minerals, although there were decreases in serum levels of organic compounds including bilirubin, lipids and some enzyme activities, but serum levels of uric acid, GOT and LDH activities tended to increase, because of bias due to use of different auto-analyzers.

Serum values of IGF-I, IGF-II, IGFBP-3, TGF-β, and sFas in individual sera after storage for 9 years at -80℃, which were separately collected from Hokkaido inhabitants in 1990, were similar to those of fresh sera newly collected from Saitama healthy inhabitants in 1999 (Table 3). In a study of other serum samples collected from 100 healthy individuals (46 males and 64 females) in 1991 and 1999, and stored at -80℃ until 2000, there tended to be similarity in distribution of serum values of IGF-I, IGF-II, IGFBP-3 and sFas between serum samples collected in 1991 and 1999 from different individuals, although those of TGF β 1 and SOD activity tended to change during storage (Table 4).

DISCUSSION

In the JACC Study, sera collected from 39,242 subjects and separated from blood cells were stocked in deep freezers at -80℃ until 1999; serum of each participant was divided into 3 to 5 tubes (100 to 500 μ L per tube). We were unable to assess the stability of serum samples stored for about 10 years, because the volume of serum samples for the JACC Study was insufficient for measurements of many constituents using different various protocols. Therefore, we evaluated the stability of frozen and stored sera using serum samples separately collected from other inhabitants and pooled serum.

Results of this study, in which values were compared between fresh and frozen samples of pooled serum prepared for quality control of determination of JACC Study samples, demonstrate that serum levels of proteins such as albumin and total protein tend to remain steady during frozen storage for several years.

Some reports indicate that there is little change in serum levels of constituents such as proteins, minerals, glucose and uric acid during storage at -70℃,[14] although serum levels of creatinine and lipids such as triglyceride tended to decrease during storage for 6 years at -80℃ in this study. The difference in enzyme activities in the present study may be due to the estimation methods used for each auto-analyzer, although it has been reported that serum AST (GOT) activity changes during storage.[14,15] It has also been reported that the plasma protein fraction can be safely used after storage for 5 years at room temperature.[17] There have been no previous detailed reports about the stability of cytokines such as IGFs, TGF-β 1 and sFas in frozen serum samples examined after long-term storage. In the present study, the mean values of cytokines such as TGF-β 1 in sera separately collected from inhabitants varied over a range of about 14% after 9 years of storage at -80℃. However, we also obtained that serum values of other cytokines such as IL-6 (but not TNF-α) tended to decrease (more than 60%) after 9 years of storage at -80℃, in comparison between fresh and frozen samples. The range of variation was similar to the reported coefficients of variation for determinations of IGFs, TGF-β, sFas and SOD activity: 1.1 to 7.3% for intra-assay, and 1.6 to 11.7% for inter-assay.[18-25] Moreover, in the present study, serum SOD activity was stable during long-term storage at -80℃. In previous studies of SOD activity, there was no significant change related to storage time or temperature,[12,26] erythrocyte-SOD activity was unstable,[27] and protein levels were unusually stable.[28]

The present results indicate that serum levels of IGF-I, IGF-II, IGFBP-3, sFas, and TGF-β 1 remain stable during long-term storage at -80℃, because distributions of serum levels of these constituents were nearly equal between fresh and frozen specimens separately collected from different inhabitants. They also suggest that SOD activity is a useful biomarker for cancer prevention research such as a nested case-control study.

MEMBER LIST OF THE JACC STUDY GROUP

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REFERENCES

1. Aoki K. Report by the Research Committee of the Ministry of Education, Science, Sports and Culture on evaluation of risk factors for cancer. J Epidemiol 1996; 6: S107-S113.
2. Ohno Y, Tamakoshi A, JACC Study Group. Japan Collaborative Cohort Study for evaluation of cancer risk sponsored by Monbusho (JACC study). J Epidemiol 2001; 11: 144-50.
3. Rinderknecht E, Humbel RE. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 1978; 253: 2769-76.
4. LeRoith D, Roberts CT Jr. Insulin-like factors. Ann NY Acad Sci, 1993; 692: 1-9.
5. Thissen JP, Ketelsegers JM, Underwood LE. Nutritional regulation of the insulin-like growth factors. Endocrine Rev 1994; 15: 80-101.
6. Baxter RC. Biochemical characterization of insulin-like growth factor binding proteins. Act Endocrine 1991; 124: 33-40.
7. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Transforming growth factor-β in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem 1983; 258: 7155-60.
8. Lawrence DA. Transforming growth factor-β: a general review. Eur Cytokine Netw 1996; 7: 363-74.
9. Seishima M, Takemura M, Saito K, Sano H, Minatoguchi S, Fujiwara H, et al. Highly sensitive ELISA for soluble Fas in serum: increased soluble Fas in the elderly. Clin Chem 1996; 42: 1911-4.
10. Oberley LW, Buettner GR. Role of superoxide dismutase in cancer: a review. Cancer Res 1979; 39: 1141-9.
11. Taniguchi N. Clinical significances of superoxide dismutases: changes in aging, diabetes, ishemia, and cancer. Adv Clin Chem 1992; 29: 1-59.
12. Casado A, de la Torre R, Lopez-Fernandez ME, Carrascosa D, Casado MC, Ramirez MV. Superoxide dismutase and catalase blood levels in patients with malignant diseases. Cancer Lett 1995; 93: 187-92.
13. Ito Y, Suzuki K, Sasaki R, Otani M, Aoki K. Mortality rates from cancer or all causes and SOD activity level and Zn/Cu ratio in peripheral blood: population-based follow-up study. J Epidemiol 2002; 12: 14-21.
14. Wilson SS, Guillan RA, Hocker EV. Studies of the stability of 18 chemical constituents of human serum. Clin Chem 1972; 18: 1498-503.
15. DiMagno EP, Corle D, O'Brien JF, Masnyk IJ, Go VLW, Aamodt R. Effect of long-term freezer storage, thawing, and refreezing on selected constituents of serum. Mayo Clin Proc 1989; 64: 1226-34.
16. Willett WC. Nutritional Epidemiology, second edition, Oxford University Press, New York, Oxford, pp 189-342, 1998.
17. Hink JH Jr, Pappenhagen AR, Lundblad J, Johnson FF. Plasma protein fraction (human) physical and chemical properties after storage for 7-8 years. Vox Sang 1970; 18: 527-41.
18. Takasu S, Tsutiya M, Mori K, Iwamoto H, Kasahara H, Horikawa H. Development and foundamental studies of non-extract IGF-I/IGF-II IRMA. Horumonn To Rinsyou 1996; 44: 49-57. (in Japanese)
19. Simatsu A, Fujieda K. Simultaneous measurement of IGF-I, IGF-II and IGFBP-3 levels in patients with growth hormone disorders. Hormone To Rinsyou 1996; 44: 59-66. (in Japanese)
20. Terazone M, Iwamoto K, Kasahara H, Horikawa S. Fundamental studies of IGFBP-3 IRMA kit. Igaku To Yakugaku, 1996; 35: 893-7. (in Japanese)
21. Danielpour D. Improved sandwich enzyme-linked immunosorbent assays for transforming growth factor β1. J Immuno Methods 1993; 158: 17-25.
22. R&D System, Inc Quantikine. Human TGF-β1 Immunoassay Manual, Minneapolis, USA, 1998.
23. Kobayashi S, Koike T. Apoptosis in autoimmune diseases. Ryumachi 1995; 35: 712-25. (in Japanese)
24. Medical & Biological Laboratories (MBL) Co. Ldt. sFas (s) ELISA Kit Manual, Code No. 5251, Nagoya, Japan, 1996. (in Japanese)
25. Oyanagni Y. Establishment of nitrite-kit for SOD activity determination. Ensho 1984 4: 63-73. (in Japanese)
26. Fridovich I. Superoxide dismutase. Adv Enzym 1974; 41: 35-97.
27. Jozwik M, Jozwik M, Jozwik M, Szczypka M, Gajewska J, Laskowska-Klita T. Antioxidant defence of red blood cells and plasma in stored human blood. Clin Chim Acta 1997; 267: 129-42.
28. Di Mambro VM, Borin MF, Fonseca MJ. Topical formula-

tions with superoxide dismutase: influence of formulation composition on physical stability and enzymatic activity. J Pharm Biomed Analy 2003; 32: 97-105.

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Detection of plasma *hnRNP B1* mRNA, a new cancer biomarker, in lung cancer patients by quantitative real-time polymerase chain reaction

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Summary Circulating cell-free nucleic acids are noninvasive diagnostic tools for cancer detection. Heterogeneous nuclear ribonucleoprotein (hnRNP) B1, an RNA binding protein, has been found overexpressed in the early stage of lung cancer, including bronchial dysplasia, a premalignant lesion of lung squamous cell carcinoma. To determine the utility of plasma hnRNP B1 RNA and as cancer detection markers for lung cancer, we analyzed plasma *hnRNP B1* mRNA of lung cancer patients by real-time RT-PCR. Plasma RNA was extracted from plasma of 44 lung cancer patients, 7 lung neoplasm patients, 24 benign lung diseases and 25 healthy volunteers. Mean concentration of plasma *hnRNP B1* mRNA in lung cancer patients was 0.99 pg/μg RNA, whereas that in healthy volunteers and in benign lung diseases was 0.23 pg/μg RNA and 0.30 pg/μg RNA, respectively ($p < 0.05$). Twenty of 44 (45.5%) lung cancer patients showed more than 0.70 pg/μg RNA of plasma *hnRNP B1* mRNA, compared with only 3 of 25 (12.0%) healthy volunteers. Looking at histological subtype, squamous cell carcinoma patients showed higher *hnRNP B1* mRNA in the plasma than did adenocarcinoma patients, which is consistent with our previous immunohistochemistry results. These results indicate that plasma *hnRNP B1* mRNA is a useful non-invasive markers for detection of lung cancer.

1. Introduction

Lung cancer is the major cause of cancer-related death in Japan [1]. In spite of advances in early detection technology, the number of lung cancer patients has increased remarkably [1]. Since 1998, over 50,000 have died annually in Japan,

Abbreviations: hnRNP, heterogeneous nuclear ribonucleoprotein; PCR, polymerase chain reaction

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and the overall survival rate of lung cancer patients is still only 40%, 5 years after diagnosis [2]. Conventional cytological diagnosis using sputum has proved insufficient for clinical use because of its lack of efficacy [3]; a new molecular diagnostic marker for detection of the early events of lung carcinogenesis is urgently required.

hnRNP A2/B1 protein is the major component of a nuclear core complex which regulates RNA maturation, including RNA splicing and mRNA transport from nucleus to cytoplasm [4,5]. Drs. Mulshine and Tockmann were the first to report that hnRNP A2/B1 was overexpressed in lung cancer tissues with high frequency [6,7]. We subsequently identified hnRNP B1, a splicing variant of hnRNP A2, and found that it was overexpressed in nuclei of human lung cancer cells from the early stage to the advanced [8–10]. Moreover, hnRNP B1 was overexpressed in squamous cell carcinoma in various organs, such as oral cavity and esophagus, in addition to lung [11,12].

For clinical use of hnRNP B1 as an early detection marker of lung cancer as well as squamous cell carcinoma in various organs, a sensitive and non-invasive method for detection of hnRNP B1 was next required. We focused on circulating nucleic acids containing RNA free from endonuclease, detected in the plasma of patients. It was previously reported that the mRNAs were released from cells as a complex with ribonucleoproteins and thought to escape from degradation by serum RNase [13,14]. Previous studies had demonstrated that *tyrosinase*, *telomerase RNA template* (*hTR*) and *telomerase reverse transcriptase protein* (*hTERT*) mRNA were present in the sera/plasma of patients with malignant melanoma and breast cancer [15,16]. And Fleischhacker et al. [17] recently reported that *hnRNP B1* mRNA was detectable in the sera of a high percentage of lung cancer patients, an observation consistent with our conception that the detection of *hnRNP B1* mRNA would be a promising method for evaluation of the usefulness of *hnRNP B1* in cancer detection.

