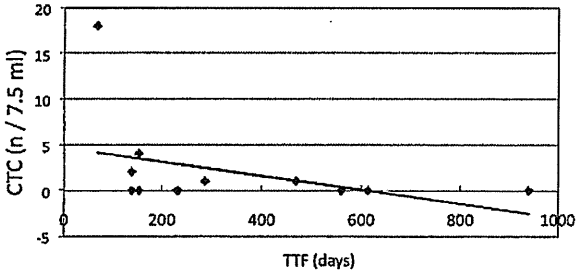

Figure 1. *Correlation between the number of circulating tumor cells (CTCs) during therapy and time to treatment failure (TTF).*

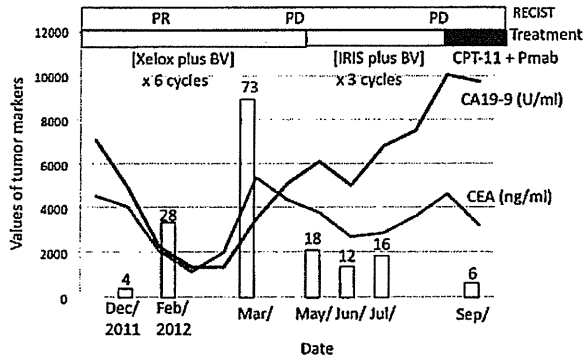

Figure 2. *Change in number of circulating tumor cells (CTCs) during the treatment of case 3. The number of CTCs is indicated by white bar. Carcinoembryonic antigen (CEA) is indicated in blue and carbohydrate antigen 19-9 (CA19-9) in red. The result of response evaluation criteria in solid tumors (RECIST) in each timing is indicated at the top. Xelox, Capecitabine + oxaliplatin; BV, bevacizumab; IRIS, irinotecan + S1; CPT-11, irinotecan; Pmab, panitumumab; PR, partial response; PD, progress disease.*

Table II. *Number of circulating tumor cells detected during therapy and treatment outcome.*

Case	CTC (n/7.5 ml)	TTF (days)
1	0	940 (FOLFOX+BV)
2	2	139 (Xelox)
3	4	153 (Xelox+BV)
4	18	69 (IRIS+BV)
5	0	232 (CPT-11+Pmab)
6	1	468 (FOLFIRI+Pmab)
7	0	139 (SOX)
8	0	230 (SOX)
9	0	613 (FOLFIRI+BV)
10	0	153 (CTP-11+Cmab)
11	0	559 (FL)
12	0	287 (no therapy, TTP)

TTF, time to treatment failure; FOLFOX, 5-fluorouracil + leucovorin + oxaliplatin; BV, bevacizumab; Xelox, Capecitabine + oxaliplatin; IRIS, irinotecan + S1; Pmab, panitumumab; FOLFIRI, 5-fluorouracil + leucovorin + irinotecan; SOX, S1 + oxaliplatin; CPT-11, irinotecan ; FL, 5-fluorouracil + leucovorin, TTP, time to progression.

Potential use of CTCs as a predictive biomarker for outcome of chemotherapy for CRC. Comparison of the number of CTCs before and after chemotherapy could predict the treatment outcome. In case 3, we detected CTCs several times. We compared the change in CTC number with other evaluative methods, such as Response Evaluation Criteria in Solid Tumors (RECIST) and the tumour markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). As shown in Figure 2, an increase in the number of CTCs was observed during Xelox plus BV treatment, three months prior to RECIST evaluation, and one month prior to the increase in tumour markers. The same trend was observed for treatment with irinotecan plus S1 (IRIS) plus BV. In case 13, the number of CTCs declined from 1 to 0 during capecitabine plus oxaliplatin (XELOX) therapy. A decrease in CTCs was associated with partial response (PR; RECIST) evaluated at two-month intervals beginning with the initiation of therapy and was also associated with a decrease in tumour markers between the baseline measurement and during therapy of CEA (from 1636.1 to 187.5 ng/ml) and CA19-9 (from 2137.0 to 411.8 U/ml). The number of CTCs did not increase for four months, and the disease kept within stable disease (SD; RECIST) criteria during this period. These observations demonstrate that if CTCs are detectable, changes in the number present after treatment may be useful for predicting therapeutic outcomes much earlier than that with the current methods.

In the cases where CTCs were not present initially, they were not detected even after the disease progression (cases 1, 4, and 11; Table I). In the CTC-negative cases, we did not obtain any predictive values.

Utility of CTCs as a sample source for molecular analysis. We attempted to analyse *KRAS* in the DNA derived from CTCs collected in cases 2, 3 (twice), 5, 12, and 13 using the Scorpion-ARMS method. No DNA was amplified in case 3 or case 12, where the number of CTCs was 4 and 1 per 7.5 ml of whole blood, respectively (Table III). In the other four cases, where the number of CTCs ranged from 1 to 28 per 7.5 ml of whole blood, the DNA was insufficiently amplified, and no *KRAS* mutants were amplified. For cases 3 and 13, we compared the results of Scorpion-ARMS analysis from surgically removed tissue samples and CTCs. While analysis of the tissue samples identified both cases as having

Table III. *Molecular analysis of circulating tumor cell (CTCs).*

Case	CTC (n/7.5 ml)	KRAS		RNAS extraction
		In CTC	In tissue sample	
2	2	Wild-type*	O Wild-type*	nd
3	4	NA		nd
	28	Wild-type*		nd
	73	nd	× (G13D)	NO
	18	nd		NO
	12	nd		NO
5	1	Wild-type*	O Wild-type	nd
12	1	NA	(G12V)	nd
13	1	Wild-type*	× (G13D)	nd

NA, Not amplified; nd, not done; NO, not extracted; O, match; X, no match.

the *KRAS* G13D mutation, analysis of CTC DNA from the same cases did not yield any result (Table III). The CTC DNA obtained from our examination seemed to be inadequate for *KRAS* Scorpion-ARMS analysis. We also made three attempts to obtain RNA from the CTCs captured in case 3, where the number of CTCs was 12, 18, and 73, but all failed (Table III).

Discussion

CTCs have been recently detected in various types of cancers, including colonic, breast, and prostatic cancer (11, 12). The importance of CTC analysis has been proposed, including its use as a prognostic or predictive biomarker. In this study, we examined the practical availability of CTC analysis using the CellSearch system, which involves outsourcing the analysis to a commercial laboratory. The detection rate and the number of cells identified were rather low, even in stage IV CRC. Previous studies reported detection rates of over two CTCs per 7.5 ml of blood in 30-40% of patients with metastatic CRC (4-6); in patients with metastatic breast and prostate cancer, the same rate was observed in 60% of the patients (11, 12). Our observations are similar to the former.

In general, the number of CTCs in patients with metastatic CRC seems to be lower than that observed in patients with metastatic breast cancer. The cell surface markers used in the CellSearch system (*i.e.* cytokeratin and adhesion-related EpCAM) may be less abundant in patients with metastatic CRC compared with those with metastatic breast cancer. Another possibility is that a fraction of the CTCs may transform to mesenchymal cells through epithelial mesenchymal transition (EMT). This EMT may be more frequent in CTCs from metastatic CRC than from those in metastatic breast cancer. The method used to collect CTCs may require modification according to the type of cancer. Immunomagnetic separation has been reported to improve CTC detection rates. For example, cytokeratin 20 was positive in CTCs in 92.9% of patients with metastatic CRC after column immunomagnetic separation (5).

Many reports describe a relationship between therapeutic outcomes and baseline number of CTCs or number of CTCs during therapy (5, 6, 9). However, in this study, there was no correlation between the number of CTCs during therapy and the outcomes. This observation may be due to the low detection rate of CTCs in metastatic CRC. Once CTCs are detected, the change in the number of CTCs could be a good predictive marker of ongoing treatment, as shown in our cases. In contrast to single measures of CTC number (either baseline or during therapy), changes in CTC counts during therapy could be used to determine whether to continue or change the therapy. Prospective studies should be conducted in the future to clarify these points.

CTCs are viewed as a good source of DNA and RNA for analyses (13-15). However, the DNA obtained using the CellSearch system was not suitable for *KRAS* Scorpion-ARMS analysis in this study. The PCR conditions, such as primer sequences, composition of reaction buffer, and annealing temperature, may require modification. RNA was not recovered from CTCs using the CellSearch system.

Recently, circulating DNA was shown to be useful for identifying acquired resistance to antibodies to EGFR in metastatic CRC (16). This method seems to be much more potent than CTC analysis for *KRAS* mutation detection. However, a next generation sequencer is necessary to use this method, and the balance between cost and effectiveness should be discussed before choosing this method for daily clinical use. Furthermore, CTCs may be rich in molecular information derived from RNAs or proteins rather than DNA. Analysis of these molecules may be advantageous over that of circulating DNA.

