trend p=0.05) and 1.67 (95% CI: 0.97, 2.88; trend p=0.36), respectively.

Table 2. Hazard ratios and 95% confidence intervals for colon and rectal cancer according to quintiles of meat consumptions for men and women: the JPHC Study, 1995 and 1998–2006 (n=38,462 and 42,196 for men and women, respectively)

	Men						Women					
	Colon (481 cases)				Rectal (233 cases)			Colon (307 cases)			Rectal (124 cases)	
	Median (g/d)	Cases	HR†	(95%CI‡)	Cases	HR† (95%CI‡)	Median (g/d)	Cases	HR†	(95%CI‡)	Cases	HR† (95%CI‡)
Total meat												
Q1	20	98	1.00	(reference)	60	1.00 (reference)	18	63	1.00	(reference)	31	1.00 (reference)
Q2	39	107	1.25	(0.95, 1.65)	43	0.78 (0.53, 1.16)	36	65	1.14	(0.80, 1.62)	19	0.63 (0.35, 1.12)
Q3	56	99	1.27	(0.95, 1.69)	46	0.89 (0.60, 1.32)	52	46	0.82	(0.56, 1.21)	25	0.89 (0.52, 1.53)
Q4	77	82	1.12	(0.83, 1.52)	47	0.94 (0.63, 1.40)	70	67	1.26	(0.88, 1.81)	28	1.02 (0.59, 1.74)
Q5	117	95	1.44	(1.06, 1.98)	37	0.83 (0.52, 1.30)	107	66	1.35	(0.92, 1.98)	21	0.78 (0.41, 1.46)
trend p			0.07			0.64			0.10			0.83
Red meat												
Q1	15	103	1.00	(reference)	53	1.00 (reference)	14	63	1.00	(reference)	31	1.00 (reference)
Q2	31	103	1.14	(0.87, 1.50)	46	0.96 (0.64, 1.43)	29	67	1.19	(0.84, 1.69)	20	0.67 (0.38, 1.19)
Q3	46	90	1.08	(0.81, 1.44)	48	1.06 (0.71, 1.58)	43	39	0.70	(0.47, 1.06)	30	1.08 (0.65, 1.81)
Q4	65	94	1.19	(0.89, 1.60)	50	1.16 (0.78, 1.74)	60	68	1.30	(0.91, 1.86)	21	0.77 (0.43, 1.37)
Q5	102	91	1.27	(0.93, 1.74)	36	0.93 (0.58, 1.49)	93	70	1.48	(1.01, 2.17)	22	0.81 (0.43, 1.52)
trend p			0.15			0.99			0.03			0.63
Beef												
Q1	0.2	102	1.00	(reference)	53	1.00 (reference)	0.1	59	1.00	(reference)	27	1.00 (reference)
Q2	6.0	83	0.88	(0.65, 1.18)	46	0.89 (0.60, 1.33)	3.9	67	1.37	(0.96, 1.94)	30	1.27 (0.75, 2.15)
Q3	11	101	1.23	(0.93, 1.63)	38	0.82 (0.54, 1.25)	8.8	61	1.31	(0.91, 1.89)	24	1.06 (0.61, 1.86)
Q4	19	108	1.35	(1.02, 1.78)	46	1.02 (0.68, 1.53)	15	54	1.26	(0.86, 1.84)	20	0.94 (0.52, 1.72)
Q5	34	87	1.15	(0.85, 1.55)	50	1.16 (0.77, 1.74)	28	66	1.62	(1.12, 2.34)	23	1.11 (0.61, 2.02)
trend p			0.10			0.28			0.04			0.95
Pork												
Q1	6.5	112	1.00	(reference)	54	1.00 (reference)	6.1	65	1.00	(reference)	24	1.00 (reference)
Q2	15	95	0.94	(0.71, 1.24)	54	1.08 (0.74, 1.58)	15	54	0.92	(0.64, 1.32)	28	1.18 (0.68, 2.05)
Q3	24	86	0.89	(0.67, 1.18)	34	0.71 (0.46, 1.10)	24	62	1.04	(0.73, 1.49)	23	0.97 (0.54, 1.73)
Q4	36	96	1.01	(0.77, 1.34)	50	1.08 (0.72, 1.60)	35	48	0.81	(0.55, 1.20)	25	1.06 (0.60, 1.90)
Q5	62	92	1.06	(0.78, 1.42)	41	0.97 (0.63, 1.51)	59	78	1.42	(0.99, 2.04)	24	1.06 (0.57, 1.97)
trend p			0.53			0.97			0.05			0.97
Processed meat												
Q1	0.2	106	1.00	(reference)	66	1.00 (reference)	0.4	61	1.00	(reference)	27	1.00 (reference)
Q2	1.9	106	1.11	(0.85, 1.46)	49	0.84 (0.58, 1.21)	2.2	69	1.26	(0.89, 1.79)	27	1.09 (0.64, 1.87)
Q3	3.9	81	0.91	(0.68, 1.22)	35	0.64 (0.42, 0.97)	4.3	60	1.10	(0.76, 1.58)	21	0.85 (0.47, 1.52)
Q4	7.3	89	1.05	(0.79, 1.41)	48	0.91 (0.62, 1.33)	7.6	58	1.12	(0.77, 1.62)	27	1.19 (0.68, 2.08)
Q5	16	99	1.27	(0.95, 1.71)	35	0.70 (0.45, 1.09)	15	59	1.19	(0.82, 1.74)	22	0.98 (0.53, 1.79)
trend p			0.10			0.25			0.64			1.00
Chicken												
Q1	0.5	103	1.00	(reference)	59	1.00 (reference)	0.5	66	1.00	(reference)	21	1.00 (reference)
Q2	4.3	95	0.99	(0.75, 1.31)	47	0.82 (0.55, 1.20)	4.0	55	0.90	(0.62, 1.29)	29	1.35 (0.76, 2.38)
Q3	7.4	106	1.13	(0.86, 1.49)	40	0.72 (0.48, 1.08)	6.8	75	1.26	(0.90, 1.77)	28	1.33 (0.75, 2.37)
Q4	11	88	1.06	(0.79, 1.42)	48	0.90 (0.61, 1.34)	11	50	0.83	(0.57, 1.21)	20	0.97 (0.52, 1.82)
Q5	21	89	1.11	(0.83, 1.49)	39	0.72 (0.47, 1.09)	19	61	1.01	(0.70, 1.46)	26	1.27 (0.69, 2.32)
trend p			0.44			0.22			0.91			0.80