Considering the above-mentioned studies, we analyzed and quantified plasma *hnRNP B1* mRNA by real-time RT-PCR in the plasma of lung cancer patients, and the results were compared with levels in patients with benign lung diseases and healthy volunteers. This paper reports that *hnRNP B1* mRNA was apparently elevated in the plasma of lung cancer patients, thus, showing that quantification of nucleic acid of hnRNP B1 gene is a plausible plasma marker for detection of lung cancer.

2. Materials and methods

2.1. Patients' profiles and collection of plasma samples

Plasma samples were obtained from lung cancer patients (before chemotherapy or radiation) and non-lung cancer patients who had been admitted to Saga Medical School Hospital, as well as from volunteers, after informed consent was given. Blood samples containing one-tenth volume of 3.8% citric acid were subjected to centrifugation of 3500 rpm at 4 °C, and then the samples were stored at −80 °C until assays were performed.

2.2. Detection of *hnRNP B1* mRNA in the plasma

Total RNA was isolated from 500 μl of patient's plasma by ISOGEN reagent (Nippon gene, Japan) with some modification: We treated the plasma with ISOGEN reagent twice to remove protein fraction before isopropanol precipitation. Calculation of the RNA concentration is based on the absorbance at 260 nm. The levels of *hnRNP B1* mRNA were determined by real-time RT-PCR. One μg total RNA was applied to RT with MuLV reverse transcriptase (Roche Molecular Systems, NJ) at 37 °C for 60 min, and the obtained cDNAs were subjected to quantitative SYBR Green real-time PCR. The quality of cDNA obtained by RT was confirmed by PCR using β-actin primer sets as a control gene. Each 20 μl SYBR Green reaction consisted of 2 μl cDNA (100 μg/μl), 2 μl 10× LightCycler-DNA Master SYBR Green I (Roche Diagnostics Corporation, IN), and 1 μM forward and reverse primers. hnRNP B1 specific primers were as follows: Sense, 5′-TGTTCCTTTGGAGAGGAAAAAG-3′ and antisense, 5′-TTGATCTTTTGCTTGCAGGA-3′ (amplicon size, 164 bp). Quantitative PCR was performed on LightCycler™ V3. System (Roche Diagnostics Corporation, IN) using three-stage program parameters provided by the manufacturer: standard curve was obtained from the results with serial dilution of full length cDNA of hnRNP B1 subcloned into pTYB2 vector. Sixty cycles of 2 s at 95 °C, 10 s at 55 °C, 15 s at 72 °C. Specificity of the amplified PCR product was assessed by performing melting curve analysis. Agarose gel electrophoresis of PCR products, followed by staining with ethidiumbromide, was then performed to confirm the specificity of the amplification. Quantification focused on the initial exponential phase of amplification above baseline according to the Light-Cycler software. All the

results were obtained from the thrice repeated extraction and reverse transcription of RNAs from one patient's plasma, and four times PCR amplification.

2.3. Statistical analysis

The association between the levels of *hnRNP B1* mRNA and clinicopathological parameters were examined by chi-square test for contingency tables.

3. Results

3.1. *hnRNP B1* mRNA was detectable, and elevated, in the plasma of lung cancer patients as well as other neoplasm patients

A total of 75 patients – 44 lung cancer patients (19 adenocarcinoma, 14 squamous cell carcinoma, 7 small cell carcinoma and 4 large cell carcinoma), 7 cancer patients (except primary lung cancer), and 24 benign lung diseases, such as bacterial pneumonia, collagen disease, interstitial pneumonitis, pneumothorax, – along with 25 healthy volunteers, were studied, and these clinical parameters are shown in Table 1 and Table 2. The seven cancer patients were two lymphoma, three metastatic lung cancer, one invasive thymoma, and one thymic carcinoid. We first confirmed that *hnRNP B1* mRNA was present in the plasma of lung cancer patients, as Dr. Fleischhacker had recently reported (Fig. 1). We then extracted total RNA from both plasma and sera, and quantified mRNA level of each patient using real-time RT-PCR. The results were compared with those of non-lung cancer patients and healthy volunteers. Mean and median plasma RNA concentration of all samples was 0.78 μg/ml plasma (0.02–3.60), and no apparent difference in RNA concentration among healthy volunteers, benign pulmonary disease patients, cancer patients (except primary lung cancer), and lung cancer patients was observed (Table 2). Forty-nine samples out of 100 (49.0%) showed detectable *hnRNP B1* mRNA by real-time RT-PCR, and the mean value of *hnRNP B1* mRNA is shown in Table 3. No clear correlation between *hnRNP B1* mRNA levels and clinical parameters – such as clinical stage, white blood cell count, or C-reactive protein level in the peripheral blood – was observed (data not

Table 1 Clinical parameters of lung cancer patients

Parameters	Ad	Sq	Sm	La	Total
Age	69.0 (50–83)	66.3 (43–80)	68.9 (63–77)	67.3 (58–77)	
Sex					
M	15	13	6	4	38
F	4	1	6	0	6
Stage					
I	3	2	0	2	7
II	3	2	0	0	5
III	3	7	3	1	14
IV	10	3	4	1	18
BT (°C)	36.5 ± 0.13	36.3 ± 0.15	36.6 ± 0.15	36.7 ± 0.60	
WBC (/ml)	8027 ± 1294	9407 ± 1952	5685 ± 538	6175 ± 2523	
CRP (mg/dl)	2.53 ± 1.43	3.39 ± 1.56	1.29 ± 0.77	8.87 ± 4.86	

Table 2 Plasma nucleic acids and clinical parameters

	Normal	Benign	Neoplasm	Lung cancer	Total
No. of patients	25	24	7	44	100
Male	17	18	5	38	78
Female	8	6	2	6	22
Age	30.5 (23–47)	60.2 (22–86)	50.1 (19–74)	67.8 (43–83)	55.6 (19–86)
B.I.	92.9 (0–500)	637.9 (0–2760)	479.0 (0–1920)	1036.3 (0–3800)	540 (0–3800)
Total RNA (μg/ml plasma)	0.71 (0.02–2.74)	0.78 (0.04–1.90)	0.66 (0.10–1.34)	0.86 (0.02–3.60)	0.78 (0.02–3.60)

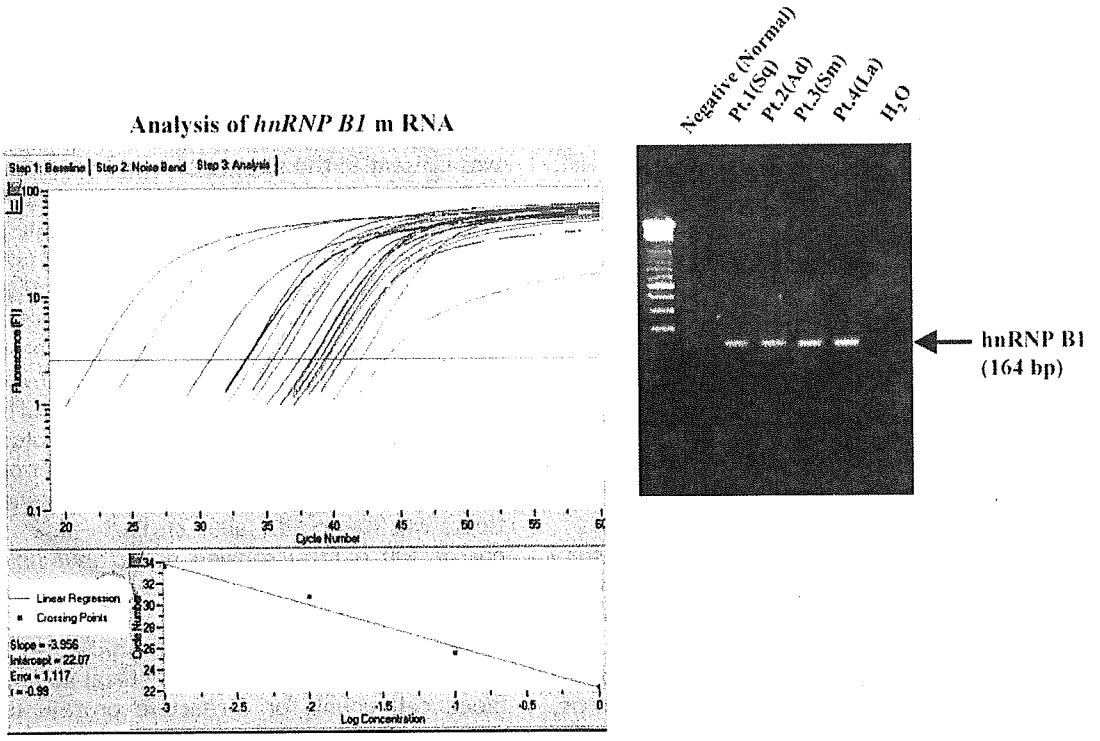

Fig. 1 *hnRNP B1* mRNA in the plasma of lung cancer patients was measured by real-time RT-PCR. Quantitative PCR was performed on LightCycler™ V3. System using three-stage program parameters provided by the manufacturer. Specificity of the amplification of PCR products were confirmed by agarose gel electrophoresis visualizing with ethidium bromide staining.

shown). The mean value of *hnRNP B1* mRNA among lung cancer patients was 0.99 ± 0.26 pg/μg plasma RNA, which was significantly higher than that of normal volunteers (0.23 ± 0.07 pg/μg plasma RNA) or benign lung disease patients (0.30 ± 0.11 pg/μg plasma RNA) (Table 3, Fig. 2). From these results, we determined that the tentative cut off value of plasma *hnRNP B1* mRNA as 0.70 pg/μg RNA. Positivity of *hnRNP B1* mRNA in plasma was 12.0% (3/25) for healthy volunteers, 20.8% (5/24) for benign lung diseases, 57.1% (4/7) for non-lung cancer neoplasm, and 45.5% (20/44) for lung cancer patients (Fig. 3, Table 4).

3.2. Plasma *hnRNP B1* mRNA in each clinical parameter of lung cancer patients

Positivity of *hnRNP B1* mRNA was significantly high in heavy smokers (Brinkman Index; ≥600, $p < 0.01$) among lung cancer patients. Looking at pathological type of lung cancer, the positivity of *hnRNP B1* mRNA was 57.1% (8/14) for squamous cell carcinoma, 33.3% (6/18) for adenocarcinoma, 42.9% (3/7) for small cell carcinoma, and 75.0% (3/4) for large cell carcinoma (Table 4), consistent with our previous results in which hnRNP B1 overexpression was more frequently observed in lung squamous cell carcinoma than in adenocarcinoma, by immunohistochemical analysis. It is important to note that

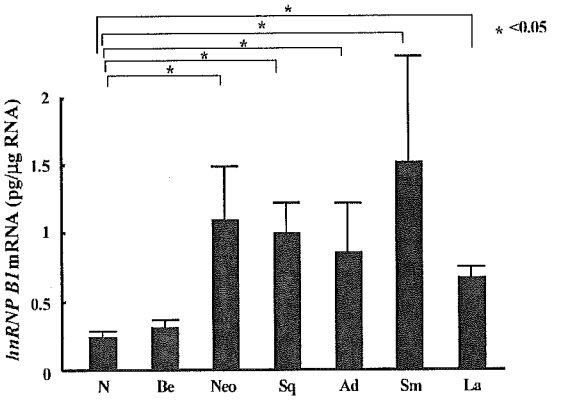

Fig. 2 Concentration of plasma *hnRNP B1* mRNA in patients with (N) normal healthy volunteers; (Be) benign lung diseases; (Neo) neoplasm except for lung cancer; (Sq) squamous cell carcinoma; (Ad) adenocarcinoma; (Sm) small cell carcinoma; (La) large cell carcinoma. *hnRNP B1* mRNA levels in each group were presented as mean value ± S.D.