Acknowledgements

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Updated overall survival results from a randomized phase III trial comparing gefitinib with carboplatin–paclitaxel for chemo-naïve non-small cell lung cancer with sensitive EGFR gene mutations (NEJ002)

A. Inoue[1]*, K. Kobayashi[2], M. Maemondo[3], S. Sugawara[4], S. Oizumi[5], H. Isobe[6], A. Gemma[7], M. Harada[8], H. Yoshizawa[9], I. Kinoshita[10], Y. Fujita[11], S. Okinaga[12], H. Hirano[13], K. Yoshimori[14], T. Harada[15], Y. Saijo[16], K. Hagiwara[17], S. Morita[18] & T. Nukiwa[1] for the North-East Japan Study Group

[1]*Department of Respiratory Medicine, Tohoku University Hospital, Sendai;* [2]*Department of Respiratory Medicine, Saitama Medical University International Medical Center, Saitama;* [3]*Department of Respiratory Medicine, Miyagi Cancer Center, Miyagi;* [4]*Department of Pulmonary Medicine, Sendai Kousei Hospital, Sendai;* [5]*First Department of Medicine, Hokkaido University School of Medicine, Sapporo;* [6]*Department of Medical Oncology, KKR Sapporo Medical Center, Sapporo;* [7]*Department of Internal Medicine, Division of Pulmonary Medicine, Infections Disease and Oncology, Nipppon Medical School, Tokyo;* [8]*Department of Respiratory Medicine, National Hospital Organization Hokkaido Cancer Center, Sapporo;* [9]*Bioscience Medical Research Center, Niigata University Medical & Dental Hospital, Niigata;* [10]*Department of Medical Oncology, Hokkaido University Graduate School of Medicine, Sapporo;* [11]*Department of Respiratory Medicine, National Hospital Organization Asahikawa Medical Center, Asahikawa;* [12]*Department of Respiratory Medicine, Kesennuma City Hospital, Miyagi;* [13]*Department of Respiratory Medicine, Iwate Prefectural Central Hospital, Morioka;* [14]*Division of Pulmonary Medicine, Anti-Tuberculosis Association Fukujuji Hospital, Tokyo;* [15]*Center for Respiratory Diseases, Hokkaido Social Insurance Hospital, Sapporo;* [16]*Department of Medical Oncology, Graduate School of Medicine, Hirosaki University, Hirosaki;* [17]*Department of Respiratory Medicine, Saitama Medical University, Saitama;* [18]*Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, Yokohama, Japan*

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Background: NEJ002 study, comparing gefitinib with carboplatin (CBDCA) and paclitaxel (PTX; Taxol) as the first-line treatment for advanced non-small cell lung cancer (NSCLC) harboring an epidermal growth factor receptor (EGFR) mutation, previously reported superiority of gefitinib over CBDCA/PTX on progression-free survival (PFS). Subsequent analysis was carried out mainly regarding overall survival (OS).

Materials and methods: For all 228 patients in NEJ002, survival data were updated in December, 2010. Detailed information regarding subsequent chemotherapy after the protocol treatment was also assessed retrospectively and the impact of some key drugs on OS was evaluated.

Results: The median survival time (MST) was 27.7 months for the gefitinib group, and was 26.6 months for the CBDCA/PTX group (HR, 0.887; $P = 0.483$). The OS of patients who received platinum throughout their treatment ($n = 186$) was not statistically different from that of patients who never received platinum ($n = 40$). The MST of patients treated with gefitinib, platinum, and pemetrexed (PEM) or docetaxel (DOC, Taxotere; $n = 76$) was around 3 years.

Conclusions: No significant difference in OS was observed between gefitinib and CBDCA/PTX in the NEJ002 study, probably due to a high crossover use of gefitinib in the CBDCA/PTX group. Considering the many benefits and the risk of missing an opportunity to use the most effective agent for EGFR-mutated NSCLC, the first-line gefitinib is strongly recommended.

Key words: EGFR mutation, gefitinib, individualized treatment, lung cancer

introduction

Two pivotal studies have revealed that somatic mutations in the kinase domain of the epidermal growth factor receptor (EGFR) strongly correlate with responsiveness to gefitinib, the first EGFR tyrosine kinase inhibitor (EGFR-TKI) used to treat non-small cell lung cancer (NSCLC) [1, 2]; subsequently, several phase II studies have demonstrated the promising efficacy of individualized treatment for advanced NSCLC patients with EGFR-TKI on the basis of EGFR gene mutation status [3–10]. Subsequently, we have conducted a phase III

*Correspondence to: Dr A. Inoue, Department of Respiratory Medicine, Tohoku University Hospital, 1-1, Seiryomachi, Aobaku, Sendai, 980-8574, Japan. Tel: +81-22-717-8539; Fax: +81-22-717-8549; E-mail: akinoue@idac.tohoku.ac.jp

study comparing gefitinib with the standard platinum doublet regimen, carboplatin (CBDCA, Nippon Kayaku, Tokyo) and paclitaxel (PTX, Bristol-Myers Squibb, Tokyo), as the first-line treatment for advanced NSCLC harboring EGFR gene mutations (NEJ002) [11]. The study revealed that gefitinib provided significantly longer progression-free survival (PFS), the primary endpoint of the study, than CBDCA/PTX. Other phase III studies also have demonstrated the superiority of EGFR-TKI over the platinum doublet regimen [12, 13]; thus EGFR-TKIs are now globally recognized as the standard first-line treatment for advanced NSCLC with sensitive EGFR mutations [14].

Regarding overall survival (OS), one of the secondary endpoints of NEJ002, the rate of events was <40% in the previous report, for which the data cutoff point was December 2009. Although our study was not powered for OS, we proceeded with this OS analysis to evaluate the long-term survival result for each treatment group. We updated the data for PFS, OS, and safety examined in a longer follow-up period and also assessed the impact of subsequent chemotherapy on OS in patients with EGFR-mutated NSCLC.

materials and methods

study design and treatment

Full details of the NEJ002 study have been published previously. Eligible patients had chemo-naïve advanced NSCLC with a sensitive EGFR mutation detected by the highly sensitive peptide nucleic acid-locked nucleic acid PCR clamp method [15]. Patients were randomly assigned (1:1) to gefitinib (250 mg/day) or CBDCA (AUC 6.0)/paclitaxel (Taxol, 200 mg/m^2) on day 1 every 3 weeks (up to six cycles). The primary endpoint of NEJ002 was to evaluate the superiority of gefitinib over CBDCA/PTX in PFS. The secondary endpoints included response rate, OS, quality of life (QOL), and safety profiles (see Supplementary data, available at *Annals of Oncology* online). Patients provided a written informed consent. The study was conducted in accordance with the Helsinki Declaration of the World Medical Association. The protocol was approved by the institutional review board of each participating institution.

updated evaluation

PFS, OS, and safety data evaluated by the Common Terminology Criteria for Adverse Events version 3.0 were re-evaluated at the data cutoff point in December 2010 for the entire intent-to-treat population ($n = 228$), which was initially unplanned. Detailed information on subsequent chemotherapy carried out after the protocol treatment was also assessed for all patients retrospectively.

statistical analysis

The Kaplan–Meier survival curves were drawn for PFS and OS and compared using a two-sided non-stratified log-rank test with a significance level of 0.05. The hazard ratio (HR, gefitinib:CBDCA/PTX) and its two-sided 95% confidence interval (CI) were calculated by Cox regression analysis including only the treatment arm as a covariate. Subgroup analyses for OS, which were shown in a forest plot, were carried out to examine the interaction effect of treatment arm with age, gender, performance status, smoking status, type of histology, and type of EGFR mutation using a Cox regression model including treatment arm, each of the clinical factors, and their interaction effects as covariates. We did not account for adjustment for multiplicity due to the repetition of subgroup analyses, because we carried out them as exploratory analyses. Other comparative analyses were evaluated on the basis of a two-sided 5% significance level and 95% CI. All analyses were carried out using SAS for Windows release 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

updated PFS

Among the 224 patients assessable, the updated median PFS of the gefitinib group and that of the CBDCA/PTX group were 10.8 months and 5.4 months, respectively (HR, 0.322; 95% CI 0.236–0.438; $P < 0.001$), which was quite similar to the previous results (Table 1). The number of events for PFS at the last data cutoff (December 2010) was 98 in the gefitinib group and 101 in the CBDCA/PTX group. The rate of events for PFS slightly increased from the previous report (from 83% to 88%).

updated OS

At the last data cutoff point, the median follow-up time was 704 days (range 30–1659) and 69 death events were observed in each arm. The rate of events for OS increased from 36% in the previous report to 61% in the current study (Table 1). The MST and the 2-year survival rate were 27.7 months and 58%,

