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ORIGINAL ARTICLE – COLORECTAL CANCER

OSNA-Based Novel Molecular Testing for Lymph Node Metastases in Colorectal Cancer Patients: Results from a Multicenter Clinical Performance Study in Japan

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ABSTRACT

Background. Lymph node (LN) metastasis in colorectal cancer (CRC) is a critical factor in making accurate prognoses and therapeutic decisions. This study evaluated the clinical performance of the one-step nucleic acid amplification (OSNA) assay in accurately diagnosing LN metastases in CRC patients through the specific detection of cytokeratin 19 mRNA levels in LNs.

Methods. The OSNA assay was performed on 121 LNs dissected from early-stage CRC patients (pStage 0 or I) or from patients with benign colorectal disease (study 1). Separately, 385 LNs were dissected from 85 CRC patients (any stage); the OSNA assay was performed on half of each LN, and the results were compared with histopathological examination in 2-mm intervals of the other LN half (study 2).

Results. In study 1, all 121 histopathologically negative LNs were also negative by the OSNA assay (concordance rate for metastasis negative: 1.0, 95% confidence interval [95% CI]: 0.976–1.0). In study 2, the concordance rate between the OSNA assay and the 2-mm-interval histopathological examination was 0.971 (95% CI: 0.950–0.984), with a sensitivity of 0.952 (95% CI: 0.881–0.987) and a specificity of 0.977 (95% CI: 0.953–0.991).

Conclusions. The OSNA assay provided a judgment performance equivalent to a 2-mm-interval histopathological examination, a more detailed assay than the common pathological examination. Therefore, the OSNA assay is considered a new molecular examination method for the diagnosis of LN metastases in CRC patients in clinical settings.

Lymph node (LN) metastasis in colorectal cancer (CRC) is a critical factor in predicting the prognosis of patients lacking distant metastases; it is also a key factor in determining the applicability of postoperative adjuvant chemotherapy.[1–4] The postoperative LN metastasis examination commonly used for CRC patients relies on microscopic examination of hematoxylin and eosin (H&E)

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stained histopathological specimens prepared from the LN section with the largest cutting surface. In this method, however, some of the metastases are unavoidably overlooked because of their localization in the LN. In 2004, the 5-year survival rate of stage IIB (T4N0M0; negative for LN metastases but the tumor directly invades other organs) patients was inferior to that of stage IIIA (T1 or 2N1M0; positive for fewer than 4 LN metastases) patients (72.2% vs 83.4%, respectively), and it is suggested that the high recurrence rate for stage IIB patients could be due to the nondetection of metastases during histopathological examination.[5–8] Accordingly, a new, highly accurate, clinically relevant method to detect LN metastasis is needed to ensure accurate diagnosis in CRC patients following surgery.

The reverse-transcription polymerase chain reaction (RT-PCR) has been used to analyze tumor-specific mRNA as molecular biology based techniques are reportedly shown to be more accurate for detecting LN metastases.[9,10] However, these methods have not yet come into clinical practice, possibly due to the complexity and time-consuming nature of the tests. The one-step nucleic acid amplification (OSNA) assay is a novel technique using the reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method for gene amplification. OSNA is already in clinical use for the diagnosis of LN metastases in breast cancer and is in research use for LN metastases in CRC, using cytokeratin 19 (CK19) mRNA as a molecular marker.[11–15] In this method, the supernatant of a homogenized LN solution is directly analyzed without the mRNA purification process that is usually required in RT-PCR. The use of an automated gene amplification detector (the RD-100i) offers rapidity and simplicity of detection of LN metastases.

The objective of the present study was to determine whether the OSNA assay for CK19 mRNA provided sufficient diagnoses of LN metastases in CRC patients. The multicenter clinical study should clarify the accurate diagnostic power provided by the pathological practice and by the OSNA method.

MATERIALS AND METHODS

Study Design

The present study was conducted in 2 phases, study 1 and study 2. Study 1 was designed to test (through concordance) whether the OSNA assay would yield false positives for histopathologically negative LNs in which no tumor cells were detected by the extensive 0.1-mm-interval histopathological examination with H&E staining and immunohistochemistry (IHC). The goal of study 2 was to investigate (through concordance) whether the OSNA assay would exhibit judgment performance equivalent to the 2-mm-interval histopathological examination. The entire study was conducted with the approval of the institutional review boards and independent ethics committees of each of the 6 institutes between October 22, 2007 and November 5, 2008.