Table 3 Elevation of plasma *hnRNP B1* mRNA in lung cancer patients

	Normal	Benign	Neoplasm	Lung cancer					Total
				Sq	Ad	La	Sm	Total	
No. of patients	25	24	7	14	19	4	7	44	100
hnRNP B1 mRNA (pg/μg RNA)	0.23 ± 0.07	0.30 ± 0.11	1.09 ± 0.43	0.99 ± 0.33	0.85 ± 0.42	0.67 ± 0.07	1.51 ± 1.04	0.99 ± 0.26	0.67 ± 0.12

Sq: squamous cell carcinoma; Ad: adenocarcinoma; Sm: small cell carcinoma and La: large cell carcinoma.

non-lung cancer neoplasm patients also showed a high concentration of *hnRNP B1* mRNA (Table 3), suggesting that plasma *hnRNP B1* mRNA might also be elevated in other malignant diseases. As for clinical stage, elevated plasma *hnRNP B1* mRNA was observed in 42.9% (3/7) of stage I, and in 60% (3/5) of stage II patients, suggesting that plasma *hnRNP B1* mRNA is elevated from early clinical stages.

4. Discussion

Detection of cell-free circulating nucleic acid in the plasma or serum looks increasingly reliable as a non-invasive, simple method for diagnosis of cancer. Several reports have presented plausible markers for detection of cancer cells in the plasma/serum: transmembrane glycoprotein *5T4* mRNA in lung cancer (positivity = 42.8%) [18]; *hTR* (28%) [16], *hTERT* (25%) [17], *mammaglobin* (60%) [19] and *cytokeratin 19* (49%) mRNA [20] in breast cancer; and *tyrosinase* mRNA [15] in melanoma patients. We first determined the extraction condition of RNA from the blood samples to obtain the high quality and quantity RNA. After comparison of several preliminary experiments, we used plasma for extraction of RNA, rather than serum, because of the minimum effect from the contamination of aggregated lymphocyte was observed. We also adjusted time after collection of the blood until centrifugation. These quality control was quite important for obtain the stable RNA concentrations from a sample and the high quality cDNAs after reverse transcription.

We previously reported that hnRNP B1 protein was not detectable in normal bronchial cells or alveolar cells but was overexpressed in lung cancer cells, by immunohistochemistry [8–10]: hnRNP B1 was therefore thought to be a candidate marker for detecting cancer cells in blood. Fleischhacker et al. [17] first found that *hnRNP B1* mRNA was detectable in the sera of lung cancer patients: 18/18 lung cancer patients in this study showed positive for *hnRNP B1* mRNA or *Her2/neu* mRNA. Our quantitative analysis revealed that positivity of *hnRNP B1* mRNA using our cut off value of 0.70 pg/μg RNA was 44.5% (20/45) for lung cancer patients and 12.0% (3/25) for healthy volunteers, approximately equivalent to the numbers for serum *mammaglobin* mRNA in breast cancer [19], and *5T4* mRNA in lung cancer [18]. It is important to note that *hnRNP B1* mRNA in the plasma was elevated from clinical stage I patients (3/5), although the number of subjects was small. These results were consistent with our previous immunohistochemical results, which showed that hnRNP B1 was overexpressed from the early

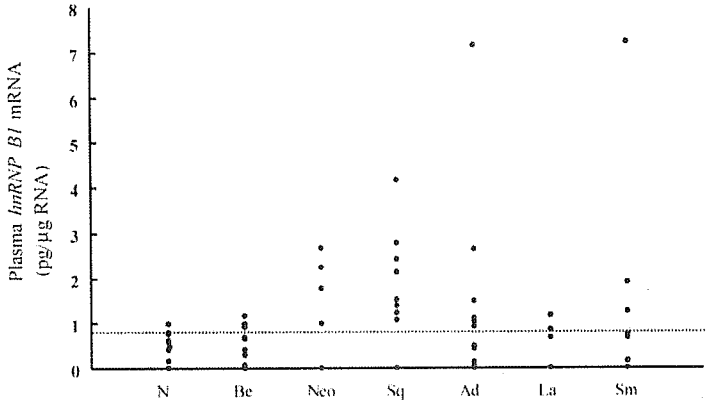

Fig. 3 Concentration of plasma *hnRNP B1* mRNA fragments in each patient with (N) normal healthy volunteers; (Be) benign lung diseases; (Neo) neoplasm except for lung cancer; (Sq) squamous cell carcinoma; (Ad) adenocarcinoma; (Sm) small cell carcinoma; (La) large cell carcinoma. Dashed line represents tentative cut off value as 0.7 pg/μg plasma RNA.

stage of lung caner; thus, further evaluation of plasma *hnRNP B1* mRNA levels in the early stage of lung cancer is required. We are now considering an age- and sex-adjusted study comparing plasma *hnRNP B1* mRNA of healthy persons and cancer patients. We think it quite suggestive that *hnRNP B1* mRNA was elevated in neoplasms (in addition to primary lung cancer), including metastatic squamous cell carcinoma of laryngeal cancer, malignant lymphoma, and invasive thymoma. And since hnRNP B1 was overexpressed not only in lung cancer, but also in oral cancer, esophageal cancer, and malignant lymphoma [11,12,21], plasma *hnRNP B1* mRNA could be a molecular tumor marker of various neoplasms.

These results encourage us to proceed to the next step: analysis of plasma *hnRNP B1* mRNA as a molecular marker for detection of the early stage of lung cancer and also for early detection of recurrence after surgery. Although a large scale comparison of lung cancer and a healthy population is needed before results are conclusive, this is the first report that the quantification of plasma *hnRNP B1* mRNA is a promising molecular marker for first step evaluation of lung cancer as well as other types of cancers. Since cancer prognosis improves the earlier it is detected, these molecular markers could be the beginning of a vital new strategy in the battle against cancer.

Table 4 Positivity for plasma *hnRNP B1* mRNA in lung cancer patients of each clinical parameters

Parameters	No. of patients	Positivity (%)	
Sex			
Male	38	50.0 (19/38)	$p=0.06$
Female	6	16.7 (1/6)	
Brinkman index			
<600	10	10.0 (1/10)	$p<0.01$
>600	34	55.9 (19/34)	
Stage			
I	7	42.9 (3/7)	
II	5	60.0 (3/5)	
III	14	28.6 (4/14)	
IV	18	55.6 (10/18)	
Histological types			
Squamous cell carcinoma	14	57.1 (8/14)	
Adenocarcinoma	18	33.3 (6/18)	
Large cell carcinoma	5	60.0 (3/5)	
Small cell carcinoma	7	43.0 (3/7)	

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References

[1] The Editorial Board of Cancer Statistics in Japan. Mortality from malignant neoplasms by age group and sex in Japan. In: Kakizoe T, Yamaguchi N, Mitsuhashi F, Koshiji M, Oshima A, Ohtaka M, editors. Cancer statistics in Japan. Tokyo: Foundation for Promotion of Cancer Research; 2001.

[2] Lung Cancer Registration Committee. Time trends and survival rate of lung cancer. In: Watanebe S, Tominaga W, Kakizoe T, editors. Gann monograph on cancer research 43. Tokyo: Japan Scientific Societies Press; 1995. p. 119–27.

[3] Hirsch FR, Bunn PA Jr, Dmitrovsky E, Field JK, Franklin WA, Greenberg RE, et al. Proceedings of the IV International Conference on Prevention and Early Detection of Lung Cancer, Reykjavik, Iceland, 9–12 August 2001. Lung Cancer 2002;37:325–44.

[4] Burd CG, Dreyfuss G. Conserved structures and diversity of functions of RNA-binding proteins. Science 1994;265: 615–21.

[5] Mayeda A, Munroe SH, Caceres JF, Krainer AR. Function of conserved domains of hnRNP A1 and other hnRNP A/B proteins. EMBO J 1994;13:5483–95.

[6] Tockman MS, Gupta PK, Myers JD, et al. Sensitive and specific monoclonal antibody recognition of human lung cancer antigen on preserved sputum cells: a new approach to early lung cancer detection. J Clin Oncol 1988;6:1685–93.

[7] Zhou J, Mulshine JL, Unsworth EJ, Scott FM, Avis IM, Vos MD, et al. Purification and characterization of a protein that permits early detection of lung cancer. J Biol Chem 1996;18:10760–6.

[8] Sueoka E, Sueoka N, Goto Y, et al. Heterogeneous nuclear ribonucleoprotein B1 as early cancer biomarker for occult cancer of human lungs and bronchial dysplasia. Cancer Res 2001;61:1896–902.

[9] Tominaga M, Sueoka N, Irie K, Iwanaga K, Tokunaga O, Hayashi S, et al. Detection and discrimination of preneoplastic and early stages of lung adenocarcinoma using hnRNP B1 combined with the cell cycle-related markers p16, cyclin D1, and Ki-67. Lung Cancer 2003;40:45–53.

[10] Wu S, Sato M, Endo C, Sakurada A, Dong B, Aikawa H, et al. hnRNP B1 protein may be a possible prognostic factor in squamous cell carcinoma of the lung. Lung Cancer 2003;41:179–86.

[11] Goto Y, Sueoka E, Chiba H, Fujiki H. Significance of heterogeneous nuclear ribonucleoprotein B1 as a new early detection marker for oral squamous cell carcinoma. Jpn J Cancer Res 1999;90:1358–63.

[12] Matsuyama S, Goto Y, Sueoka N, et al. Heterogeneous nuclear ribonucleoprotein B1 expressed in esophageal squamous cell carcinoma as a new biomarker for diagnosis. Jpn J Cancer Res 2000;91:658–63.

[13] Stroun M, Anker P, Beljanski M, Henri J, Lederrey C, Ojha M, et al. Presence of RNA in the nucleoprotein complex spontaneously released by human lymphocytes and frog auricles in culture. Cancer Res 1978;38:3546–54.

[14] Wieczorek AJ, Rhyner C, Block LH. Isolation and characterization of an RNA–proteolipid complex associated with the malignant state in humans. Proc Natl Acad Sci USA 1985;82:3455–9.

[15] Kopreski MS, Benko FA, Kwak LW, Gocke CD. Detection of tumor messenger RNA in the serum of patients with malignant melanoma. Clin Cancer Res 1999;5:1961–5.

[16] Chen XQ, Bonnefoi H, Pelte MF, Lyautey J, Lederrey C, Movarekhi S, et al. Telomerase RNA as a detection marker in the serum of breast cancer patients. Clin Cancer Res 2000;6:3823–6.

[17] Fleischhacker M, Beinert T, Ermitsch M, Seferi D, Possinger K, Engelmann C, et al. Detection of amplifiable messenger RNA in the serum of patients with lung cancer. Ann N Y Acad Sci 2001;945:179–88.

[18] Kopreski MS, Benko FA, Gocke CD. Circulating RNA as a tumor marker: detection of 5T4 mRNA in breast and lung cancer patient serum. Ann N Y Acad Sci 2001;945:172–8.

[19] Gal S, Fidler C, Lo YM, Chin K, Moore J, Harris AL, et al. Detection of mammaglobin mRNA in the plasma of breast cancer patients. Ann N Y Acad Sci 2001;945:192–4.

[20] Silva J, Silva JM, Garcia V, Garcia JM, Dominguez G, Bonilla F. RNA is more sensitive than DNA in identification of breast cancer patients bearing tumor nucleic acids in plasma. Genes Chromosomes Cancer 2002;35:375–56.

[21] Tani H, Ohshima K, Haraoka S, Hamasaki M, Kamma H, Ikeda S, et al. Overexpression of heterogeneous nuclear ribonucleoprotein B1 in lymphoproliferative disorders: high expression in cells of follicular center origin. Int J Oncol 2002;21:957–63.