Table 1. Previous and updated results of survival

	Previous results (in 2009)		Updated results (in 2010)	
First-line treatment group	Gefitinib	CBDCA/PTX	Gefitinib	CBDCA/PTX
PFS				
Median PFS, months	10.8	5.4	10.8	5.4
Hazard ratio (95% CI)	0.296 (0.215–0.408)		0.322 (0.236–0.438)	
One-year PFS rate	42.1%	3.2%	43.8%	4.2%
Number of events (%)	87 (76%)	100 (91%)	98 (86%)	101 (92%)
Overall survival				
Median survival time, months	30.5	23.6	27.7	26.6
Hazard ratio (95% CI)	0.798 (0.517–1.232)		0.887 (0.634–1.241)	
1-year survival rate	84.7%	86.4%	85.0%	86.8%
2-year survival rate	61.4%	46.7%	57.9%	53.7%
Number of events (%)	39 (34%)	43 (38%)	69 (61%)	69 (61%)

CBDCA/PTX, carboplatin plus paclitaxel; CI, confidence interval; PFS, progression-free survival.

respectively, for the gefitinib group, and 26.6 months and 54% for the CBDCA/PTX group (HR, 0.887; 95% CI 0.634–1.241; $P = 0.483$) (Figure 1). No factor, including the type of EGFR mutation, had a substantial impact on OS between the groups (Figure 2).

safety

No additional serious adverse event (NCI-CTC grade ≥3) was reported in either group after the previous report. Briefly, the most common adverse events reported were rash and diarrhea with gefitinib, and appetite loss, sensory neuropathy, and myelotoxicities with CBDCA/PTX. The combined incidence of serious adverse events combined was significantly higher in the CBDCA/PTX group than in the gefitinib group (71.7% versus 41.2%; $P < 0.001$).

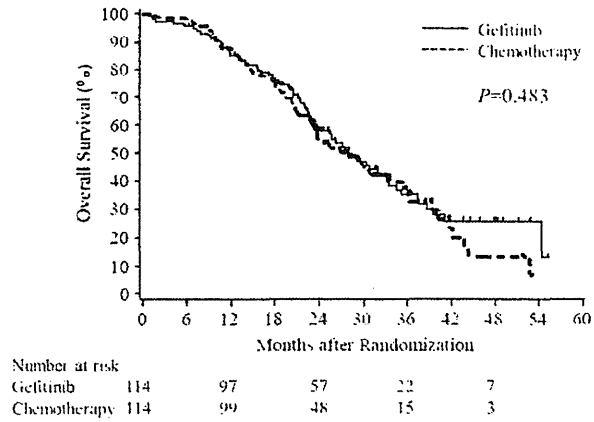

Figure 1. Kaplan–Meier curves for updated overall survival (OS) in the intent-to-treat population of NEJ002.

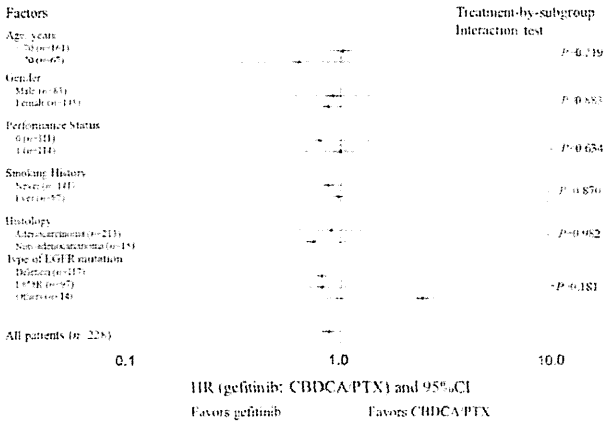

Figure 2. Forest plot of updated overall survival (OS) by clinical factors and the type of epidermal growth factor receptor (EGFR) mutation. Hazard ratio (HR) <1 implies a lower risk of death for patients treated with first-line gefitinb.

post-protocol chemotherapy

The chemotherapy regimens employed in NEJ002 are summarized in Table 2. Regarding the number of subsequent regimens, >50% of patients had received third-line chemotherapy or more, which was quite compatible with general practice in Japan (Figure 3A).

In the gefitinib group, 82 patients (72%) received at least one subsequent regimen. Among these, 74 patients (65%) were treated with the platinum doublet regimen including a crossover use of CBDCA/TXL in 59 patients (52%). Some patients received pemetrexed (PEM) combined with a platinum agent because it became available for the treatment of NSCLC in Japan in May 2009. Twelve patients went back on gefitinib and 32 received erlotinib in one of their later-line treatments. Among the 32 patients who received no subsequent regimen, 12 (11%) had been still treated with their first-line gefitinib at the data cutoff point (8 patients had still maintained their response to gefitinib, while 4 had continued gefitinib after the documentation of disease progression, in accordance with the patient's wishes). There were various reasons why the other 20 patients (18%) did not receive any subsequent regimens: deterioration of PS due to the progression of NSCLC ($n = 11$), interstitial lung disease due to gefitinib treatment ($n = 3$), exacerbation of co-morbidities ($n = 2$), or in accordance with the patient's wishes ($n = 4$). On the other hand, 113 patients (99%) in the CBDCA/PTX group had received at least one subsequent regimen, of whom 112 (98%) had moved to gefitinib.

The standard second-line chemotherapeutic agents PEM or docetaxel (DOC, Sanofi-Aventis K.K., Tokyo), which are used for advanced NSCLC, were used in 29% and 25% of patients in the gefitinib group, respectively, and in 16% and 19% of those in the CBDCA/PTX group, respectively. More than >20% of patients in both the arms received other agents such as irinotecan, S-1, gemcitabine, vinorelbine, or amrubicin as third- or later-line chemotherapy.

evaluation of the impact of key drugs on OS

To examine the impact of the platinum agent on OS of patients with EGFR-mutated NSCLC, we compared the OS of patients who received both gefitinib and a platinum agent in their treatment ($n = 186$) with that of patients who had never received a platinum agent ($n = 40$) in NEJ002. We found no significant difference between the OS of each group (Figure 3B). The number of patients who received a platinum agent but had not received gefitinib was only two in NEJ002.

We then assessed the impact of standard second-line agents (PEM and DOC) on OS. We divided patients who had received third-line or more in NEJ002 ($n = 131$) into two groups: the first group received EGFR-TKI, platinum agent, and PEM or DOC (P/D group, $n = 76$), and the second group received EGFR-TKI, platinum agent, but neither PEM nor DOC (no P/D group, $n = 55$). The MST of the P/D group was significantly longer than that of the no P/D group (34.8 months versus 22.6 months, $P = 0.003$) (Figure 3C).

Table 2. Summary of regimens for entire treatment in NEJ002

	Second-line *n* (%)	Third- or later-line *n* (%)	Total *n* (%)
First-line gefitinib group (*n* = 114)			
EGFR-TKI	8 (7.0)	34 (29.8)	114 (100)
Gefitinib	2 (1.8)	10 (8.8)	114 (100)
Erlotinib	6 (5.3)	26 (22.8)	32 (28.1)
Chemotherapy	74 (64.9)	52 (45.6)	76 (66.7)
Platinum based	71 (62.3)	11 (9.6)	74 (64.9)
CBDCA/PTX[a]	56 (49.2)	3 (2.6)	59 (51.8)
Platinum/PEM[b]	11 (9.6)	4 (3.5)	15 (13.2)
PEM (monotherapy)	2 (1.8)	16 (14.0)	18 (15.8)
DOC	0	28 (24.6)	28 (24.6)
Others[c]	1 (0.9)	26 (22.8)	27 (23.7)
First-line CBDCA/PTX group (*n* = 114)			
EGFR-TKI	109 (95.6)	42 (36.8)	112 (98.2)
Gefitinib	109 (95.6)	8 (7.0)	112 (98.2)
Erlotinib	0	33 (28.9)	33 (28.9)
BIBW2992	0	2 (1.8)	2 (1.8)
Chemotherapy	3 (2.7)	52 (45.6)	114 (100)
Platinum based	2 (1.8)	9 (7.9)	114 (100)
CBDCA/PTX	1 (0.9)	1 (0.9)	114 (100)
Platinum/PEM	0	4 (3.5)	4 (3.5)
PEM (monotherapy)	0	14 (12.3)	14 (12.3)
DOC	1 (0.9)	21 (18.4)	22 (19.3)
Others[c]	0	26 (22.8)	26 (22.8)

CBDCA/PTX, carboplatin plus paclitaxel; PEM, pemetrexed; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; DOC, docetaxel.

[a]Includes two CBDCA/PTX plus bevacizumab.

[b]Includes one CBDCA/PEM plus bevacizumab.