Patient Samples

In preparation for study 1, 173 LNs with a minor axis 4 mm or smaller were dissected from 29 patients who had provided written informed consent for participating in the study. Patients who had been diagnosed as clinical stage 0/I or benign colorectal disease at age 18 or older were enrolled. In study 2, 434 LNs with a minor axis 8 mm or smaller were dissected from 91 CRC patients aged 18 years or older who were enrolled in the study.

Patients who had received preoperative or intraoperative adjuvant therapy and who were suffering or had suffered from any other cancer were excluded from this study, since no basic assessment had been performed for these patients using the OSNA assay.

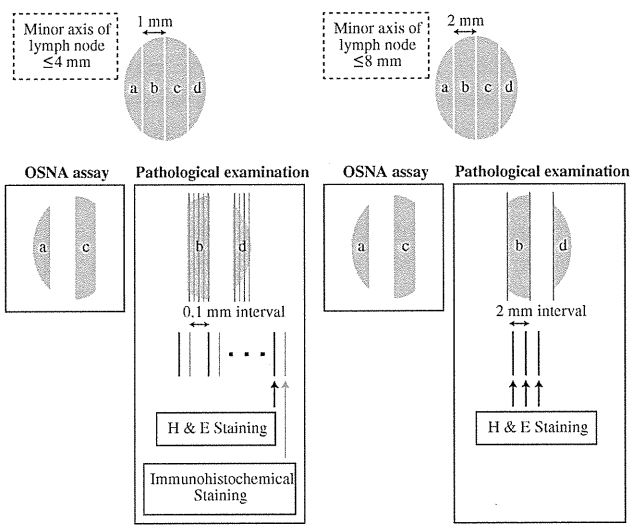

FIG. 1 Lymph node processing in study 1 and study 2. Lymph nodes were divided at 1-mm intervals (study 1) or 2-mm intervals (study 2), and nonadjacent blocks were alternatively subjected to histopathological examination or the OSNA assay. In study 1, the blocks for histopathological examination were processed as pairs of serial sections taken entirely at 0.1-mm intervals. Each pair was stained with hematoxylin and eosin (H&E) and anticytokeratin antibody. In study 2, a subset of the sections prepared from the cut surfaces were stained with H&E

LN Processing

To determine inclusion for analysis in study 1, 173 LNs were divided at 1-mm intervals, and nonadjacent blocks were alternatively subjected to histopathological examination and the OSNA assay (Fig. 1). The blocks for histopathological examination (blocks *b* and *d* in Fig. 1) were processed as pairs of 5-μm thick serial sections taken at 0.1-mm intervals. Subsequently, 1 section of each pair was stained with H&E and the other with anticytokeratin antibody (DAKO AE1/AE3); LNs in which all sections were found to be free of tumor cells by microscopy were selected as analytical samples. The remaining blocks (blocks *a* and *c* in Fig. 1) were subjected to the OSNA assay. Of the 173 LNs, 40 (23%) were derived from pathological stage II or III patients diagnosed by postoperative pathology and were excluded from the analysis, and 12 samples that were not confirmed as LNs were also excluded. The remaining 121 LNs derived from 18 patients (Table 1) were finally designated as histopathologically negative LNs for study 1 and were used in the concordance analysis with the OSNA assay.

TABLE 1 Patient demographics and baseline characteristics

	Study 1 (n = 18)		Study 2 (n = 85)	
	No. of patients	%	No. of patients	%
Age				
Mean	60		66	
Standard deviation	12.0		11.9	
Median	62		64	
Range	36–80		40–93	
Sex				
Male	11	61.1	44	51.8
Female	7	38.8	41	48.2
Tumor site				
Colon	11	61.1	62	72.9
Rectum	3	16.6	23	27.1
(benign colorectal disease)	4	22.2	–	–
Histological types				
Well/moderately	14	77.7	76	89.4
Poor/mucinous	0	0	8	9.4
Adenosquamous carcinoma	0	0	1	1.2
(benign colorectal disease)	4	22.2	–	–
Pathological Stage; TNM 6th edition				
0	4	22.2	1	1.1
I	10	55.5	14	16.4
IIA	–	–	16	18.8
IIB	–	–	0	0
IIIA	–	–	8	9.4
IIIB	–	–	21	24.7
IIIC	–	–	22	25.8
IV	–	–	3	3.5
(benign colorectal disease)	4	22.2	–	–