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Single nucleotide polymorphisms in the *EXO1* gene and risk of colorectal cancer in a Japanese population

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EXO1 is a member of the RAD2 nuclease family and functions in DNA replication, repair and recombination. We investigated the relationship of single nucleotide polymorphisms (SNPs) at exon 10 (T439M) and exon 13 (P757L) of the *EXO1* gene with development, progression and metastasis of colorectal cancer. For T439M, the *Thr/Met* genotype [odds ratio (OR) = 2.03, 95% confidence interval (CI) 1.04–3.98] and *Thr/Met* and *Met/Met* genotypes combined (OR = 2.37, 95% CI 1.23–4.56) demonstrated significant association with the development of colorectal cancer after adjusting for age, gender and smoking status. For P757L, patients with the *Leu/Leu* genotype showed a reduced risk of colorectal cancer (adjusted OR = 0.398, 95% CI 0.183–0.866) when the *Pro/Leu* and *Pro/Pro* genotypes were combined and used as the reference. The *Leu/Leu* genotype also had a reduced risk (adjusted OR = 0.373, 95% CI 0.164–0.850) when the *Pro/Leu* genotype was used as the reference. Individuals who carried both putative risk genotypes (*Thr/Met* and *Met/Met* for T439M and *Pro/Leu* for P757L) showed an adjusted OR of 4.95 (95% CI 1.56–15.7) compared with those who carried both low risk genotypes. Analysis of microsatellite instability (MSI) revealed that tumors from individuals who carried both putative risk genotypes tended to have a higher frequency of MSI positives than those from patients who carried both low risk genotypes, although a significant correlation was not found between *EXO1* genotype and MSI status. This is the first report to provide evidence for an association of *EXO1* gene polymorphisms with colorectal cancer risk. The *EXO1* genotypes were not associated with any clinicopathological characteristics in colorectal cancer patients.

Introduction

The exonuclease 1 (*EXO1*) gene, located at chromosome 1q42–q43, contains one untranslated exon followed by 13 coding exons and encodes an 846 amino acid protein (1,2). The gene product is a member of the RAD2 nuclease family and functions in DNA replication, repair and recombination (3–5). Recently it has been shown that it plays a role in both 5′→3′ and 3′→5′ mispair-dependent excision repair *in vitro* (6). Wei *et al.* (7) found that mammalian EXO1 functions in mutation avoidance and is essential for male and female meiosis. They also reported that EXO1 inactivation predisposes mice to the development of tumors late in life and specifically increases the risk of lymphoma. EXO1 can interact physically with the DNA mismatch repair (MMR) proteins MSH2 and MLH1 in both yeast and human cells and with MSH3 in human cells (2,8–12). Tishkoff *et al.* (13) reported that the expression of a 3.0 kb *EXO1* transcript was detected in significantly higher level in testis, thymus, colon and placenta. Elevated expression of the *EXO1* gene in the colon is intriguing because hereditary non-polyposis colorectal cancer (HNPCC), which is characterized primarily by the development of early onset colorectal cancer and a number of other epithelial malignancies, can be attributed to inherited defects in MSH2/MLH1-dependent mismatch repair (14). Wu *et al.* (15) proposed that the *EXO1* gene could be associated with HNPCC predisposition because EXO1 protein strongly interacts with MSH2 protein. More recently, the *EXO1* gene showed negative association with HNPCC, although it is involved in DNA MMR (16,17). However, we cannot exclude a role of *EXO1* as a low penetrance cancer susceptibility or modifying gene, because the studies performed to date have only focused on suspected HNPCC cases.

Genetic polymorphisms of DNA repair genes have been reported to determine susceptibility to several cancers, including lung, esophageal, bladder, nasopharyngeal and non-melanoma skin cancers (18–22). Although polymorphisms in some genes have been studied in relation to colorectal cancer (23–25), no study has been conducted on the association between polymorphisms in the *EXO1* gene and colorectal cancer risk. Wu *et al.* (15) identified 12 missense single nucleotide polymorphisms (SNPs) in the *EXO1* gene in exon 6 (D249N), exon 7 (G274R and N279S), exon 9 (R354H), exon 10 (T439M, V458M and V460L), exon 11 (K589E and G670E), exon 12 (C723R and S725S) and exon 13 (P757L), while the function of these polymorphisms remains unclear.

Microsatellite instability (MSI) is caused by a failure of the MMR system. Such MMR defects may be caused either by a germline MMR gene mutation, affecting mainly *MLH1* or *MSH2*, or by somatic MMR gene inactivation, most commonly through epigenetic silencing via methylation of the *MLH1* promoter. *EXO1* genotype may be associated with cancer incidence and with MSI status because EXO1 protein strongly interacts with the MSH2 and MLH1 proteins. In our present

Abbreviations: CI, confidence interval; EXO1, exonuclease 1; HNPCC, hereditary non-polyposis colorectal cancer; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, MSI high; MSI-L, MSI low; MSS, microsatellite stable; OR, odds ratio; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; TNM, UICC Tumor–Node–Metastasis classification.

study we have investigated whether SNPs at T439M and P757L of the *EXO1* gene are associated with the risk of development, progression and metastasis of colorectal cancer. We also evaluated an association between *EXO1* SNPs and MSI status using five microsatellite markers (*BAT25*, *BAT26*, *D5S346*, *D2S123* and *D17S250*) that the National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome proposed for MSI assessment in HNPCC, collectively known as the NCI panel (26).

Materials and methods

Patients and control groups

We analyzed 102 Japanese patients chosen from those who were histologically diagnosed as having primary colorectal cancer and underwent surgical operation at Okayama University Hospital in 1994–2003, which gathers patients from not only Okayama but also various regions, mainly the Chugoku and Shikoku Districts (around Okayama), in Japan. We confirmed microscopically that all colorectal cancer patients have primary colorectal carcinomas. Clinical stage and pathological grade in all colorectal cancer patients were confirmed by operation and pathology. The clinicopathological staging and histological classification were according to the criteria of the UICC Tumor-Node-Metastasis Classification of Malignant Tumours (TNM), 6th edition, 2002, colon and rectum (ICD-O C 18-C 20). The 110 controls were randomly selected from a prospective cohort study among a general Japanese population. Written informed consent was obtained from all colorectal cancer patients and controls. The characteristics of the 102 colorectal cancer patients and 110 controls are shown in Table I. There were no significant differences in gender and age at recruitment between the colorectal cancer patients and controls ($P > 0.05$). Pack-year equivalents ([cigarettes/day ÷ 20] × [smoking years]) were used for smoking status (we could not obtain smoking status for 3 of 102 patients). Patients who smoked for ≥20 years were less frequent than controls compared with never smokers ($P = 0.001$).

DNA extraction

Genomic DNA of 102 patients was isolated from freshly frozen non-neoplastic colorectal mucosae using SDS/proteinase K treatment, phenol–chloroform extraction and ethanol precipitation. Tumor DNA was also isolated from freshly frozen neoplastic colorectal mucosae. Genomic DNA of 110 controls was extracted from peripheral lymphocytes.

Genotyping at T439M

Genotype at T439M was analyzed by PCR–restriction fragment length polymorphism (RFLP). In brief, PCR was performed in a 20 μl reaction mixture with 20 ng genomic DNA, 2.5 mM each dNTP, 1× PCR buffer, 8 pmol each primer (forward primer, 5′-TCT CTA AGT ACA GGT GAA ACA AAG; reverse primer, 5′-GAG CTA TTT TTC TTG GTC TTC TAC) and 0.5 U rTaq DNA polymerase (Takara, Kyoto, Japan). Amplification conditions were 3 min of initial denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, with a final extension of 7 min at 72°C. The 125 bp PCR products were digested overnight at 37°C with BsaAI (New England Biolabs, Beverly, MA). BsaAI digestion of the PCR product gives rise to 100 and 25 bp fragments for the *Thr* (ACG) allele and a single 125 bp fragment for the *Met* (ATG) allele. Digested fragments were subjected to electrophoresis on 3% agarose gel and visualized under UV light.

Table I. Characteristics of colorectal cancer patients and healthy controls

	Patients ($n = 102$)	Controls ($n = 110$)
Gender[a]		
Male	62 (60.8%)	79 (71.8%)
Female	40 (39.2%)	31 (28.2%)
Age (years ± SD)	64.5 ± 11.5	66.4 ± 8.28
Smoking status		
Never	44 (43.1%)	26 (23.6%)
Current	55 (53.9%)	84 (76.4%)
<20 pack-years	14 (25.5%)	14 (16.7%)
≥20 pack-years[b]	41 (74.5%)	70 (83.3%)
Unknown	3 (2.9%)	0 (0.0%)

[a]$P > 0.05$ for differences between patients and controls.
[b]$P = 0.001$ compared with controls.

Genotyping at P757L

Genotyping at P757L was also conducted by PCR–RFLP. PCR was performed with forward primer 5′-CAG AAT GGT CTT AAA ATG GGT GT and reverse primer 5′-TTC AGA ATA AGA AAC AAG GCA AC. Amplification conditions were 3 min initial denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C, with a final extension of 7 min at 72°C. The 255 bp PCR products were digested overnight at 37°C with MnlI (New England Biolabs). MnlI digestion gives 153 and 102 bp fragments for the *Pro* (CCT) allele and a single 255 bp fragment for the *Leu* (CTT) allele.

Analysis of MSI

Tumor and normal DNA was analyzed for MSI using the NCI panel of five microsatellite markers: *BAT25*, *BAT26*, *D5S346*, *D2S123* and *D17S250*. Oligonucleotide forward primers were fluorescently 5′-labeled. PCR was performed in a 20 μl reaction mixture with 20 ng genomic DNA, 2.5 mM each dNTP, 1× PCR buffer, 4 pmol each primer and 0.5 U rTaq DNA polymerase (Takara, Kyoto, Japan). Each primer sequence was as follows; *BAT25*, forward primer 5′-TCG CCT CCA AGA ATG TAA GT, reverse primer 5′-TCT GCA TTT TAA CTA TGG CTC; *BAT26*, forward primer 5′-TGA CTA CTT TTG ACT TCA GCC, reverse primer 5′-AAC CAT TCA ACA TTT TTA ACC C; *D5S346*, forward primer 5′-ACT CAC TCT AGT GAT AAA TCG G, reverse primer 5′-GTT TCC ATT GTA GCA TCT TGA C; *D2S123*, forward primer 5′-ACA TTG CTG GAA GTT CTG GC, reverse primer 5′-CCT TTC TGA CTT GGA TAC CA; *D17S250*, forward primer 5′-GCT GGC CAT ATA TAT ATT TAA ACC, reverse primer 5′-GGA AGA ATC AAA TAG ACA AT. Each amplification condition was as follows: *BAT25*, *BAT26* and *D2S123*, 3 min initial denaturation at 94°C, followed by 28 cycles of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C, with a final extension of 7 min at 72°C; *D5S346* and *D17S250*, 3 min initial denaturation at 94°C, followed by 28 cycles of 30 s at 94°C, 1 min at 58°C and 1 min at 72°C, with a final extension of 7 min at 72°C. Then MSI was analyzed in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using GeneScan Analysis 3.7 software (Applied Biosystems). MSI was indicated by the presence of novel peaks in the tumor tissue that were not seen in normal control tissue from the same patient or by a difference in microsatellite lengths between the two samples. Tumors exhibiting MSI at two or more markers were defined as MSI high (MSI-H). Tumors showing instability at only one marker were defined as MSI low (MSI-L). Tumors in which no markers exhibited MSI were considered to be microsatellite stable (MSS).