[c]Includes irinotecan, S-1, gemcitabine, vinorelbine, and amrubicine.

discussion

Although the NEJ002 study met its primary endpoint, in that gefitinib was superior to CBDCA/PTX in PFS, OS data were also important in evaluating the efficacy of the entire treatment including the regimens investigated. The current updated analysis demonstrated that the treatment course initiated with gefitinib achieved OS at least equivalent to a traditional treatment course initiated with a platinum doublet regimen for patients with advanced NSCLC harboring a sensitive EGFR mutation. Since the median follow-up time increased from 17 months in the previous report to 23 months in the current analysis, the OS results should become more accurate. We have already reported that the QOL was significantly better in the gefitinib group than in the CBDCA/PTX group in NEJ002 [16]. Moreover, gefitinib attained a high response rate, rapid improvement of symptoms, and exhibited low toxicity. Taking these factors together, we recommend the use of gefitinib as the first-line treatment.

There is a conservative opinion which states that the platinum doublet regimen should still be used as the first-line treatment for advanced NSCLC. This is because there has been no prospective study showing superiority of first-line EGFR-TKI over platinum doublet regimens for OS. Furthermore, some retrospective analyses have suggested that EGFR-TKI might be similarly effective in EGFR-mutated NSCLC regardless of the line at which it is used [17]. However, it is very important to recognize from our study that, though almost 100% of patients in the CBDCA/PTX group crossed over to gefitinib, the OS curve of the first-line gefitinib group was not inferior to that of the CBDCA/PTX group. While the risk associated with missing the administration of platinum agents after first-line gefitinib may be of concern, our *post-hoc* analysis suggested that the impact of the platinum agent on OS would not be larger than that of EGFR-TKI for patients with EGFR-mutated NSCLC. Figure 3B shows the MST of patients treated without platinum to be >2 years, which is a quite favorable result compared with previous historical data obtained when EGFR-TKI was not available. Thus, we feel that it is a concern if the chance to use gefitinib is missed when chemotherapy is carried out as the first-line treatment. The extremely high crossover rate in NEJ002 is hard to attain in general practice. In fact, only 51.5% of patients in the first-line CBDCA/PTX group received subsequent EGFR-TKI in the IPASS study [12]. Thus, we strongly recommend that the best drug should be used in the first instance.

Patients in the first-line gefitinib group tend to be treated with PEM or DOC monotherapy more intensively; this was because we supposed that some of these did not receive platinum doublet treatment for various reasons. However, we consider that the ideal treatment strategy for appropriate patients is to make use of available standard drugs. The most important finding in the *post-hoc* analysis shown in Figure 3C was that patients treated with EGFR-TKIs, platinum, and

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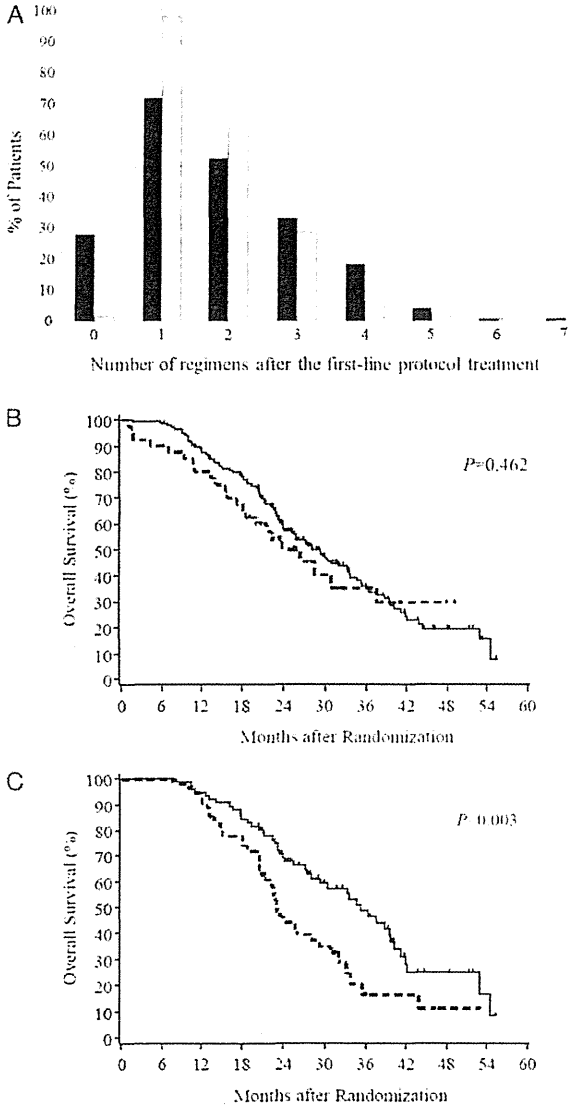

Figure 3. Evaluation of the impact of subsequent treatment on overall survival (OS) in NEJ002. The number of regimens that patients received after the first-line treatment with gefitinib (black bar) and that with chemotherapy (white bar) (A). The OS of patients treated with whichever line of gefitinib but not platinum (a dotted line) and those treated with both gefitinib and platinum (a solid line) (B). The OS of patients treated with gefitinib, platinum, with pemetrexed (PEM) and/or docetaxel (DOC; a solid line), and those treated with gefitinib, platinum but neither pemetrexed nor docetaxel (a dotted line) (C).

PEM/DOC achieved MST of around 3 years even though they had systemically advanced disease; however, the analysis may not conclusively show the difference between the two groups because they were not randomly assigned. This suggests that patients with EGFR-mutated NSCLC and with good PS enough to complete many lines of treatment may further benefit from a proper use of the above mentioned 'key drugs'. Although PEM and DOC were equally recognized as standard second-line agents at the time of the NEJ002 study [18], we now consider PEM to be more appropriate for EGFR-mutated NSCLC where adenocarcinoma is much common [14]. Since at least 14 patients (12%) failed to move to subsequent chemotherapy and ~20% of patients had never received platinum agents or PEM after their disease progressed in the gefitinib group, we think there may be a room for improvement of OS in these populations. Thus, we are now investigating a new treatment strategy, in which the first-line gefitinib is combined with CBDCA and PEM, for patients with EGFR-mutated NSCLC (UMIN000002789).

There are some limitations in the current analysis. First, the sample size of NEJ002 had inadequate power for evaluation of the difference in OS between the two groups. Since death events in one-third of patients have not yet occurred, the true OS curve may change slightly from that shown in this report. A meta-analysis combining several phase III studies and comparing EGFR-TKI with platinum doublet in an EGFR-mutated NSCLC population would be warranted. Second, the *post-hoc* analysis on subsequent chemotherapies may have been biased, because post-protocol treatments were not restricted under the NEJ002 protocol; however, they were very similar to those used in general practice in Japan. In addition, the unplanned comparative analysis between the subgroups shown in Figure 3B and C cannot draw definitive conclusions. It may be difficult to find whether the additive effect of platinum agents or PEM/DOC or good PS itself, that enabled patients to receive those agents irrespective of chemotherapy effects, influenced survival prolongation in the superior group more directly. However, we believe that they give us some interesting suggestions for future investigations such as that underway in our new study.

The reason there was no significant difference in OS between the first-line gefitinib group and the first-line CBDCA/PTX group in NEJ002 was very likely a high rate of crossover use of gefitinib in the CBDCA/PTX group. Considering the many benefits from EGFR-TKI use and the risk of missing an opportunity to use the most effective agent for treatment of EGFR-mutated NSCLC, the first-line gefitinib is strongly recommended in general practice for this population.

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disclosure

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Pemetrexed-based chemotherapy in patients with advanced, ALK-positive non-small cell lung cancer

A. T. Shaw[1]*, A. M. Varghese[2], B. J. Solomon[3], D. B. Costa[4], S. Novello[5], M. Mino-Kenudson[6], M. M. Awad[1], J. A. Engelman[1], G. J. Riely[2], V. Monica[5], B. Y. Yeap[1] & G. V. Scagliotti[5]

[1]Department of Medicine Hematology/Oncology, Massachusetts General Hospital Cancer Center, Boston; [2]Department of Thoracic Oncology, Memorial Sloan-Kettering Cancer Center, New York, USA; [3]Department of Medical Oncology, Peter MacCallum Cancer Centre, East Melbourne, Australia; [4]Division of Hematology/Oncology, Beth Israel Deaconess Medical Center, Boston, USA; [5]Department of Clinical and Biological Sciences, University of Torino, Torino, Italy; [6]Department of Pathology, Massachusetts General Hospital, Boston, USA

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Background: Anaplastic lymphoma kinase (ALK)-positive non-small-cell lung cancer (NSCLC) is highly responsive to crizotinib. To determine whether ALK-positive NSCLC is also sensitive to pemetrexed, we retrospectively evaluated progression-free survival (PFS) of ALK-positive versus ALK-negative patients who had been treated with pemetrexed-based chemotherapy for advanced NSCLC.

Patients and methods: We identified 121 patients with advanced, ALK-positive NSCLC in the USA, Australia, and Italy. For comparison, we evaluated 266 patients with advanced, ALK-negative, epidermal growth factor receptor (EGFR)-wild-type NSCLC, including 79 with KRAS mutations and 187 with wild-type KRAS (WT/WT/WT). We determined PFS on different pemetrexed regimens.