To determine inclusion for analysis in study 2, 434 LNs were divided into blocks at equal 2-mm intervals (Fig. 1). The 5-μm thick sections that had been prepared from the cut surfaces of nonadjacent alternating blocks (blocks *b* and *d*) were subjected to histopathological examination and stained with H&E. The remaining blocks (*a* and *c*) were subjected to the OSNA assay. There were 49 LNs excluded from the study, because 40 of the samples were not confirmed as LNs, and OSNA data for 9 LNs were not available because of quality-control errors. A total of 385 LNs from 85 patients (Table 1) were finally evaluated by comparing judgments resulting from the OSNA assay with those resulting from the 2-mm-interval histopathological examination. For blinding in both study 1 and study 2, identification codes were assigned separately to LN blocks analyzed by either histopathological examination or OSNA assay, so that the judgment results of one method would not influence those of the other.

Histopathological Evaluation

LNs with at least 1 observed tumor cell were judged to be positive, and LNs lacking 1 observed tumor cell were judged to be negative. The final histopathological results were determined by the judgments of 2 independent pathologists to ensure objectivity.

OSNA Assay

The OSNA assay used CK19 mRNA as a marker. The cutoff value between positive and negative LN for metastases in CRC was set at 250 copies/μl based on the logarithmic midpoint between the maximum value of the CK19 mRNA copy number in LNs from pN0 patients and −2SD value from the average of CK19 mRNA copy number in histopathologically positive LNs (our unpublished observation).

Surgically excised and divided LN blocks (*a* and *c*, Fig. 1) were homogenized using LYNORHAG lysis buffer (Sysmex Corp.). CK19 mRNA in each lysate was amplified using the LYNOAMP BC gene amplification reagent (Sysmex Corp.) and detected by measuring the rise time required to exceed a predetermined threshold turbidity caused by the by-product magnesium pyrophosphate. The rise times were analyzed using a previously generated standard curve, and CK19 mRNA concentrations in the LN

were calculated using the RD-100i automated gene amplification detector (Sysmex Corp.).[11]

Statistical Analysis

In study 1, the target value for the negative concordance rate of the OSNA assay was set at 0.99 on the assumption that 1% localization of metastases was the most unlikely level. The 95% confidence interval (95% CI) for the negative concordance rate was calculated from the results of study 1; study 1 was judged to be effective if its lower limit was below 0.89 (target value 0.99–Δ0.1). In study 2, the target value for the concordance rate of the 2 methods was established at 0.95; this value was obtained by defining the 2 methods as sufficiently equivalent if the discordance rate between the OSNA assay and the 2-mm-interval histopathological examination was not more than 5%. The 95% CI for the concordance rate was calculated from the result of study 2; study 2 was judged to be effective if its lower limit was not below 0.85 (target value 0.95–Δ0.1).

The diagnostic ability for LN metastasis by 2-mm-interval pathology and 1-level pathology was assessed by McNemar's test.

CK19 Protein Levels in Primary Tumors

Primary tumors from the 85 patients in study 2 (Table 1) were evaluated by staining with the anti-CK19 antibody (DAKO RCK108). For the primary tumors in which the stained area accounted for less than 10% of the total area, CK19 mRNA copy numbers were assessed by the OSNA assay if the LNs had been judged positive by histopathological examination.

Further Analysis of Discordant LN Samples

In study 2, to confirm the localization of tumor cells in the LNs, the remaining LN blocks (*b* and *d*) used in the histopathological examination were prepared as pairs of 5-μm thick serial sections taken at 0.1-mm intervals and stained with H&E and the anti-CK19 antibody. The samples were examined for the presence or absence of tumor cells and their distribution. When the OSNA assay was negative but the pathological method was positive, CK19 protein expression in tumor cells was confirmed by staining the IHC specimen with the anti-CK19 antibody.