Statistical analysis

All statistical analyses in this study were performed using SPSS software version 12.0 (SPSS Inc., Japan). Odds ratio (OR) and 95% confidence interval (CI) were adjusted for age, gender and smoking status using an unconditional logistic regression model. Accordance with the Hardy–Weinberg equilibrium was examined for colorectal cancer patients and controls using the χ^2 test. In colorectal cancer patients the correlation between the genotype and clinicopathological characteristics was examined by χ^2 and Fisher's exact probability tests. Values of $P < 0.05$ were considered significant.

Results

Risk of colorectal cancer by genotype at T439M and P757L in the EXO1 gene

Figure 1 shows the representative PCR–RFLP patterns of the T439M and P757L genotypes in the *EXO1* gene. Digestion of the PCR product (125 bp) with BsaAI at T439M resulted in a single fragment of 125 bp for the *Met* allele and two fragments of 100 and 25 bp for the *Thr* allele. Digestion of the PCR product (255 bp) with MnlI at P757L resulted in a single fragment of 255 bp for the *Leu* allele and two fragments of 153 and 102 bp for the *Pro* allele.

EXO1 genotypes in colorectal cancer patients and healthy controls are shown in Table II. For T439M, the *Thr/Thr*, *Thr/Met* and *Met/Met* genotypes were found in 63 (63.6%), 30 (30.3%) and 6 (6.1%) of 102 colorectal cancer patients (the genotypes of three patients could not be identified because of unsuccessful PCR) and in 88 (80.0%), 22 (20.0%) and 0 (0%) of 110 controls, neither of which deviated from those expected

from the Hardy–Weinberg equilibrium ($P = 0.649$ and 0.507, respectively). *Met* allele frequencies were 21.2 and 10.0% in patients and controls, respectively ($P = 0.00148$). For P757L, the genotypes *Pro/Pro*, *Pro/Leu* and *Leu/Leu* were found in 35 (34.3%), 53 (52.0%) and 14 (13.7%) of 102 colorectal cancer patients and in 36 (32.7%), 47 (42.7%) and 27 (24.5%) of 110 controls. The distributions of genotypes at P757L in patients and controls also fitted the Hardy–Weinberg equilibrium ($P = 0.690$ and 0.342, respectively). *Leu* allele frequencies did not show a significant difference ($P = 0.197$) between patients and controls (39.7 and 45.9%, respectively). For T439M, the *Thr/Met* genotype (OR = 2.03, 95% CI 1.04–3.98) and *Thr/Met* and *Met/Met* genotypes combined (OR = 2.37, 95% CI 1.23–4.56) demonstrated a significant OR after adjusting for age, gender and smoking status when the *Thr/Thr* genotype (wild-type) was defined as the reference. An OR for the *Met/Met* genotype could not be calculated. For P757L, none of the *Pro/Leu* genotypes (OR = 1.17, 95% CI 0.610–2.24), *Leu/Leu* genotypes (OR = 0.436, 95% CI 0.183–1.04) or *Pro/Leu* and *Leu/Leu* genotypes combined (OR = 0.893, 95% CI 0.485–1.65) demonstrated a significant OR when the *Pro/Pro* genotype (wild-type) was defined as the reference. However, when the *Pro/Leu* and *Pro/Pro* genotypes were combined and used as the reference, the patients with the *Leu/Leu* genotype showed a reduced risk of colorectal cancer (adjusted OR = 0.398, 95% CI 0.183–0.866). The *Leu/Leu* genotype also had a reduced risk (adjusted OR = 0.373, 95% CI 0.164–0.850) when the *Pro/Leu* genotype was defined as the reference. Therefore, the relative risk of developing colorectal cancer did not show an allele-dose relationship for P757L.

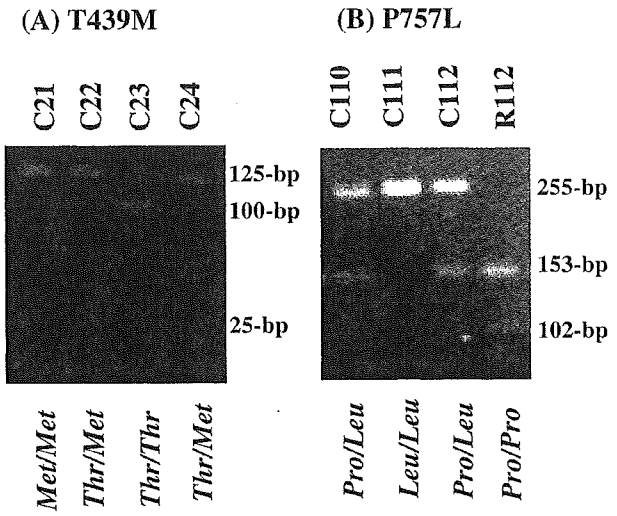

Fig. 1. Polymorphisms of *EXO1* exon 10 (T439M) and exon 13 (P757L). (**A**) T439M locus. (**B**) P757L locus. Numbers above the panel are case numbers. Genotypes are shown below each panel.

Table II. *EXO1* genotypes in colorectal cancer patients and healthy controls

Amino acid	Genotype	Patients[a] ($n = 102$)	Controls[a] ($n = 110$)	P[b]	OR (95% CI)	
					Crude	Adjusted[c]
T439M	*Thr/Thr*	63 (63.6%)	88 (80.0%)		1 (Reference)	1 (Reference)
	Thr/Met	30 (30.3%)	22 (20.0%)	0.038	1.91 (1.01–3.61)	2.03 (1.04–3.98)
	Met/Met	6 (6.1%)	0 (0%)		Not calculated	Not calculated
	Thr/Met and *Met/Met*	36 (36.4%)	22 (20.0%)	0.010	2.29 (1.23–4.25)	2.37 (1.23–4.56)
	Unknown	3	0			
	Allele frequencies					
	Thr	156 (78.8%)	198 (90.0%)	0.00148		
	Met	42 (21.2%)	22 (10.0%)			
P757L	*Pro/Leu* and *Pro/Pro*	88 (86.3%)	83 (75.5%)		1 (Reference)	1 (Reference)
	Leu/Leu	14 (13.7%)	27 (24.5%)	0.020	0.489 (0.240–0.996)	0.398 (0.183–0.866)
	Pro/Leu	53 (52.0%)	47 (42.7%)		1 (Reference)	1 (Reference)
	Leu/Leu	14 (13.7%)	27 (24.5%)	0.019	0.460 (0.216–0.979)	0.373 (0.164–0.850)
	Unknown	0	0			
	Allele frequencies					
	Pro	123 (60.3%)	119 (54.1%)	0.197		
	Leu	81 (39.7%)	101 (45.9%)			

[a]The observed genotype distributions of patients and controls were in agreement with the Hardy–Weinberg equilibrium.
[b]*P* values were for the difference in genotype frequencies between patients and controls.
[c]ORs were adjusted for age, gender and smoking status. Three patients whose smoking status was not known were omitted when adjusted ORs were calculated.

Table III. Joint effect of T439M and P757L and colorectal cancer risk

T439M	P757L	Patients	Controls	P[a]	OR (95% CI)		MSI (+)/cases
					Crude	Adjusted[b]	
Thr/Thr	*Leu/Leu*	12	24		1 (Reference)	1 (Reference)	1/12 (8.3%)
Thr/Met and *Met/Met*	*Leu/Leu*	2	3	0.277	1.33 (0.196–9.08)	0.168 (0.007–4.19)	0/2 (0.0%)
Thr/Thr	*Pro/Leu*	32	37	0.161	1.73 (0.747–4.00)	1.88 (0.778–4.55)	5/32 (15.6%)
Thr/Met and *Met/Met*	*Pro/Leu*	21	10	0.007	4.20 (1.51–11.7)	4.95 (1.56–15.7)	4/21 (19.0%)

[a]*P* values were for the difference in genotype frequencies between patients and controls.
[b]ORs were adjusted for age, gender and smoking status. Three patients whose smoking status was not known were omitted when adjusted ORs were calculated.

Table IV. Association between *EXO1* genotype and clinocopathological characteristics

Characteristic	Factor[a] (+) (n = 21)	Factor[a] (−) (n = 78)	P value
Histology			0.912
Well	4	13	
Moderate	15	59	
Poor	1	2	
Other	1	4	
T			0.272
Tis, T1, T2	1	12	
T3, T4	20	66	
N			0.795
N0	10	35	
N1	6	29	
N2	5	12	
Unknown	0	2	
M			0.180
M0	13	54	
M1	8	24	
TNM stage			0.260
0, I, II	9	32	
III, IV	12	46	

T, primary tumor; N, lymph node metastasis; M, distant metastasis.
[a]Factor = *Thr/Met* and *Met/Met* (T439M) and *Pro/Leu* (P757L).

We also analyzed the joint effect of polymorphisms at T439M and P757L (Table III). There was no linkage disequilibrium between T439M and P757L polymorphisms. We considered carriers of the genotypes found to be at the lowest risk of disease (*Thr/Thr* for T439M and *Leu/Leu* for P757L) as the reference for this analysis. As shown in Table III, individuals who carried only one of the two polymorphisms associated with colorectal cancer risk (i.e. *Thr/Met* and *Met/Met* for T439M or *Pro/Leu* for P757L) were not at significant risk for development of colorectal cancer, whereas individuals who carried both putative risk genotypes showed an adjusted OR of 4.95 (95% CI 1.56–15.7). Adjustment for age, gender and smoking status was not significantly effective in all these estimates.

Association between EXO1 genotypes and MSI status

Among tumors from the 102 colorectal cancer patients analyzed, 6 (5.9%) were MSI-H, 11 (10.8%) were MSI-L and the remaining 85 were MSS. We evaluated association between *EXO1* SNPs and MSI status (Table III). As shown in Table III, individuals who carried both putative risk genotypes tended to have a higher frequency of MSI-positive (MSI-H and MSI-L) tumors than those who carried both low risk genotypes, although no significant correlation was found between *EXO1* genotype and MSI status (P = 0.422).

Association between EXO1 genotype and clinicopathological characteristics

We analyzed the association between the *EXO1* genotype and clinicopathological characteristics in colorectal cancer patients (Table IV). We compared the patients with *Thr/Met* and *Met/Met* for T439M and *Pro/Leu* for P757L with the other patients. However, there were no significant differences between these two groups.

Discussion

The *EXO1* gene has been investigated in association with HNPCC (14–17,27). Wu *et al.* (15) found several *EXO1* mutants in HNPCC patients who were shown to be negative for germline mutations in the *MLH1*, *MSH2* and *MSH6* genes. Among a total of 30 alterations in the *EXO1* exons, 14 were unique to HNPCC patients. However, the other 16 were thought to be polymorphisms because they occurred at similar frequencies in both patients and controls. Thompson *et al.* (17) reported that the *EXO1* gene does not appear to be associated with HNPCC, but did not refer to these polymorphisms. Therefore, we investigated T439M and P757L among these polymorphisms.

In the present study we have examined whether SNPs at T439M and P757L in the *EXO1* gene are associated with the risk for development, progression and metastasis of colorectal cancer. We found significant differences in genotype distribution of the *EXO1* gene between colorectal cancer patients and controls. We recognize that this population of colorectal cancer patients does not seriously deviate from the general Japanese population because Japan is an almost racially homogeneous nation and Okayama has had population influxes from other areas such as Tokyo and Osaka (urban cities representing Japan) and the Chugoku and Shikoku Districts (around Okayama). For T439M of *EXO1*, an OR of 2.03 was observed among individuals with the *Thr/Met* genotype and an OR of 2.37 was observed among individuals with the *Thr/Met* and *Met/Met* genotypes combined. For P757L of *EXO1*, patients with the *Leu/Leu* genotype had a reduced risk of colorectal cancer when the *Pro/Leu* and *Pro/Pro* genotypes combined or *Pro/Leu* genotype was defined as the reference (OR = 0.398 and 0.373, respectively). Interestingly, individuals with putative risk genotypes for both T439M (*Thr/Met* and *Met/Met*) and P757L (*Pro/Leu*) were at a 5-fold higher risk of colorectal cancer. As for an association between *EXO1* SNPs and MSI, individuals who carried both putative risk genotypes tended to have higher frequency of MSI-positive tumors than those who carried both low risk genotypes, although no significant correlation was found. This fact may support the proposal that high risk genotypes of the *EXO1* gene cause colorectal cancer through incomplete MMR. Our findings are the first to suggest an association between polymorphisms in the *EXO1* gene and risk of colorectal cancer. No other reports have found an association between other cancers and *EXO1* SNPs. Although intriguing, however, these joint effects should be interpreted with caution, given the modest size of the present study to evaluate joint effects. The *EXO1* genotype was not significantly associated with clinicopathological characteristics. Therefore, the *EXO1* gene does not appear to be associated with progression or metastasis of colorectal cancer.