*Correspondence to: Dr A. T. Shaw, Thoracic Oncology, Massachusetts General Hospital Cancer Center, Yawkey 7B-7508, 32 Fruit Street, Boston, MA 02114, USA.
Tel: +1-617-724-4000; Fax: +1-617-726-0453; E-mail: ashaw1@partners.org

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RESEARCH ARTICLE Open Access

Methylation of the *KEAP1* gene promoter region in human colorectal cancer

Naoyuki Hanada[1,2], Takenori Takahata[1], Qiliang Zhou[1], Xulu Ye[1], Ruowen Sun[1,5], Jugoh Itoh[1], Atsushi Ishiguro[1], Hiroshi Kijima[3], Junsei Mimura[4], Ken Itoh[4], Shinsaku Fukuda[2] and Yasuo Saijo[1*]

Abstract

Background: The Keap1-Nrf2 pathway has been reported to be impaired in several cancers. However, the status of Keap1-Nrf2 system in human colorectal cancer (CRC) has not been elucidated.

Methods: We used colorectal cancer (CRC) cell lines and surgical specimens to investigate the methylation status of the *KEAP1* promoter region as well as expression of Nrf2 and its downstream antioxidative stress genes, *NQO-1* and *AKR1C1*.

Results: DNA sequencing analysis indicated that all mutations detected were synonymous, with no amino acid substitutions. We showed by bisulfite genomic sequencing and methylation-specific PCR that eight of 10 CRC cell lines had hypermethylated CpG islands in the *KEAP1* promoter region. HT29 cells with a hypermethylated *KEAP1* promoter resulted in decreased mRNA and protein expression but unmethylated Colo320DM cells showed higher expression levels. In addition, treatment with the DNA methyltransferase inhibitor 5-Aza-dC combined with the histone deacetylase inhibitor trichostatin A (TSA) increased *KEAP1* mRNA expression. These result suggested that methylation of the *KEAP1* promoter regulates its mRNA level. Time course analysis with the Nrf2-antioxidant response element (ARE) pathway activator t-BHQ treatment showed a rapid response within 24 h. HT29 cells had higher basal expression levels of *NQO-1* and *AKR1C1* mRNA than Colo320DM cells. Aberrant promoter methylation of *KEAP1* was detected in 53% of tumor tissues and 25% of normal mucosae from 40 surgical CRC specimens, indicating that cancerous tissue showed increased methylation of the *KEAP1* promoter region, conferring a protective effect against cytotoxic anticancer drugs.

Conclusion: Hypermethylation of the *KEAP1* promoter region suppressed its mRNA expression and increased nuclear Nrf2 and downstream ARE gene expression in CRC cells and tissues.

Background

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in most Western countries [1]. Over the past decade, molecular-targeted drugs have been applied in combination with cytotoxic agents. Consequently, the median overall survival for patients with advanced CRC has become longer than 24 months. Although the spectrum of therapeutic agents is becoming broader, many issues remain to be solved regarding cancer progression and acquisition of resistance to chemotherapy in CRC.

The Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor-erythroid 2 -related factor 2 (Nrf2) pathway is one of the master regulators of cellular defense against oxidative and electrophilic stresses [2-4]. Nrf2 is a basic region-leucine zipper (bZip)-type transcription factor, which was identified as a binding protein of the β-globin gene locus [5,6]. Subsequently, Nrf2 was recognized to be a major transactivation factor for antioxidant response element (ARE)-dependent gene transcription [7]. The ARE is a *cis*-acting regulatory element of genes encoding phase II detoxification enzymes and antioxidant proteins, such as NAD(P)H quinone oxidoreductase-1 (NQO-1), glutathione S-transferases (GST), heme oxygenase-1 (HO-1), and aldo-keto reductase family 1 member C1 (AKR1C1). Keap1 is a negative regulator of Nrf2 and its main function is to serve as an adaptor for

* Correspondence: yasosj@cc.hirosaki-u.ac.jp
[1]Department of Medical Oncology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan
Full list of author information is available at the end of the article

cullin3/ring box1 (Cul3/Rbx1) E3 ubiquitin ligase complex [8-12]. Under physiological conditions, Keap1 maintains a low basal level of Nrf2 by constantly targeting Nrf2 for ubiquitin-mediated protein degradation [13,14]. Once a cell is exposed to oxidative stress, Keap1 acts as a sensor and its cysteine residues are modified. This modification prevents rapid degradation of Nrf2, and the accumulated Nrf2 translocates into the nucleus, leading to active transcription of downstream cytoprotective genes.

The Keap1-Nrf2 signaling pathway is impaired in lung cancer, which is caused by mutations within functionally important domains of the *KEAP1* or *NRF2* gene [15-17]. Impaired Keap1 activity and somatic mutation of Nrf2 lead to full Nrf2 activation, and cancer cells may acquire a protective mechanism against the surrounding microenvironment, resulting in cancer cell proliferation, differentiation, and chemoresistance [15,17]. Similar *KEAP1* mutations have been reported in patients with gall bladder cancer and in breast cancer cell lines [18,19].

Recently, Wang *et al.* reported that the promoter region of *KEAP1* is aberrantly hypermethylated and *KEAP1* mRNA expression levels are low in some lung cancer cell lines and lung cancer tissues [20]. Aberrant methylation of the *KEAP1* promoter region was also reported in prostate cancer [21] and malignant glioma [22]. However, the methylation status of *KEAP1* in CRC has not been elucidated.

As an impaired Keap1-Nrf2 system is induced by mutation or hypermethylation in several types of human cancer, we hypothesized that mutation or epigenetic changes of *KEAP1* may decrease Keap1 expression and increase Nrf2 activity and transactivation of its downstream genes in CRC. In the present study, we investigated the methylation status of *KEAP1* in 10 CRC cell lines and 40 surgically excised CRC tissue specimens. We found frequent hypermethylation of the *KEAP1* gene promoter region in human CRC. In addition, the levels of Nrf2 target gene expression were upregulated in hypermethylated cells.

Methods

CRC cell lines and patient tissue samples

Human CRC cell lines were obtained from cell banks. The HT29 cell line was from American Type Culture Collection, while WiDr, LoVo, DLD-1, SW837, and Colo320DM cell lines were from the Human Science Research Resources Bank (Osaka, Japan). HCT15 and SW480 were from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University. TT1TKB and CW-2 were from RIKEN BioResource Center (Ibaraki, Japan). HT29, WiDr, LoVo, DLD-1, SW480, and SW837 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS). HCT15, CW-2, and Colo320DM were cultured in RPMI1640 medium containing 10% FBS. Forty CRC tissues and adjacent normal colorectal tissue samples were collected with written informed consent at Hirosaki University Hospital. The tissues were immediately frozen and stored at -80°C after surgical resection. The study of CRC tissues samples was approved by the Ethics Committee of Hirosaki University School of Medicine.

Cell treatment

HT29 cells were plated at 5×10^6 cells/10-cm dish 24 h prior to treatment. Cells were treated with 10 μM 5-aza-2'-deoxycytidine (5-Aza-dC) for 96 h to block CpG methylation, followed by treatment with 1 μM trichostatin A (TSA), a reversible inhibitor of histone deacetylase, for 24 h. To evaluate downstream gene expression of Nrf2, HT29 and Colo320DM cells were treated with 50 μM *tert*-butylhydroquinone (t-BHQ), a potent inducer of Nrf2-dependent gene expression, and cells were harvested at 2, 4, 8, 12, and 24 h after treatment. RNA was then extracted, and real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed as described below to measure the expression of *NQO1* and *AKR1C1*. The TaqMan Gene Expression Assay ID for the *NQO1* mRNA is Hs00168547_m1, and that for *AKR1C1* is Hs00413886_m1.

DNA and RNA extraction and DNA sequencing of the *KEAP1* gene

Genomic DNA was extracted from CRC cell lines using a QIAmp DNA Mini kit (Qiagen, Valencia, CA), and RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's protocols. The DNA/RNA concentration and their quality were evaluated by measuring the ratio of optical density at 260/280 nm with NanoDrop (NanoDrop Technologies Wilmington, DE). For detection of *KEAP1* mutation, DNA extracted from cell lines was amplified using AmpliTaq Gold® Fast PCR Master Mix (Applied Biosystems, Carlsbad, CA). Direct sequencing was performed using the primer sets reported previously by Shibata *et al.* [18].

Methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) of the *KEAP1* gene

The primer sets of MSP and BSP used to target the CpG islands located in the putative promoter region of *KEAP1* [20] are shown in Table 1 and Figure 1. These primer sets were designed using Methyl Primer Express Software v1.0 (Applied Biosystems), and PCR conditions for MSP and BSP are shown in Table 1. Aliquots of 2 μg of extracted DNA from CRC cell lines were

Table 1 PCR primers and thermal cycling conditions

Methods	Primers	Sequence
BSP		Forward: 5'-AAGAAAAGAAAAGAAAAGAAAATTTAG-3'
		Reverse: 5'-TTTAGTGAGGTAGATAATTTTTT-3'
PCR conditions		Initial denaturation at 95°C (10 min) and 35 cycles at 95°C (3 s), 52°C (3 s), 72°C (5 s), and a final extension at 72°C (10 s)
MSP	Methylation-	Forward: 5'-TAGATAATTTTTTTTAGATTTTGCGGTCG-3'
	Specific	Reverse: 5'-TCCTCGCGAAACTACGC-3'
PCR condition		Initial denaturation at 95°C (10 min) and annealing temperature decrement of 0.5°C every cycle (from 70°C to 66.5°C) followed by 32 cycles of 66°C (3 s), 72°C (5 s), and a final extension at 72°C (10 s)
MSP	Non-methylation	Forward: 5'-TAGATAATTTTTTTTAGATTTTGTGGTTG-3'
	-specific	Reverse: 5'-TCCTCACAAAACTACAC-3'
PCR condition		Initial denaturation at 95°C (10 min) and annealing temperature decrement of 0.5°C every cycle (from 64°C to 60.5°C) followed by 32 cycles of 60°C(3 s), 72°C (5 s), and a final extension at 72°C (10 s)

converted using an Epitect Bisulfite kit (Qiagen) in accordance with the manufacturer's instructions. Direct DNA sequencing by dye terminator cycle sequencing was performed after bisulfite treatment using an ABI 310 Genetic analyzer (Applied Biosystems). PCR amplification with MSP primers was then performed using 10 μl of AmpliTaq Gold® Fast PCR Master Mix and 20 ng of template DNA (the PCR conditions are shown in Table 1). CpG-methylated HeLa genomic DNA and 5-Aza-dC-treated Jurkat genomic DNA (New England Biolabs Japan, Tokyo, Japan) were used as controls for methylated and unmethylated sequence detection, respectively. MSP products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized with a UV transilluminator.

Real-time RT-PCR

Expression of *KEAP1* mRNA was measured by quantitative real-time PCR in triplicate using TaqMan Gene Expression Assays (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). Intact total RNA was extracted as described above. Reverse transcriptase reactions were performed on aliquots of 2 μg of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. The

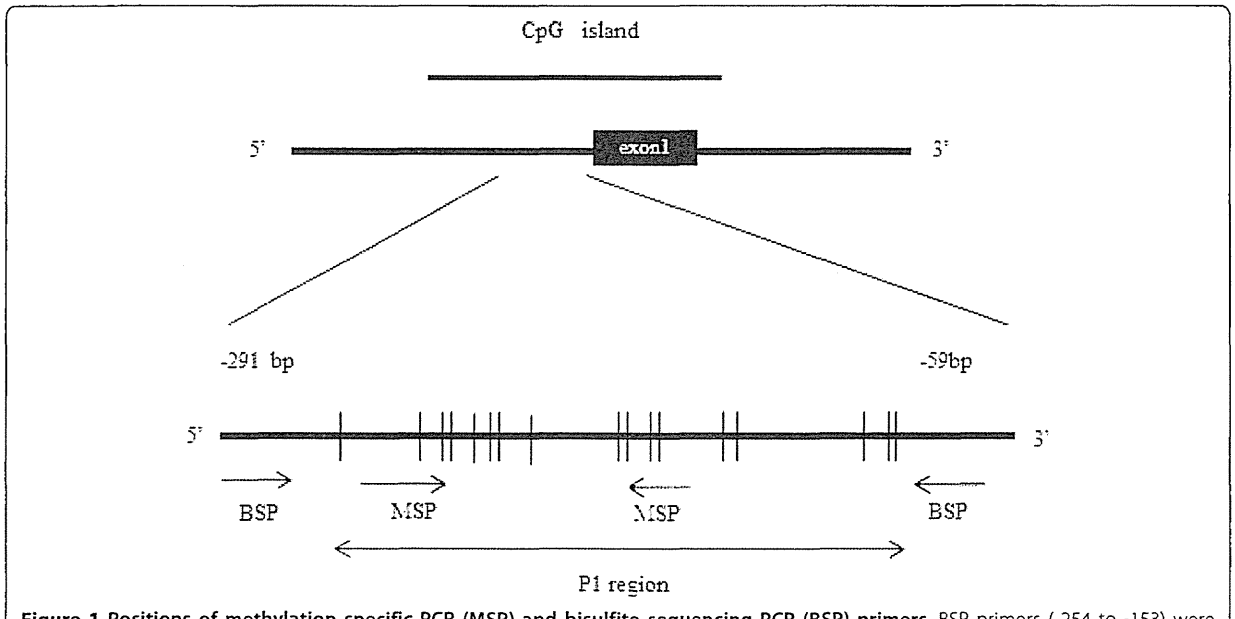

Figure 1 Positions of methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) primers. BSP primers (-254 to -153) were designed to include the 12 CpG sites. MSP forward and reverse primers contained two and three CpG sites, respectively.

conditions for reverse transcription were 25°C (10 min), 37°C (120 min), and 85°C (5 min). The TaqMan Gene Expression Assay ID of the *KEAP1* mRNA is Hs00202227_m1. Calculations were performed using the comparative C_T method. GAPDH (Assay ID Hs99999905_m1) was used as an endogenous control gene for normalization of PCR for the amount of RNA added to the reverse transcription reactions. The mRNA levels are expressed as fold induction relative to the control. The conditions for real-time PCR were 50°C (2 min), 95°C (10 min), followed by 40 cycles of 95°C (15 s) and 60°C (1 min).

Western blotting analysis

Whole-cell, cytoplasmic, and nuclear extracts from HT29 and Colo320DM cells were prepared using a Nuclear Extract kit (Active Motif, Tokyo, Japan) according to the manufacturer's instructions. The protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Whole-cell lysates containing 5 μg of protein from HT29 cells and 12.5 μg of protein from Colo320DM cells were loaded in each lane, run on a NuPAGE 4%-12% Bis-Tris gel (Invitrogen, Carlsbad, CA), and transferred onto PVDF iBlot Gel Transfer Stacks (Invitrogen). After blotting, membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and 1% non-fat dried milk for 1 h. After blocking, membranes were probed overnight at 4°C with a rat monoclonal antibody against Keap1 (dilution 1:5,000; clone#144), a rabbit polyclonal antibody against Nrf2 (1:200; Santa Cruz, #sc-722), a mouse monoclonal antibody against NQO-1 (1:1,000; Santa Cruz Biotechnology, #sc-32793), and a mouse monoclonal antibody against AKR1C1 (1:1,000; ATGen,#ATGA0201). Membranes were washed four times (10 min per wash) with antibody dilution buffer and then incubated with goat anti-rabbit IgG (1:2,000; Santa Cruz Biotechnology) for 1 h at room temperature. A rabbit monoclonal antibody against β-actin (1:2,000; Cell Signaling Technologies, Danvers, MA) and a mouse monoclonal Antibody against histone H1 (1:500; Santa Cruz Biotechnology, #sc-8030) were used as controls. After extensive washing (4 × 10 min with TBS-T), antibody detection was performed with SuperSignal West Pico Chemiluminescent Substrate Kits (Pierce, Rockford, IL).

Statistical analysis

Data are presented as the means ± standard deviation. Student's *t* test was used to assess the significance of three independent experiments. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

Genetic alteration of *KEAP1* in CRC cell lines

As *KEAP1* gene mutations have been reported in other types of human cancer, we sequenced all protein-coding exons in 10 CRC cell lines. We detected a C-to-T transition (G157G) in exon 2 of LoVo cells, a C-to-G transition (L470L) in exon 4 of LoVo, DLD-1, TT1TKB, HCT15, and CW-2 cells, and a C-to-T transition (Y537Y) in exon 5 of CW-2 cells. All mutations were single-nucleotide polymorphisms and had been reported previously. No missense or nonsense mutations were observed.

Analysis of the methylation status of the *KEAP1* promoter region in 10 CRC cell lines

The *KEAP1* promoter region was hypermethylated in lung cancer cell lines and lung cancer tissues, as reported previously by Wang *et al.* [20]. They reported that the P1 region, including 12 CpGs (-291 to -89), was heavily hypermethylated in the CpG islands around the transcriptional initiation site of *KEAP1*. Therefore, we investigated the methylation status of the P1 region in *KEAP1* using MSP and BSP primers designed as shown in Figure 1. MSP analysis indicated that the P1 region was hypermethylated in HT29, WiDr, LoVo, DLD-1, SW480, TT1TKB, HCT15, and CW-2 cells, but not in SW837 or Colo320DM (Figure 2A). Furthermore, we determined the methylation status of each of the 12 CpG dinucleotide sites in the P1 region by BSP. As shown in Figure 2B, most of CpG sites were methylated in HT29, WiDr, LoVo, DLD-1, SW480, TT1TKB, HCT15, and CW-2, but not in SW837 or Colo320DM. Representative results of methylation analysis of CpG islands in the promoter region of the *KEAP1* gene are shown in Figure 2B. All cytosines in the P1 region were converted to thymidine in Colo320DM cells, although in HT29 cells the 5'-methylcytosines of CpG sites remained as cytosines. In contrast, both cytosines and thymidines in the 5'-methylcytosines of CpG sites were observed in HCT15 cells. Aberrant hypermethylation in the *KEAP1* promoter region was frequently observed in human CRC cell lines.