RESULTS

Study 1

Based on the results of the 0.1-mm-interval histopathological examination with H&E staining and IHC with the anticytokeratin antibody, 121 LNs were selected as histopathologically negative (see the section Materials and Methods). For each of these samples, the copy number of CK19 mRNA was less than 250 copies/μl as assayed by OSNA, with no amplification observed during the reaction time in 115 of the 121 LN samples (95.0%) (Fig. 2).

The concordance rate of the OSNA assay (cutoff value 250 copies/μl) for judgment of histopathologically negative LNs was 1.0, providing a sufficiently lower limit of 0.976

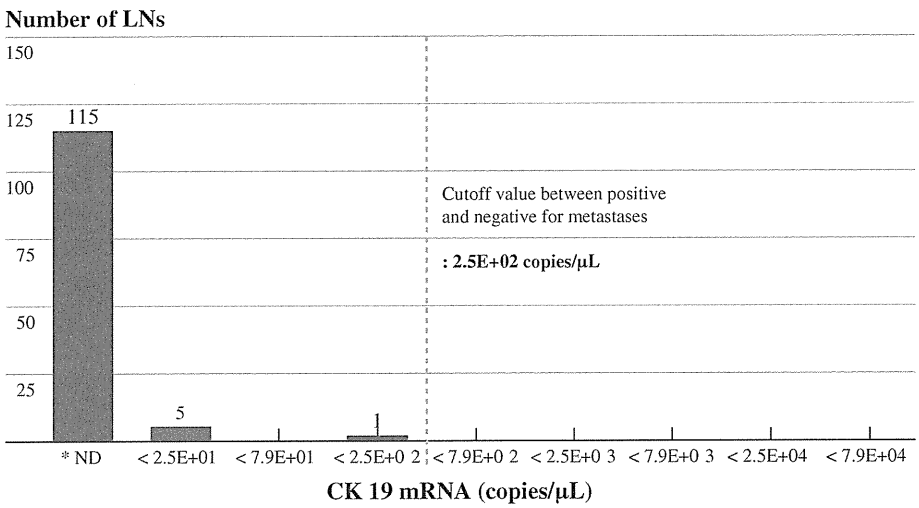

FIG. 2 CK19 mRNA copies/μl detected in histopathologically negative lymph nodes (LNs). Among the LNs derived from patients with benign colorectal disease or colorectal cancer (pStage 0/I), LNs that were found to be tumor-free by examination at 0.1-mm intervals with hematoxylin and eosin and immunohistochemistry were defined as "histopathologically negative." The CK19 mRNA cutoff level to separate "positive for metastasis" and "negative for metastasis" was 250 (2.5E+02) copies/μl (see the section Materials and Methods). *ND, the CK19 mRNA amplification reaction was not detected

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 *TaqI* 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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Tutorial videos of bioinformatics resources: online distribution trial in Japan named TogoTV

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Abstract

In recent years, biological web resources such as databases and tools have become more complex because of the enormous amounts of data generated in the field of life sciences. Traditional methods of distributing tutorials include publishing textbooks and posting web documents, but these static contents cannot adequately describe recent dynamic web services. Due to improvements in computer technology, it is now possible to create dynamic content such as video with minimal effort and low cost on most modern computers. The ease of creating and distributing video tutorials instead of static content improves accessibility for researchers, annotators and curators. This article focuses on online video repositories for educational and tutorial videos provided by resource developers and users. It also describes a project in Japan named TogoTV (http://togotv.dbcls.jp/en/) and discusses the production and distribution of high-quality tutorial videos, which would be useful to viewer, with examples. This article intends to stimulate and encourage researchers who develop and use databases and tools to distribute how-to videos as a tool to enhance product usability.