As functional motifs of EXO1 protein, an EXO1 nuclease domain was identified in the N-terminal 391 amino acid residues (27) and a protein–protein interaction domain with MMR proteins was identified on the basis of sequence alignment and functional domain analysis (2,9–12,28) (Figure 2). T439M is located in the MLH1 interaction domain and P757L is located within the region required for interaction with MSH2. It is known that mutations of MSH2 and MLH1 are associated with typical HNPCC, resulting in a total loss of MMR function (29). In the case of T439M, a polar amino acid (Thr) is replaced by a non-polar amino acid (Met). Considering that the genotype *Met/Met* were not found in the control subjects in T439M, it is possible that substitution of *Thr* by *Met* may strongly affect interaction with other MMR proteins. As for P757L, this substitution may influence protein–protein interaction because

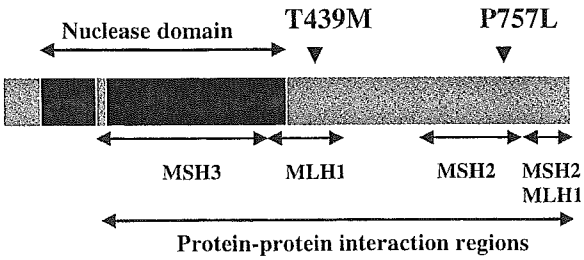

Fig. 2. Functional motifs of *EXO1* and the locations of polymorphisms. Nuclease domains and protein–protein interaction regions are shown by arrows. SNPs examined in the present study (T439M and P757L) are indicated above the motif by inverted triangles.

prolines tend to destabilize (kink) α-helices due to the lack of a backbone hydrogen bond and steric constraints (30–32). It is notable that the MMR proteins MSH2, MLH1 and MSH6 are constituents of BASC, the BRCA1-associated genome surveillance complex (33). If EXO1 is also involved in this complex through MMR proteins, the protein may play a more general role(s) in the protection of DNA in addition to MMR.

In conclusion, our data provide evidence for an association between the *EXO1* gene polymorphisms at T439M and P757L and the risk of development of colorectal cancer. It is possible that these polymorphisms may influence susceptibility to colorectal cancer through incomplete DNA repair. The association was more prominent for individuals who carried both of the two putative risk genotypes. Further study with sufficiently larger populations and functional analysis of these polymorphisms will be needed to clarify the unsolved issues.

Acknowledgement

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References

1. Wilson,D.M.,III, Carney,J.P., Coleman,M.A., Adamson,A.W., Christensen,M. and Lamerdin,J.E. (1998) Hex1: a new human Rad2 nuclease family member with homology to yeast exonuclease 1. *Nucleic Acids Res.*, **26**, 3762–3768.
2. Schmutte,C., Marinescu,R.C., Sadoff,M.M., Guerrette,S., Overhauser,J. and Fishel,R. (1998) Human exonuclease I interacts with the mismatch repair protein hMSH2. *Cancer Res.*, **58**, 4537–4542.
3. Lee,B.I., Nguyen,L.H., Barsky,D., Fernandes,M. and Wilson,D.M.,III (2002) Molecular interactions of human Exo1 with DNA. *Nucleic Acids Res.*, **30**, 942–949.
4. Qiu,J., Qian,Y., Chen,V., Guan,M.X. and Shen,B. (1999) Human exonuclease 1 functionally complements its yeast homologues in DNA recombination, RNA primer removal and mutation avoidance. *J. Biol. Chem.*, **274**, 17893–17900.
5. Szankasi,P. and Smith,G.R. (1995) A role for exonuclease I from *S. pombe* in mutation avoidance and mismatch correction. *Science*, **267**, 1166–1169.
6. Genschel,J., Bazemore,L.R. and Modrich,P. (2002) Human exonuclease I is required for 5′ and 3′ mismatch repair. *J. Biol. Chem.*, **277**, 13302–13311.
7. Wei,K., Clark,A.B., Wong,E. *et al.* (2003) Inactivation of exonuclease 1 in mice results in DNA mismatch repair defects, increased cancer susceptibility and male and female sterility. *Genes Dev.*, **17**, 603–614.
8. Tishkoff,D.X., Boerger,A.L., Bertrand,P., Filosi,N., Gaida,G.M., Kane,M.F. and Kolodner,R.D. (1997) Identification and characterization of *Saccharomyces cerevisiae EXO1*, a gene encoding an exonuclease that interacts with MSH2. *Proc. Natl Acad. Sci. USA*, **94**, 7487–7492.
9. Rasmussen,L.J., Rasmussen,M., Lee,B.I., Rasmussen,A.K., Wilson,D.M.,III, Nielsen,F.C. and Bisgaard,H.C. (2000) Identification of factors interacting with hMSH2 in the fetal liver utilizing the yeast two-hybrid system. *In vivo* interaction through the C-terminal domains of hEXO1 and hMSH2 and comparative expression analysis. *Mutat. Res.*, **460**, 41–52.
10. Jager,A.C., Rasmussen,M., Bisgaard,H.C., Singh,K.K., Nielsen,F.C. and Rasmussen,L.J. (2001) HNPCC mutations in the human DNA mismatch repair gene *hMLH1* influence assembly of hMutLα and hMLH1–hEXO1 complexes. *Oncogene*, **20**, 3590–3595.
11. Schmutte,C., Sadoff,M.M., Shim,K.S., Acharya,S. and Fishel,R. (2001) The interaction of DNA mismatch repair proteins with human exonuclease I. *J. Biol. Chem.*, **276**, 33011–33018.
12. Tran,P.T., Simon,J.A. and Liskay,R.M. (2001) Interactions of Exo1p with components of MutLα in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **98**, 9760–9765.
13. Tishkoff,D.X., Amin,N.S., Viars,C.S., Arden,K.C. and Kolodner,R.D. (1998) Identification of a human gene encoding a homologue of *Saccharomyces cerevisiae* EXO1, an exonuclease implicated in mismatch repair and recombination. *Cancer Res.*, **58**, 5027–5031.
14. Peltomaki,P. and Vasen,H.F.A. (1997) Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. *Gastroenterology*, **113**, 1146–1158.
15. Wu,Y., Berends,M.J.W., Post,J.G. *et al.* (2001) Germline mutations of *EXO1* gene in patients with hereditary nonpolyposis colorectal cancer (HNPCC) and atypical HNPCC forms. *Gastroenterology*, **120**, 1580–1587.
16. Jagmohan-Changur,S., Poikonen,T., Vilkki,S. *et al.* (2003) *EXO1* variants occur commonly in normal population: evidence against a role in hereditary nonpolyposis colorectal cancer. *Cancer Res.*, **63**, 154–158.
17. Thompson,E., Meldrum,C.J., Crooks,R., McPhillips,M., Thomas,L., Spigelman,A.D. and Scott,R.J. (2004) Hereditary non-polyposis colorectal cancer and the role of *hPMS2* and *hEXO1* mutations. *Clin. Genet.*, **65**, 215–225.
18. Ratnasinghe,D., Yao,S.X., Tangrea,J.A. *et al.* (2001) Polymorphisms of the DNA repair gene *XRCC1* and lung cancer risk. *Cancer Epidemiol. Biomarkers Prev.*, **10**, 119–123.
19. Xing,D.Y., Tan,W., Song,N. and Lin,D.X. (2001) Ser326Cys polymorphism in *hOGG1* gene and risk of esophageal cancer in a Chinese population. *Int. J. Cancer*, **95**, 140–143.
20. Stern,M.C., Umbach,D.M., van Gils,C.H., Lunn,R.M. and Taylor,J.A. (2001) DNA repair gene *XRCC1* polymorphisms, smoking and bladder cancer risk. *Cancer Epidemiol. Biomarkers Prev.*, **10**, 125–131.
21. Cho,E.Y., Hildesheim,A., Chen,C.J. *et al.* (2003) Nasopharyngeal carcinoma and genetic polymorphisms of DNA repair enzymes *XRCC1* and *hOGG1*. *Cancer Epidemiol. Biomarkers Prev.*, **12**, 1100–1104.
22. Cleaver,J.E. and Crowley,E. (2002) UV damage, DNA repair and skin carcinogenesis. *Front. Biosci.*, **7**, D1024–D1043.
23. Orimo,H., Nakajima,E., Yamamoto,M., Ikejima,M., Emi,M. and Shimada,T. (2000) Association between single nucleotide polymorphisms in the hMSH3 gene and sporadic colon cancer with microsatellite instability. *J. Hum. Genet.*, **45**, 228–230.
24. Toffoli,G., Gafa,R., Russo,A., Lanza,G., Dolcetti,R., Sartor,F., Libra,M., Viel,A. and Boiocchi,M. (2003) Methylenetetrahydrofolate reductase *677 C→T* polymorphism and risk of proximal colon cancer in North Italy. *Clin. Cancer Res.*, **9**, 743–748.
25. Adleff,V., Hitre,E., Koves,I., Orosz,Z., Hajnal,A. and Kralovanszky,J. (2004) Heterozygote deficiency in thymidylate synthase enhancer region polymorphism genotype distribution in Hungarian colorectal cancer patients. *Int. J. Cancer*, **108**, 852–856.
26. Boland,C.R., Thibodeau,S.N., Hamilton,S.R. *et al.* (1998) A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res.*, **58**, 5248–5257.
27. Sun,X., Zheng,L. and Shen,B. (2002) Functional alterations of human exonuclease 1 mutants identified in atypical hereditary nonpolyposis colorectal cancer syndrome. *Cancer Res.*, **62**, 6026–6030.
28. Lee,B.I. and Wilson,D.M.,III (1999) The RAD2 domain of human exonuclease 1 exhibits 5′ to 3′ exonuclease and flap structure-specific endonuclease activities. *J. Biol. Chem.*, **274**, 37763–37769.
29. Nystrom-Lahti,M., Perrera,C., Raschle,M. *et al.* (2002) Functional analysis of *MLH1* mutations linked to hereditary nonpolyposis colon cancer. *Genes Chromosomes Cancer*, **33**, 160–167.

30. MacArthur,M.W. and Thornton,J.M. (1991) Influence of proline residues on protein conformation. *J. Mol. Biol.*, **218**, 397–412.
31. Woolfson,D.N. and Williams,D.H. (1990) The influence of proline residues on α-helical structure. *FEBS Lett.*, **277**, 185–188.
32. Chang,D.K., Cheng,S.F., Trivedi,V.D. and Lin,K.L. (1999) Proline affects oligomerization of a coiled coil by inducing a kink in a long helix. *J. Struct. Biol.*, **128**, 270–279.
33. Wang,Y., Cortez,D., Yazdi,P., Neff,N., Elledge,S.J. and Qin,J. (2000) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev.*, **14**, 927–939.