Association between *KEAP1* methylation and *KEAP1* mRNA expression

To examine the effects of *KEAP1* methylation on its mRNA expression level, we performed real-time RT-PCR of *KEAP1* mRNA as shown in Figure 3A. Cell lines with methylated *KEAP1* (HT29, WiDr, LoVo, DLD-1, SW480, TT1TKB, HCT15, and CW-2) exhibited lower levels of *KEAP1* mRNA expression compared with the unmethylated cell lines SW837 and Colo320DM.

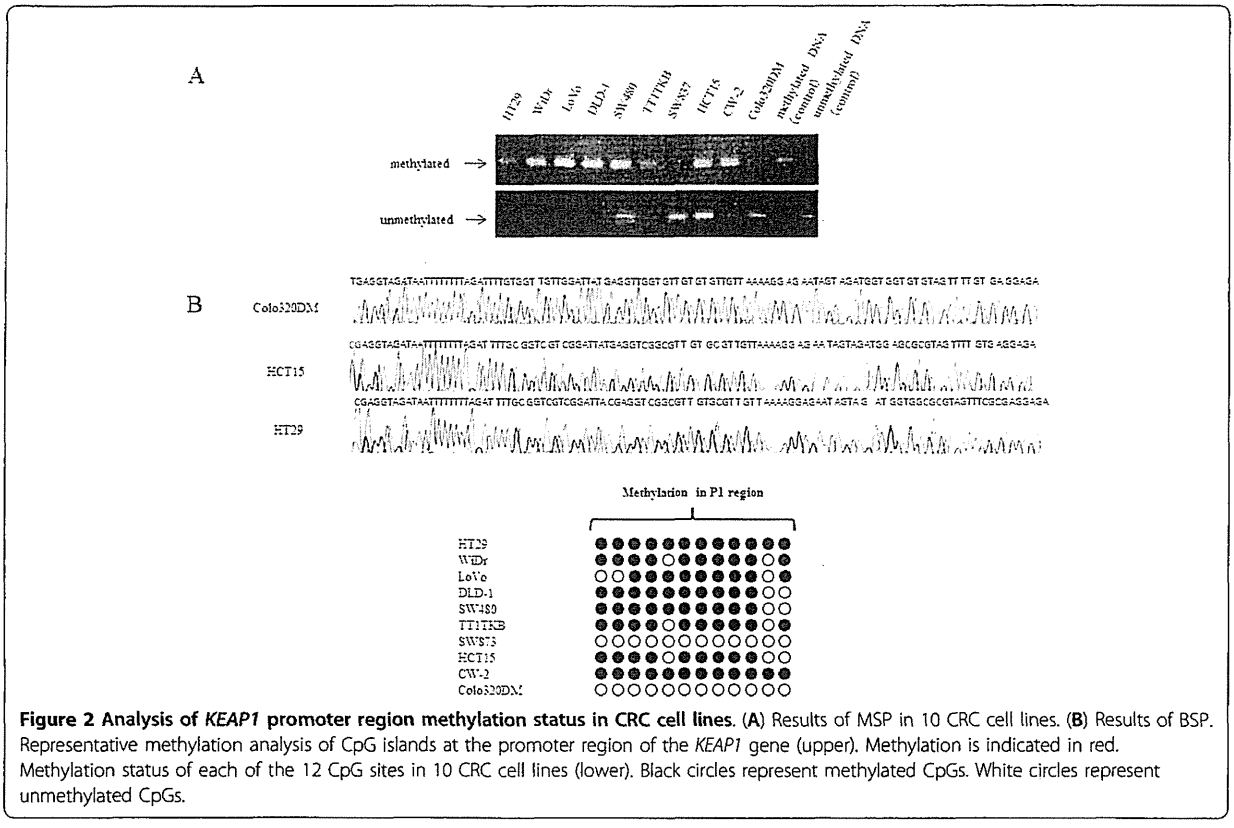

Figure 2 Analysis of *KEAP1* promoter region methylation status in CRC cell lines. **(A)** Results of MSP in 10 CRC cell lines. **(B)** Results of BSP. Representative methylation analysis of CpG islands at the promoter region of the *KEAP1* gene (upper). Methylation is indicated in red. Methylation status of each of the 12 CpG sites in 10 CRC cell lines (lower). Black circles represent methylated CpGs. White circles represent unmethylated CpGs.

To determine whether expression of *KEAP1* mRNA is epigenetically downregulated, expression of *KEAP1* mRNA was measured after treatment with the demethylating agent 5-Aza-dC at 10 μM for 4 days and/or the reversible histone deacetylase inhibitor TSA at 1 μM for 24 h in HT29 cells. The expression of *KEAP1* mRNA was markedly increased after 5-Aza-dC and TSA treatment in the methylated cell line HT29, but no changes were observed in *KEAP1* mRNA expression level in the unmethylated cell line Colo320DM (Figure 3B). MSP analysis showed that methylation of the *keap1* promoter in HT29 cells was reversed after 5-Aza-dC and TSA treatment (Figure 3B). These observations suggest that epigenetic alterations regulate Keap1 expression in CRC cell lines.

Protein levels of *Keap1* and Nrf2

To further examine whether Keap1 protein levels are different between methylated and unmethylated cells, we performed Western blotting analysis. The Keap1 protein level was reduced in HT29 cells, compared with that in Colo320DM cells, as shown in Figure 3C (left). Keap1 protein expression in the methylated cell line HT29 was reversed by treatment with 5-Aza-dC and TSA, but was unchanged in the unmethylated cell line Colo320DM (Figure 4C), mirroring similar changes in KEAP1 mRNA expression. In addition, Nrf2 protein clearly accumulated in the nuclear fraction of HT29 cells, as compared to its level in Colo320DM, whereas Nrf2 protein levels in cytoplasmic fractions were equivalent in these two cell lines (Figure 3C, right). Nrf2 protein accumulation in HT29 cells was reduced by demethylation (Figure 4C).

NQO1 and *AKR1C1* mRNA and protein levels

We measured *NQO1* and *AKR1C1* mRNA levels at different time points after treatment with t-BHQ, an activator of the Nrf2-ARE pathway, at a concentration of100 μM. *NQO1* and *AKR1C1* expression levels were higher in the methylated cell line HT29 than in the unmethylated cell line Colo320DM without stimulation (Figures 4A and 4B left). Furthermore, t-BHQ treatment significantly increased *NQO-1* and *AKR1C1* mRNA levels in HT29 cells. *AKR1C1* mRNA was below the limit of detection both at baseline and after stimulation in Colo320DM cells. The expression of *NQO1* and *AKR1C1* mRNA in the methylated cell line HT29 was reversed after treatment with 5-Aza-dC and TSA (Figures 4A and 4B (right)). NQO-1 and AKR1C1 proteins were overexpressed in methylated HT 29 cells, but their levels were reduced after demethylation (Figure 4C).