† HR, hazard ratio; ‡ CI, confidence interval. Hazard ratio was adjusted for age (continuous), Public Health Center area, Body Mass Index in kg/m^2 (<19, 19–22.9, 23–24.9, 25–26.9, and ≥27), smoking status (never, past, and current), alcohol consumption (non, occasional, 1–149, 150–299, 300–449, and ≥450g ethanol/week), physical activity in metabolic equivalent task-hours/day (<30, 30–34.9, 35–39.9, ≥40), medication use for diabetes, history of diabetes, screening examinations (fecal occult blood test; barium enema; colonoscopy), and quintiles of intake of energy, calcium, vitamin D, vitamin B_6, folate, dietary fiber, and dried and salted fish. Linear trends across quintiles of red meat or other meat intake were tested using the derived variable based on median consumption for each quintile as a continuous variable.

Table 3. Hazard ratios and 95% confidence intervals for colon cancer by sub-site according to quintiles of meat consumptions for men and women, the JPHC Study, 1995 and 1998–2006

	Men						Women					
	Proximal colon (200 cases)			Distal colon (257 cases)			Proximal colon (179 cases)			Distal colon (110 cases)		
	Cases	HR†	(95%CI‡)	Cases	HR†	(95%CI‡)	Cases	HR†	(95%CI‡)	Cases	HR†	(95%CI‡)
Total meat												
Q1	42	1.00	(reference)	52	1.00	(reference)	40	1.00	(reference)	18	1.00	(reference)
Q2	47	1.32	(0.87, 2.01)	52	1.14	(0.78, 1.69)	37	1.01	(0.65, 1.59)	25	1.54	(0.84, 2.85)
Q3	37	1.12	(0.71, 1.76)	56	1.36	(0.92, 2.00)	26	0.72	(0.43, 1.18)	17	1.07	(0.55, 2.1)
Q4	41	1.33	(0.85, 2.08)	40	1.03	(0.67, 1.58)	37	1.07	(0.67, 1.71)	28	1.78	(0.96, 3.3)
Q5	33	1.21	(0.73, 2.01)	57	1.65	(1.09, 2.52)	39	1.23	(0.75, 2.01)	22	1.41	(0.71, 2.79)
trend p		0.52			0.04			0.34			0.35	
Red meat												
Q1	47	1.00	(reference)	52	1.00	(reference)	36	1.00	(reference)	22	1.00	(reference)
Q2	43	1.06	(0.70, 1.61)	54	1.19	(0.81, 1.74)	39	1.21	(0.77, 1.91)	24	1.22	(0.68, 2.18)
Q3	36	0.96	(0.61, 1.49)	49	1.17	(0.79, 1.74)	26	0.82	(0.49, 1.37)	12	0.61	(0.30, 1.25)
Q4	40	1.12	(0.72, 1.73)	51	1.29	(0.87, 1.94)	36	1.21	(0.75, 1.96)	29	1.50	(0.84, 2.68)