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Relationship between *CDX2* gene methylation and dietary factors in gastric cancer patients

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Epigenetic gene silencing through DNA methylation is one of the important steps in the mechanism underlying tumorigenesis, including in the stomach. Past lifestyle factors of cancer patients, such as intake of vegetables, are very important in affecting gastric carcinogenesis. However, the relationship between DNA methylation and past dietary habits in cancer patients remains largely unknown. The CDX2 homeobox transcription factor plays a key role in intestinal development, but CDX2 is also expressed in most of the intestinal metaplasia and part of the carcinomas of the stomach. We analyzed the methylation status of the *CDX2* 5′ CpG island in gastric cancer cell lines by methylation-specific PCR (MSP), and then *CDX2* mRNA was found to be activated after 5-aza-2′-deoxycytidine treatment of the methylation-positive cells. We further examined the methylation status of *CDX2* in primary gastric carcinomas by MSP and compared it with the past lifestyle of the patients, including dietary habits. Methylation of *CDX2* was found in 20 (34.5%) of the 58 male patients and one (6.7%) of the 15 female patients. Since the methylation frequency was low in the female patients, the analysis was performed only on the male cases. *CDX2* methylation was correlated with the decreased intake of green tea and cruciferous vegetables, and also with full or overeating habits. These findings are consistent with epidemiological observations on gastric cancer. We also analyzed the methylation status of *p16/INK4a* and *hMLH1*, but their frequencies were not associated with dietary factors or other lifestyle factors. Thus, diet could be an important factor determining the methylation status of genes such as *CDX2* and the resultant aberrant expression of genes involved in carcinogenesis.

Abbreviations: MSP, methylation-specific PCR; RT–PCR, reverse transcription–polymerase chain reaction.

Introduction

Gastric cancer is the second most frequent cause of death from cancer in both sexes in the world (1). The precise mechanism underlying gastric carcinogenesis is not fully understood yet. However, several environmental factors, such as *Helicobacter pylori* infection, excessive intake of salt and low intake of vegetables and fruits, have been linked with gastric carcinogenesis (2–4).

Since alterations of gene functions in cancer cannot be explained by only the mutational rate, there should be a non-structural mechanism. Some cancers show hypermethylation of CpG islands in gene promoters, resulting in loss of gene function. Patterns of DNA methylation can be inherited when cells divide. This epigenetic process, as an alternative to mutations, inhibits tumor suppressor gene function (5,6).

Dietary factors are important determinants of cancer risk, including that of gastric cancer (2). Certain dietary factors and other lifestyle factors are associated with variations in DNA methylation, and these variations might underlie gastric carcinogenesis. For example, the incidence of hypermethylation of *p16/INK4a* (hereafter *p16*) in lung cancer is significantly higher in cigarette smokers than in those who have never smoked (7). Consequently, *p16* expression is silenced, resulting in the progression of carcinogenesis. The prevalence of promoter hypermethylation of six genes, such as *APC*, $p14^{ARF}$, *p16* and *hMLH1*, was higher in colorectal cancers derived from patients with a low folate/high alcohol intake than in ones with a high folate/low alcohol intake, but the differences were not statistically significant (8).

Several genes are aberrantly methylated in human primary cancers, including that of the stomach (9). *p16* and *hMLH1* have been extensively examined in gastric cancer (10–12). *p16* inhibits G_1 cyclin-dependent kinases and hence induces cell-cycle arrest, and has a tumor-suppressor gene function (13,14). Epigenetic inactivation of *hMLH1* due to promoter methylation is strongly associated with microsatellite instability and seems to be a significant event in the development of gastric cancer (10–12).

Human *CDX2* is a member of the *caudal*-related homeobox gene family (15,16). The expression of the rodent *Cdx2* homeobox gene is intestine-specific, and occurs from the early embryo to the adult stage (17), and thus it is likely that Cdx2 plays roles in both the establishment and maintenance of the intestinal epithelial phenotype. On the other hand, the ectopic expression of CDX2 has been related to intestinal metaplasia formation in the stomach. First, the CDX2 protein is not expressed in the normal stomach, but is highly expressed in nearly all of the intestinal metaplasia of the stomach (18–20). Secondly, when Cdx2 expression was directed to the gastric mucosae in transgenic mice using *cis*-regulatory elements of gastric mucosa-specific genes, ectopic Cdx2 expression induced gastric intestinal metaplasia in the mice (21,22). Gastric cancer is histologically classified into two

main types, intestinal and diffuse (23). Intestinal type gastric cancers are thought to develop from intestinal metaplasia, while diffuse type ones may mainly develop from the normal mucosae (16). CDX2 expression is stronger in intestinal type than in diffuse type gastric cancers (18).

We analyzed here the methylation status of *p16* and *hMLH1* in gastric cancers. Furthermore, since CDX2 expression is lower in gastric cancers than in intestinal metaplasia (18), we also analyzed methylation of the *CDX2* 5′ CpG island, and compared them with the dietary habits, specifically those which have previously been reported as risk or preventive factors of gastric cancer in epidemiological observation.

Materials and methods

Cell culture and drug treatment

Human gastric cancer cell lines GT3TKB and MKN74 were grown in Dulbecco's modified minimum essential medium and RPMI1640 medium, respectively, supplemented with 10% fetal bovine serum. A human colon cancer cell line, RKO, was cultured in Eagle's minimum essential medium containing 10% fetal bovine serum. For demethylation studies, cells were treated daily with 5 μM 5-aza-2′-deoxycytidine (Sigma, St Louis, MO) for 3 days.

RNA extraction and reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA). To synthesize the RT–PCR template, we used 2 μg of total RNA and reverse-transcribed it using a Superscript kit (Invitrogen). The primers used for *CDX2* amplification were described previously (24). We amplified with multiple cycle numbers (28–35 cycles) to obtain semi-quantitative differences in the expression level. As an internal control for RT–PCR analysis, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcripts were amplified for 19 cycles from the same cDNA samples (24).

Methylation analyses by the methylation-specific PCR (MSP) procedure and bisulfite DNA sequencing

We extracted genomic DNA from cultured cells or paraffin-embedded tissues by the phenol–chloroform method, and then carried out bisulfite modification and the MSP procedure as described previously (25). The primer sequences of *CDX2* for the unmethylated reaction were 5′-GAAGTTGTTGGTTTGGGGT-TTTGTAT-3′ (sense) and 5′-CCCACAATACTCCACTAACTCCTCACA-3′ (antisense), and for the methylated reaction 5′-CGTCGGTTTGGGGTTTC-GTAC-3′ (sense) and 5′-GATACTCCGCTAACTCCTCGCG-3′ (antisense), according to the GenBank sequence (AL591024). The PCR reaction for *CDX2* was performed for 35 cycles in a 25-μl mixture containing bisulfite-modified DNA (~50 ng), 2.5 μl of 10× PCR buffer, 1.25 μl of 25 mM dNTP, 10 pmol of each primer and 1 U of JumpStart Red *Taq* polymerase (Sigma). Each PCR cycle consisted of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by final extension at 72°C for 5 min. The MSP primers and conditions used for *p16* and *hMLH1* were described previously (25,26). The PCR products were electrophoresed in 2.5% agarose gels. All the MSP procedures were repeated more than twice.

Bisulfite DNA sequencing of the *CDX2* 5′ CpG island was performed as described previously (27). The primer sequences for amplification were 5′-GAAGTTTTTAATTATTGGTGTTTGTGTT-3′ (sense) and 5′-AAACCT-CACCATACTACCTAAAAACC-3′ (antisense).

Immunohistochemistry

Immunohistochemical analysis of the CDX2 protein was performed as described previously (18). Monoclonal antibody to the CDX2 protein (Bio Genex, San Ramon, CA) was diluted at 1:100.

Study population

Cancer tissue specimens were collected from 73 consecutive patients with primary gastric carcinoma in an affiliated hospital of the Tokyo Medical and Dental University, and Saitama Cancer Center Hospital during 2000–2002. Informed consent was obtained from all patients, and the study was approved by the appropriate institutional review committee. A self-administered questionnaire was used in this study to assess their lifestyle before cancer onset, covering disease history, familial history of cancer, medication, cigarette smoking, alcohol consumption, physical activity, intake frequencies of selected food groups and food items, daily consumption of tea (green tea, oolong tea and black tea), regularity of sleep and meals, eating quantity, bowel motion, height and body weight. Food groups were beef, pork, chicken, ham/sausage/bacon, grilled meat, all meat, grilled fish, salted/dried/other processed fish products, pickled vegetables, green leaf vegetables, yellow color vegetables, cruciferous vegetables, all vegetables, fruits and probiotics-fermented milk. Intake frequencies of these food groups were categorized into not eating, 1–2 times/month, 1–2 times/week, 3–4 times/week, almost every day and almost every meal. Eating quantity was categorized as full or over-eating, eating in moderation and consciously under-eating. Most lifestyle factors in this questionnaire were selected from those which have previously been reported as risk or preventive factors of gastric and colon cancers in epidemiological observation.

Tumors were reviewed by a pathologist and microdissected prior to DNA extraction. Histological classification was performed according to the general rules established by the Japanese Gastric Cancer Association (28) and the Laurén's classification (23).

Statistical analysis

The promoter methylation status of specific genes, clinicopathological parameters and lifestyle variables in the patients were computed. Differences in frequency by methylation status were tested using Fisher's exact test, and differences in mean values were tested using *t* test. Association between the methylation status and dietary variables was also analyzed by non-parametric test (Mann–Whitney *U* test). We further studied association by categorical regression analysis with optimal scaling using alternating least squares (29). In this analysis, intake frequencies of food groups and eating quantity were dichotomous: ⩽twice/week versus ⩾3 times/week for vegetable groups, ⩽twice/month versus ⩾once/week for meat and fish groups, ⩽4 times/week versus ⩾5 times/week for fruits and ⩽6 cups/day versus ⩾7 cups/day for green tea and full/over versus moderate/light for eating quantity. The statistical software used was SPSS software (version 11.0).

Results

Epigenetic silencing of CDX2 in a gastric cancer cell line

With a semi-quantitative RT–PCR assay to measure the *CDX2* mRNA level, GT3TKB did not express any *CDX2* mRNA, whereas MKN74 expressed it abundantly (Figure 1A). We then used the demethylating agent 5-aza-2′-deoxycytidine to study the epigenetic status of *CDX2* in these cell lines. *CDX2* was re-expressed in GT3TKB cells with this treatment (Figure 1A).

We have identified CpG islands associated with the *CDX2* 5′ region (Figure 1B) and thus the methylation status of *CDX2* was studied by MSP and sodium bisulfite DNA sequencing. GT3TKB cells only exhibit a methylation signal, but MKN74 cells do not exhibit any (Figure 1C), consistent with the *CDX2* expression levels in these cells. The sodium bisulfite DNA sequencing of *CDX2* in these cell lines confirmed the methylation status, i.e. *CDX2* expression-negative GT3TKB cells show densely methylated clones, while expression-positive MKN74 cells and normal gastric mucosa have unmethylated clones (Figure 1D).