Keywords: *screencast; vodcast; tutorial; YouTube; QuickTime; Flash*

INTRODUCTION

Recent advances in life sciences technology have dramatically changed the research style from hypothesis-driven research (bottom-up style) to data-driven research (top-down style). Current 'omics' projects have produced vast amounts of data that have been stored in various online databases. Simultaneously, many types of web tools have been developed to analyze the stored data. Some of them are annually featured in the *Nucleic Acid Research*'s database issue and web server issue [1, 2]. Although the increase in available resources (databases and tools) has promoted life sciences research, this situation causes the following difficulties for researchers, especially 'wet' biologists: (i) What kinds of resources exist? (ii) Where are they? (iii) How can the resources be used and combined? and (iv) How does one interpret a result? To solve these issues, development of educational content as well as a system for navigation of web resources is required [3].

Traditional methods for distributing educational content include publishing textbooks and web documents. Although the contents of a textbook are sustainable, they quickly become obsolete because of frequent updates of web interfaces and improvement in web service functions. Web documents can more easily keep up with database and tool updates. However, it has become difficult to describe current

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web services in such static documents because of the evolution of web technology. Web 2.0, Flash and AJAX (Asymmetric JavaScript + XML) have led to the development of interactive and dynamic web services. This type of content would be better expressed in an animated environment rather than in a document. As a similar example, educational videos have been distributed using videotapes, CDs/DVDs and the Internet for >30 years in some clinical fields because it was excessively difficult to describe an actual procedure in writing [4–7]. However, their creation and distribution costs were high.

Rapid improvements in recent years in computer hardware, software and the Internet have reduced the publishing cost of multimedia content. A personal computer with a high-end CPU, extensive memory and large-capacity storage space enables users to produce and encode videos with relative ease. The latest releases of major OS packages include software for recording, editing and encoding videos, such as Windows Live Movie Maker (Microsoft Corporation, Redmond, WA, USA) and QuickTime (Apple Inc., Cupertino, CA, USA). The reasonable price of such software reduces the installation cost, and its user friendliness reduces the time required. In addition to the reduction in production cost, broadband networks have also reduced the distribution cost of multimedia content and have allowed experts to readily distribute video content in their field to anyone with an Internet connection. Many recent web browsers are by default equipped with video players, such as the Adobe Flash player (Adobe Systems Incorporated, San Jose, CA, USA) and QuickTime player (Apple Inc.); thus, one can easily view a video on web browser by simply clicking the play button.

In this article, we describe online video repositories for educational purposes, worldwide movements of distributing video tutorials created by major database and tool developers, and our recent activity in Japan. In addition, we propose distributing how-to videos to the developers and users of databases and tools to promote their usability and contribute to the scientific community.

ONLINE VIDEO REPOSITORIES

Several web services are already available for video distribution. YouTube is the most popular online video sharing service, and it contains many tutorial videos and lectures in many fields [8]. Similarly, there are repository services such as Dailymotion and Vimeo (for more examples, see the Wikipedia article entitled 'List of video hosting services') [9–11]. Most services are free to use, and any registered user can upload video. Live streaming services such as Ustream, Justin.tv and Stickam also exist [12–14]. As the term 'live streaming' suggests, these services provide live streaming services for lectures, workshops, seminars and meetings that are recorded and may be played back at a later time.

In the scientific field, the *Journal of Visualized Experiments* has been published since 2006 [15]. It is a peer-reviewed, PubMed-indexed journal devoted to the publication of biological research in a video format. SciVee offers a comprehensive set of rich media solutions to enhance the discovery and collaboration of knowledge [16]. It provides Video and Podcasts (standard videos and podcasts), PubCast (synchronized video abstracts of peer-reviewed articles), PaperCast (synchronized video abstracts of non-peer-reviewed articles), SlideCast (synchronized videos of slide presentations) and PosterCast (synchronized videos of posters or other conference presentations) in collaboration with scientists and researchers, as well as journals and publishers, societies, conference organizers, universities and research institutions. Dnatube is a community-based repository of scientific videos including educational materials, seminars and lectures [17]. This site has over 5000 videos and 30 000 community members. Individual videos can be found using keyword search, category tags and topics.