Q5	34	1.07	(0.66, 1.75)	51	1.42	(0.92, 2.19)	42	1.57	(0.95, 2.58)	23	1.21	(0.63, 2.32)
trend p		0.74			0.12			0.08			0.41	
Beef												
Q1	50	1.00	(reference)	49	1.00	(reference)	28	1.00	(reference)	29	1.00	(reference)
Q2	36	0.78	(0.50, 1.20)	42	0.92	(0.60, 1.39)	42	1.95	(1.20, 3.16)	21	0.82	(0.46, 1.44)
Q3	42	1.06	(0.70, 1.61)	54	1.35	(0.91, 2.01)	39	1.91	(1.17, 3.12)	18	0.73	(0.40, 1.32)
Q4	40	1.06	(0.69, 1.63)	62	1.58	(1.07, 2.34)	26	1.39	(0.81, 2.40)	24	1.05	(0.60, 1.84)
Q5	32	0.89	(0.56, 1.41)	50	1.36	(0.90, 2.06)	44	2.52	(1.53, 4.14)	18	0.78	(0.42, 1.44)
trend p		0.95			0.04			0.01			0.69	
Pork												
Q1	45	1.00	(reference)	62	1.00	(reference)	36	1.00	(reference)	22	1.00	(reference)
Q2	41	1.02	(0.67, 1.56)	50	0.89	(0.61, 1.29)	40	1.23	(0.78, 1.93)	13	0.65	(0.32, 1.29)
Q3	38	1.01	(0.65, 1.57)	45	0.82	(0.55, 1.21)	34	1.03	(0.64, 1.65)	25	1.22	(0.68, 2.19)
Q4	37	0.99	(0.63, 1.55)	50	0.94	(0.64, 1.38)	24	0.72	(0.42, 1.22)	22	1.06	(0.58, 1.96)
Q5	39	1.17	(0.74, 1.87)	50	1.01	(0.68, 1.52)	45	1.42	(0.88, 2.30)	28	1.42	(0.77, 2.61)
trend p		0.52			0.75			0.32			0.11	
Processed meat												
Q1	36	1.00	(reference)	64	1.00	(reference)	31	1.00	(reference)	26	1.00	(reference)
Q2	51	1.60	(1.04, 2.46)	53	0.92	(0.64, 1.33)	42	1.51	(0.95, 2.42)	23	0.98	(0.55, 1.73)
Q3	37	1.20	(0.75, 1.91)	39	0.73	(0.49, 1.10)	37	1.33	(0.82, 2.16)	19	0.79	(0.43, 1.44)
Q4	39	1.31	(0.82, 2.08)	46	0.93	(0.63, 1.38)	38	1.42	(0.87, 2.31)	18	0.77	(0.42, 1.44)
Q5	37	1.38	(0.85, 2.25)	55	1.19	(0.80, 1.77)	31	1.23	(0.73, 2.07)	24	1.03	(0.57, 1.87)
trend p		0.54			0.19			0.87			0.88	
Chicken												
Q1	42	1.00	(reference)	56	1.00	(reference)	40	1.00	(reference)	21	1.00	(reference)
Q2	38	1.00	(0.64, 1.56)	51	0.95	(0.65, 1.40)	32	0.85	(0.53, 1.37)	20	1.03	(0.55, 1.91)
Q3	43	1.12	(0.73, 1.73)	57	1.14	(0.78, 1.65)	43	1.19	(0.76, 1.84)	28	1.47	(0.83, 2.62)
Q4	35	1.04	(0.66, 1.65)	49	1.08	(0.73, 1.60)	28	0.73	(0.45, 1.20)	21	1.10	(0.59, 2.06)
Q5	42	1.34	(0.85, 2.09)	44	0.99	(0.66, 1.48)	36	0.95	(0.59, 1.51)	20	1.01	(0.53, 1.92)
trend p		0.18			0.96			0.70			0.91	