Methylation status of CDX2, p16 and hMLH1 in primary gastric carcinomas

Methylation of *CDX2*, as determined by MSP, was frequent in primary gastric carcinomas, i.e. there were 21 positive cases among 73 (28.8%) total cases. Representative examples of gel analysis of MSP are shown in Figure 2. CDX2 protein expression was also analyzed in gastric carcinomas by immunohistochemistry (Figure 3 and Table I). As shown in Table I, negative or partial CDX2 expression was more frequently observed in diffuse type (28/32, 87.5%) than in intestinal type (20/35, 57.1%) gastric cancers ($P < 0.01$), which is consistent with the previous data (18). The *CDX2* methylation frequencies in gastric cancers with negative or partial expression were similar between the intestinal (40%) and diffuse types (35.7%). On the other hand, the overall methylation frequency in cancers with negative or partial CDX2 expression

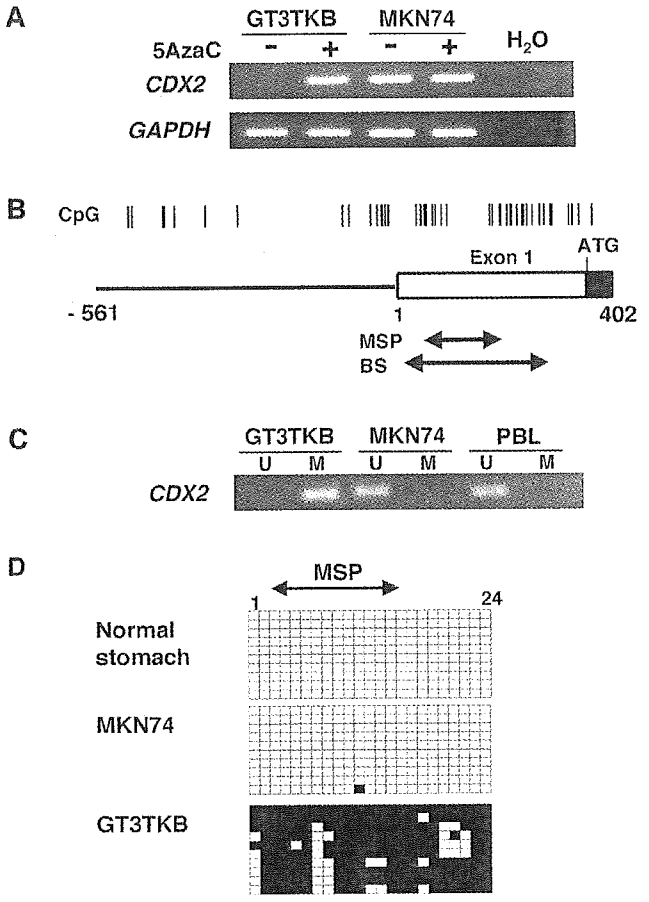

Fig. 1. *CDX2* expression and methylation status in gastric cancer cell lines. (**A**) The *CDX2* expression level was examined by RT–PCR in two cell lines with (lanes +) or without (lanes −) treatment with 5-aza-2′-deoxycytidine. *GAPDH* expression was used as an internal loading control for the RT–PCR and H_2O (no cDNA added). (**B**) Schematic representation of the *CDX2* gene promoter region. A box indicates exon 1, including coding (black) and non-coding (white) regions. Vertical bars show CpG sites. Arrows indicate the regions analyzed by MSP and bisulfite sequencing (BS). (**C**) MSP analysis of the CpG island of *CDX2* in two gastric cancer cell lines and peripheral blood lymphocytes (lane PBL). PCR products recognizing unmethylated (lanes U) and methylated (lanes M) CpG sites were analyzed in 2.5% agarose gels and stained with ethidium bromide. (**D**) Sodium bisulfite DNA sequencing of the *CDX2* CpG island in gastric cancer cell lines and normal stomach mucosa. Each horizontal row of squares represents analysis, in a single clone of bisulfite-treated DNA, of 24 CpG sites contained in the region shown. Solid and open squares represent methylated and unmethylated CpG sites, respectively.

(18/48, 37.5%) was significantly higher than that with positive expression (2/19, 10.5%) ($P = 0.03$), suggesting that methylation of the *CDX2* gene is important for gene silencing in primary gastric cancers. When we analyzed the *CDX2* methylation status by MSP in five normal and five intestinal metaplastic tissues of the stomach from independent patients, we found no methylation in any samples (Table I). Since the normal gastric mucosae did not reveal *CDX2* gene methylation, strong CDX2 expression in intestinal metaplasia compared with the normal mucosae is not attributable to demethylation but possibly to aberrant transcriptional activation.

The methylation status of the *p16* and *hMLH1* genes was also examined by MSP (Figure 2). Nine of 66 (13.6%) and seven of 67 (10.4%) gastric cancers exhibited MSP methylation signals, respectively.

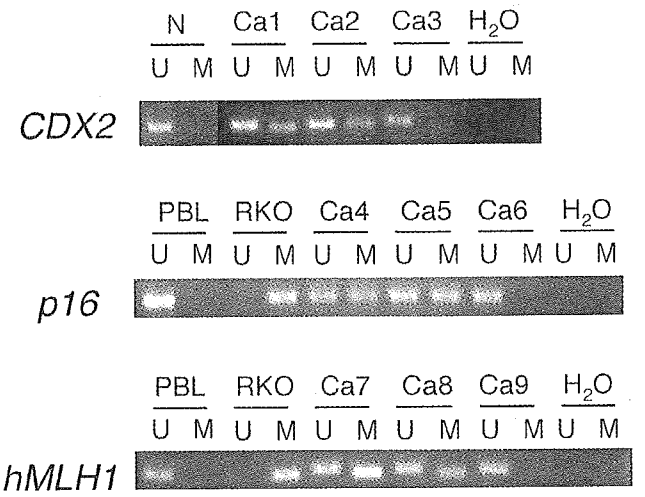

Fig. 2. Representative examples of MSP analyses of *CDX2*, *p16* and *hMLH1* in primary gastric cancer tissues (Ca), normal gastric mucosae (N), and peripheral blood lymphocytes (PBL). A colorectal cancer cell line, RKO, was used as a methylation-positive control. Lanes: U, unmethylated alleles; M, methylated alleles; H_2O, no DNA added.

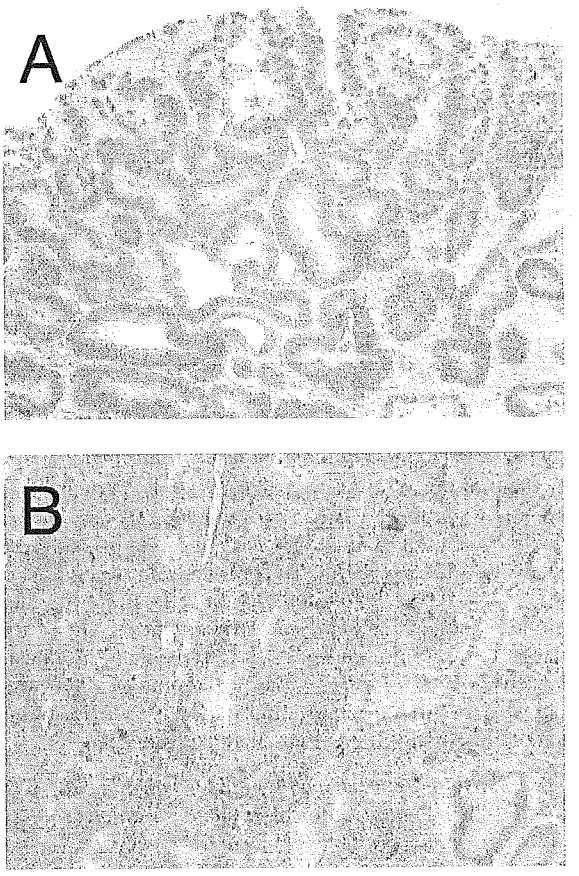

Fig. 3. Immunohistochemical staining of CDX2 in representative cancers. (**A**) A methylation-negative intestinal type gastric carcinoma showed nuclear CDX2 staining. (**B**) A diffuse type gastric carcinoma did not express the CDX2 protein, while adjacent intestinal metaplastic cells (right side) expressed CDX2. The microdissected cancer portion (Ca2) of this sample exhibited *CDX2* gene methylation (Figure 2). Original magnification ×200.

Table I. Protein expression and methylation status of the *CDX2* gene in gastric lesions

Carcinoma						Intestinal metaplasia		Normal mucosa	
Histological type	Total	− or partial expression		+ expression		Expression	Methylation	Expression	Methylation
		No.	Methylated (%)[a]	No.	Methylated (%)[a]				
Intestinal	35	20[b]	8 (40)	15	1 (6.7)	Positive[c]	0/5[d]	Negative[c]	0/5[d]
Diffuse	32	28[b]	10 (35.7)	4	1 (25)				
Total	67[e]	48	18 (37.5)	19	2 (10.5)				

[a]% of no. of methylated cases/no. of cases.
[b]The frequencies of cancers with negative or partial CDX2 expression were significantly higher in the diffuse type (28/32, 87.5%) than the intestinal type (20/35, 57.1%) ($P < 0.01$).
[c]Combined our data with references 18–20.
[d]No. of methylated/no. of cases examined.
[e]Of 73 cases examined for methylation, six cases were not informative for CDX2 expression.

Table II. Clinicopathological characteristics of study patients according to the methylation status of *CDX2*, *p16* and *hMLH1*

	CDX2 ($n = 73$)			*p16* ($n = 66$)			*hMLH1* ($n = 67$)		
	Methylated ($n = 21$)	Unmethylated ($n = 52$)	*P* value	Methylated ($n = 9$)	Unmethylated ($n = 57$)	*P* value	Methylated ($n = 7$)	Unmethylated ($n = 60$)	*P* value
Age (mean ± SD)	66.6 ± 10.5	64.8 ± 9.6	0.76	59.1 ± 10.8	66.1 ± 9.2	0.04	69.8 ± 7.9	64.6 ± 10.1	0.20
Sex									
Male	20	38		8	45		5	50	
Female	1	14	0.03	1	12	0.43	2	10	0.37
Size (cm, mean ± SD)	5.7 ± 4.1	5.5 ± 3.5	0.85	8.1 ± 3.9	5.4 ± 3.6	0.04	6.2 ± 5.1	5.6 ± 3.6	0.65
Histology									
Intestinal	9	27		1	30		3	29	
Diffuse	12	25	0.33	8	27	0.02	4	31	0.55
Depth of tumor invasion									
m, sm	11	24		2	28		4	28	
mp ~ si	10	28	0.41	7	29	0.13	3	32	0.45
Lymph node metastasis									
+	9	26		7	27		4	28	
−	12	26	0.39	2	30	0.09	3	32	0.45

The relationship between methylation frequencies of CDX2, p16 and hMLH1 and clinicopathological parameters

Clinicopathological characteristics of study patients by the methylation status of *CDX2*, *p16* and *hMLH1* are shown in Table II. The methylation of *CDX2* was significantly more frequent in males (20/58, 34.5%) than in females (1/15, 6.7%) ($P = 0.03$). *p16* methylation was more frequently found in younger patients and larger cancers ($P = 0.04$), and was more common in diffuse type than in intestinal type gastric carcinomas ($P = 0.02$). In contrast, there was no statistically significant correlation between *hMLH1* methylation and clinicopathological parameters (Table II).

The relationship between methylation frequencies of CDX2, p16 and hMLH1, and epidemiological parameters in male patients

Since *CDX2* methylation was found only in one female cancer case, the following epidemiological analyses were only performed for male patients. Methylation of *CDX2*, *p16* and *hMLH1* was found in 20/58 (34.5%), 8/53 (15.1%) and 5/55 (9.1%) male patients, respectively; those revealing methylation in any of these genes were 23/53 (43.4%). None of the epidemiological variables revealed statistical significance in relation to the methylation status of *CDX2*, *p16*, *hMLH1* and 'any of the three genes', except beef intake and *p16* methylation (Table III). Because the methylation frequency of *hMLH1* was low and was not associated with epidemiological variables, its data are not shown in Table III. Since dietary factors are closely interrelated, we further conducted categorical regression analyses of clinical and epidemiological variables, and methylation in male gastric cancer patients. A significant association was found between eating quantity or the intake of green tea and methylation of *CDX2*, and between eating quantity and methylation of 'any of the three genes' (Table III). Increased methylation frequency of *CDX2* was significantly associated with full or overeating habits, adjusting for confounding variables ($P = 0.02$). On the other hand, increased daily consumption of green tea (7 or more cups/day) showed a significant association with decreased methylation frequency of *CDX2* after adjustment ($P = 0.02$). These epidemiological factors also revealed a close association with methylation frequency of any of *CDX2*, *p16* and *hMLH1* genes ($P = 0.02$ and 0.06 for eating quantity and intake of green tea, respectively).

When we analyzed the association between the methylation status and dietary variables by non-parametric test, increased intake of cruciferous vegetables was significantly associated