Some universities and organizations also administer a video repository server, especially for providing lecture videos that are part of OpenCourseWare (OCW). The Massachusetts Institute of Technology (MIT) hosts MIT OCW and MIT World, and the University of Tokyo provides UT OCW [18–20]. Academic Earth provides online courses of the world's top scholars from Harvard University and Stanford University among other top academic institutions [21]. YouTube also has a special channel for education from colleges and universities named YouTube EDU, and another channel, Technology, Entertainment, Design (TED), delivers interesting lectures by respected individuals [22, 23]. A complete list of OCW websites is found at the OCW Consortium Website, and other useful services are listed in the Wikipedia article entitled 'List of educational video websites' [24, 25].

In addition to repository-type services, delivery-type services named vodcasts (video podcasts) are available via Really Simple Syndication (RSS) technology. If a user subscribes to a vodcast program in a vodcast player such as iTunes, the contents of the program are automatically updated when new content arrives. Since the vodcast programs can be transferred to portable devices such as the iPod, iPhone or iPad, the user can watch them anytime, anywhere. Although vodcast programs are mainly focused on news, entertainment and fashion, educational programs are also provided. Indeed, some institutes have already used the podcast/vodcast for education [26–28]. Apple collects and webcasts educational contents via the iTunes store called iTunes U [29].

VIDEO TUTORIALS PROVIDED BY RESOURCE DEVELOPERS AND USERS

As noted earlier, the publishing of tutorial videos by some providers has increased as the creation and distribution costs of videos have decreased. For example, National Center for Biotechnology Information provides tutorial videos of some services both on the YouTube channel and on their server such as dbGaP, the database of Genotypes and Phenotypes, that archives and distributes the results of studies that have investigated the interaction of genotype and phenotype and PubMed that is a database of citations and abstracts for biomedical literature from MEDLINE and additional life sciences journals [30–35]. Some projects in the European Bioinformatics Institute also distributed how-to videos for tools such as Ensembl that is genome databases for vertebrates and other eukaryotic species, QuickGO that is a fast web-based browser for Gene Ontology (GO) terms and annotations, and GOA, Gene Ontology Annotation, that provides high-quality GO annotations to proteins in the UniProt Knowledgebase and International Protein Index [36-41].

Not only service providers in national institutes but also individual service providers including relatively small communities distributed tutorial videos. Galaxy, a collaboration system for genomic research, is a highly functional and complex system, but the procedure is easily understandable because the developers provide tutorial videos on their website [42, 43]. Taverna, which is an open source and domain-independent workflow management system (a suite of tools used to design and execute scientific workflows and aid *in silico* experimentation), is also described in the tutorials in a video format [44, 45]. ATTED-II, which provides co-regulated gene relationships to estimate gene function, has YouTube channel for tutorials [46, 47]. There are many video tutorials provided by the database and tool developers.

In addition, educators and users of web resources who do not develop any databases or tools also contribute to the scientific community by providing tutorial videos. BITS, BioInformatics Tutorials Series, is a collaboration work of the MIT Engineering and Science Libraries and Harvard's Countway Library [48, 49]. BIREC, Bioinformatics Information Resource and eLearning Center, also provides tutorial videos [50]. OpenHelix provides over 100 well-organized tutorial suites including videos on web-based bioinformatics and genomic resources [3, 51]. It also has many tutorial videos in 'Tip of this week' tagging articles in the blog section [52]. In addition to videos provided by organizations, a YouTube search by database or tool name will provide many tutorial videos produced by volunteers.

TogoTV: ONLINE TUTORIAL VIDEO DISTRIBUTION TRIAL IN JAPAN

To bridge the gap between service providers and users, we created and distributed tutorial videos of databases and web tools. We describe in this article, a methodology for making and distributing videos and elaborate on this methodology with examples. TogoTV ('Togo' means 'integration' in Japanese; pronunciation symbol is [toɯgoɯ]) that is one of the services in the Integrated Database Project in Japan (Figure 1) is a portal site of tutorial and lecture videos about bioinformatics resources [53–55]. Although the original TogoTV site is mostly written in Japanese [53], there is the English interface for international users [54]. The site contains our original videos and third-party videos from publicly available website such as YouTube. All contents provided by us are distributed under the Creative Commons Attribution 2.1 Japan license and also provided as vodcasts that can be viewed using a portable device and on YouTube. Although most of the contents are described in Japanese, there are 19 original programs in English, most of which explain a service developed in the Integrated Database Project such