† HR, hazard ratio; ‡ CI, confidence interval. Hazard ratio was adjusted for age (continuous), Public Health Center area, Body Mass Index in kg/m^2 (<19, 19–22.9, 23–24.9, 25–26.9, and ≥27), smoking status (never, past, and current), alcohol consumption (non, occasional, 1–149, 150–299, 300–449, and ≥450g ethanol/week), physical activity in metabolic equivalent task-hours/day (<30, 30–34.9, 35–39.9, ≥40), medication use for diabetes, history of diabetes, screening examinations (fecal occult blood test; barium enema; colonoscopy), and quintiles of intake of energy, calcium, vitamin D, vitamin B-6, folate, dietary fiber, and dried and salted fish. Linear trends across quintiles of red meat or other meat intake were tested using the derived variable based on median consumption for each quintile as a continuous variable.

The lack of association between red meat or processed meat intake and rectal cancer did not change substantially in the decile analyses, with multivariate HRs (95% CI) for the highest versus lowest decile among men and women of 0.83 (0.42, 1.64; trend p=0.80) and 1.33 (0.60, 2.95; trend p=0.83), respectively, for red meat intake, and 0.68 (0.37,1.24; trend p=0.26) and 1.28 (0.55, 2.96; trend p=0.90), respectively, for processed meat intake.

DISCUSSION

In this population-based prospective cohort study in Japan, we observed that higher consumption of red meat, including beef and pork, was associated with an increased risk

of colon cancer among women, and that higher total meat consumption was associated with this cancer among men. By site, these positive associations were found for the risk of distal colon cancer among men and proximal colon cancer among women. No association was found between the consumption of red meat and the risk of rectal cancer in either gender, or between processed meat and the risk of either colon or rectal cancer. The highest quintile of red meat consumption in our cohort (120 and 105 g per day for men and women, respectively, based on a corrected median value according to weighed dietary records among sub-samples[28,29]) could be considered moderate by Western standards, at least.[3]

A number of mechanisms to explain the association between red meat or processed meat and colorectal cancer have been proposed. First, secondary bile acids produced by anaerobic bacteria in the large bowel from primary bile acids, which are essential to the digestion of animal fat, are thought to be colonic irritants and to have hyperproliferative effects.[34] Second, red meat is a major source of heme iron, which has high bioavailability, and iron is thought to be carcinogenic as a prooxidant.[7] Third, red meat intake enhances the production of endogenous NOC by gut bacteria, depending on pH and substrate availability.[7,18,19] Fourth, processed meat is also a candidate exogenous source of NOC, which is formed during the curing process.[7] Finally, potentially carcinogenic heterocyclic amines are formed when muscle meats such as beef, pork, or fish are cooked at high temperatures.[7]

These possible mechanisms of the association between red meat and colon cancer might also explain the association between total meat and colon cancer among men. Point estimates of multivariate HRs increased with red meat intake (but did not reach statistically significant levels), for men. Furthermore, red meat intake accounted for 85% of total meat consumption. Thus, observed results of colon cancer in men might not essentially differ from the results in women. In this study, positive associations between meat and colon cancer were clearer for the older than the younger group. These age differences in association may be partly due to changes in bacterial flora, such as the decline in beneficial bifidobacteria numbers or the increase in pH in the elderly gut,[35,36] both of which affect the production of secondary bile acids or endogenous NOC.

A number of potential differences in the impact of dietary intake on the risk of proximal or distal colon cancers have been suggested. Levels of bile acid metabolites are higher in the right than left colon, while those of a marker of exposure to potentially carcinogenic NOC are higher in the distal than proximal colonic DNA of colorectal cancer patients.[8,37] Gender differences in the risk of subsite-specific colon cancers have also been suggested [37-39] due to the higher intracolonic pH or longer bowel transit time in women than in men, which in turn affects the production of secondary bile acid or NOC. In this study, the association between meat and colon cancer were partly explained by saturated fatty acid for women and heme iron for men. On the other hand, larger number of distal colon cancer cases in men, and proximal colon in women, than opposite sub-site of colon cancer cases might possibly clearly reflect the results of total colon cancer among either gender.

To our knowledge, seven studies have independently reported associations between red meat consumption and the risk of proximal or distal colon cancer.[11,21-23,40-42] Results have shown a relatively consistent stronger positive association for the distal colon: five studies showed a stronger association for distal than proximal colon cancer[11,21-23,40] among men[21,22], women[23], or combined[11,40]; one showed a stronger association for proximal colon cancer[41]; and one found no difference for men and women combined.[42] Only a few prospective studies have evaluated the effect of red meat consumption on the risk of subsite-specific colon cancers separately by gender (men[21,22] or women[23]). Our results for the distal colon in men are consistent with one of these previous studies.[21] The observed site-specific differences in risk between genders, however, suggest possible differences in the etiology of proximal and distal colon cancers that are consistent with women's higher incidence of proximal colon tumors and adenomas in the present and Western populations.[43]

The major strength of the present study is its prospective design, which avoids exposure recall bias. Other strengths include the following: study subjects were selected from the general population; response rate to the questionnaire in this general population setting (81%) was high; and the proportion of losses to follow-up (0.3%) was negligible. Further, the number of exclusions due to missing data on red meat consumption, extreme values of energy as a proxy for dietary information, and extreme values for height and weight was not particularly large (8 percent). Although a difference in incidence among subjects with and without missing or extreme information had the potential to influence the results, no such notable difference was seen. Finally, variation among subjects in red meat consumption was sufficiently large, with a 7-fold difference in median intake between the highest (102 and 93 g for men and women, respectively) and lowest quartile groups (15 and 14 g, respectively) (Table 1). This difference was similar to or greater than those in the 7 [21,23,40,44-48] of 11 studies [21,23,40-42,44-49] which found a significant positive association between red meat intake and the risk of colon and/or rectal cancer in Western countries.

Our study has several potential limitations. First, the validity of the FFQ for meat intake was moderate at best (r=0.48-0.50 for men, r=0.44-0.45 for women),[28,29] and was not substantially different by types of meat. It could be suggested that the observed association with the risk of colon cancer might have underestimated the true magnitude of association consequent to misclassification in the FFQ. The potential attenuation might be equivalent by types of meat. However, this bias may have operated in the same direction for subsite-specific cancers between men and women. On this basis, the contrary results for subsite-specific colon cancer between men and women might not be attributable to the validity of the FFQ. Second, we did not note substantial associations for processed meat, and consumption in the highest category (median 16 and 15 g per day for men and women, respectively) was substantially lower than those for studies in Western countries which found a significant positive association with the risk of colon and/or rectal cancer.[22,40,44,46,47,49] Consumption of processed meat in our cohort was likely