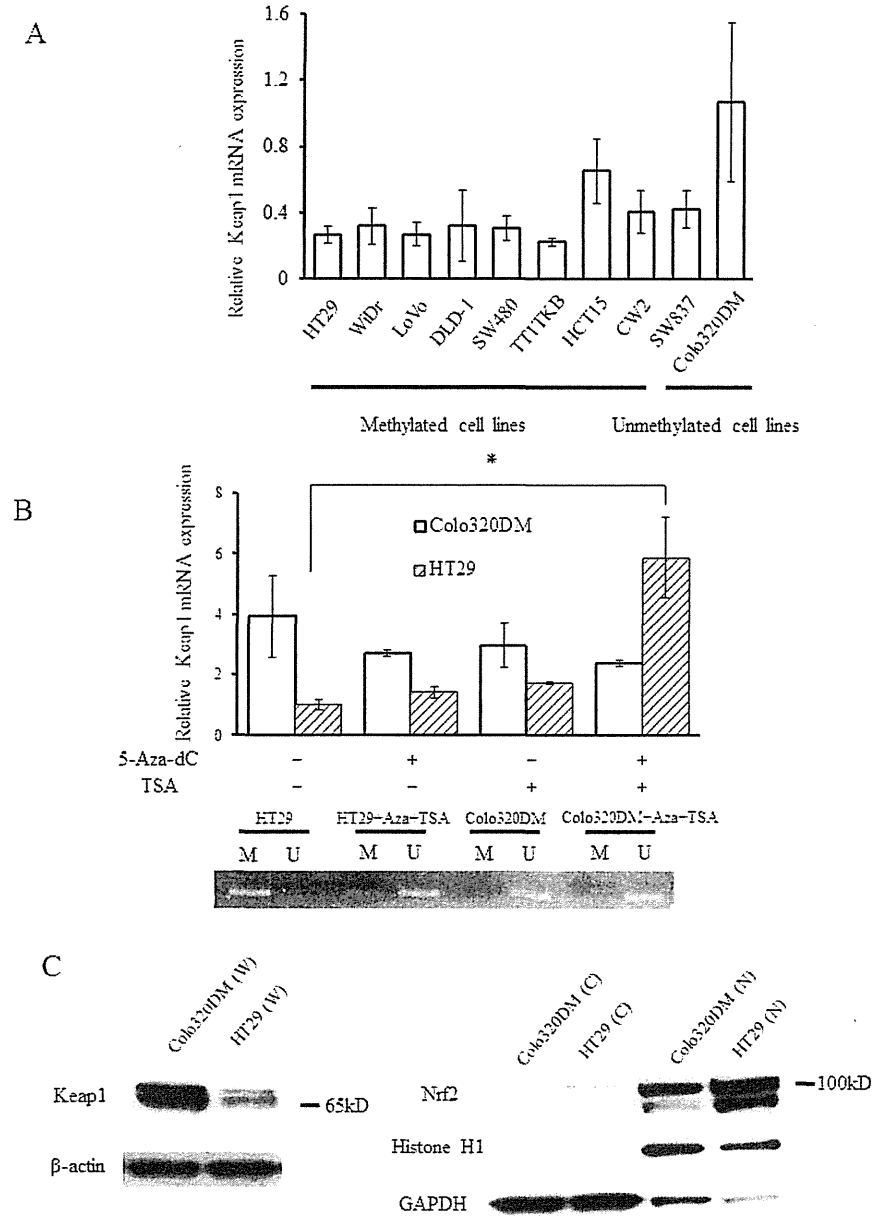

Figure 3 *KEAP1* and *Nrf2* expression. (**A**) *KEAP1* mRNA expression in 10 CRC cell lines was evaluated by real-time PCR. The expression level in Colo320DM cells was arbitrarily designated as 1. Columns, mean (n = 3); bars, standard deviation (SD). (**B**, upper) *KEAP1* mRNA levels in HT29 cells (methylated) and Colo320DM cells (unmethylated) were analyzed by real-time PCR after treatment with 5-Aza-dC, TSA, and 5-Aza-dC + TSA. The expression level in HT29 cells was arbitrarily designated as 1. Columns, mean (n = 3); bars, SD. *$P < 0.05$. (**B**, lower) MSP analysis of in HT29 cells and Colo320DM treated with 5-Aza-dC + TSA. M, methylation-specific primer; U, non-methylation-specific primer. (**C**) Western blot analysis of Keap1 and Nrf2 in methylated and unmethylated colon cancer cells. Whole-cell extracts (W), cytosolic extracts (C), and nuclear extracts (N) were prepared from Colo320DM and HT29 cells. Extracts were stained with antibody to Keap1 or Nrf2A. β-Actin, histone H1, and GAPDH antibodies were used as loading controls for whole-cell, cytosolic, and nuclear fractions, respectively.

Detection of *KEAP1* methylation using MSP in surgical samples and association between methylation status and clinicopathological features in CRC

The methylation status of each sample was confirmed by MSP and BSP. Representative MSP products for *KEAP1* in tumor tissues and normal tissues are shown in Figure 5A. Representative results of MSP sequence analysis of tumor tissues are presented in Figure 5B. Aberrant promoter methylation of *KEAP1* was detected in 21/40 (53%) tumor tissues and 10/40 (25%) normal

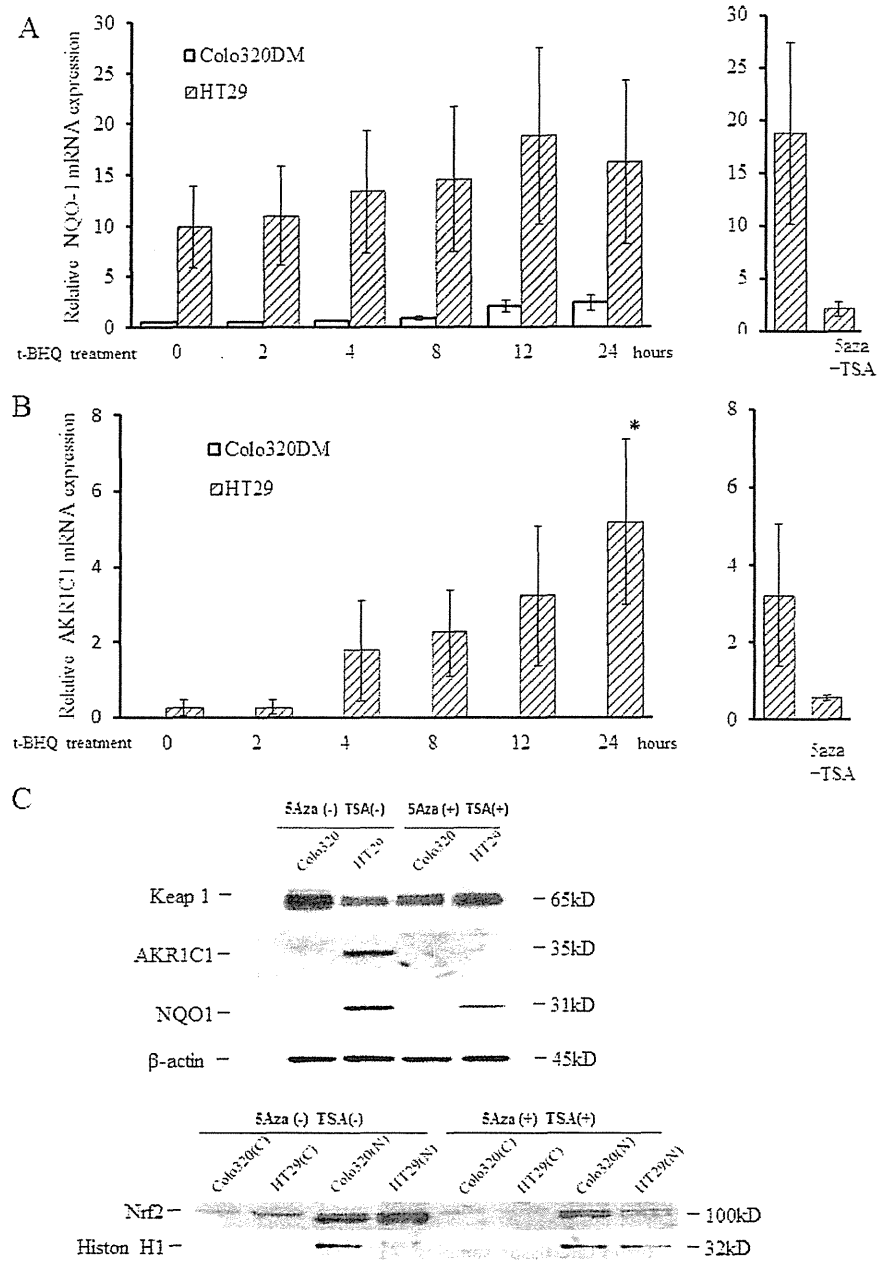

Figure 4 Expression of the *Nrf2* target genes *NQO-1* and *AKR1C1* after t-BHQ treatment. Real-time PCR analysis of the *Nrf2* target genes *NQO-1* (**A**, left) and *AKRC1* (**B**, left) in HT29 cells (methylated) and Colo320DM cells (unmethylated). Cells were treated with the Keap1 stimulator t-BHQ for 24 h. Columns, mean (n = 3); bars, SD. *P < 0.05. Real-time PCR analysis of *NQO-1* (**A**, right) and *AKRC1* (**B**, right) in HT29 cells treated with 5-Aza-dC + TSA and then t-BHQ (for 12 h). Keap1 protein levels (**C**, upper), protein expression of downstream ARE genes (**C**, upper), and changes in the subcellular distribution of Nrf2 protein (**C**, lower) after 5-Aza-dC and TSA treatment. Cells were treated with 5-Aza-dC and TSA. Expression levels of each protein were determined by Western blotting. β-actin and histone H1 were used as loading controls.

mucosal specimens (Table 2). Compared with normal mucosa, the methylation of *KEAP1* was more prominent in tumor tissues (P = 0.001). We performed statistical analyses to determine whether the *KEAP1* methylation status of colorectal tumor samples is associated with the clinicopathological features of CRC patients. In the tumor tissues, methylation of *KEAP1* was not associated with any clinicopathological features, such as primary site location, differentiation, gender, Duke's stage, clinical stage, age, lymph node

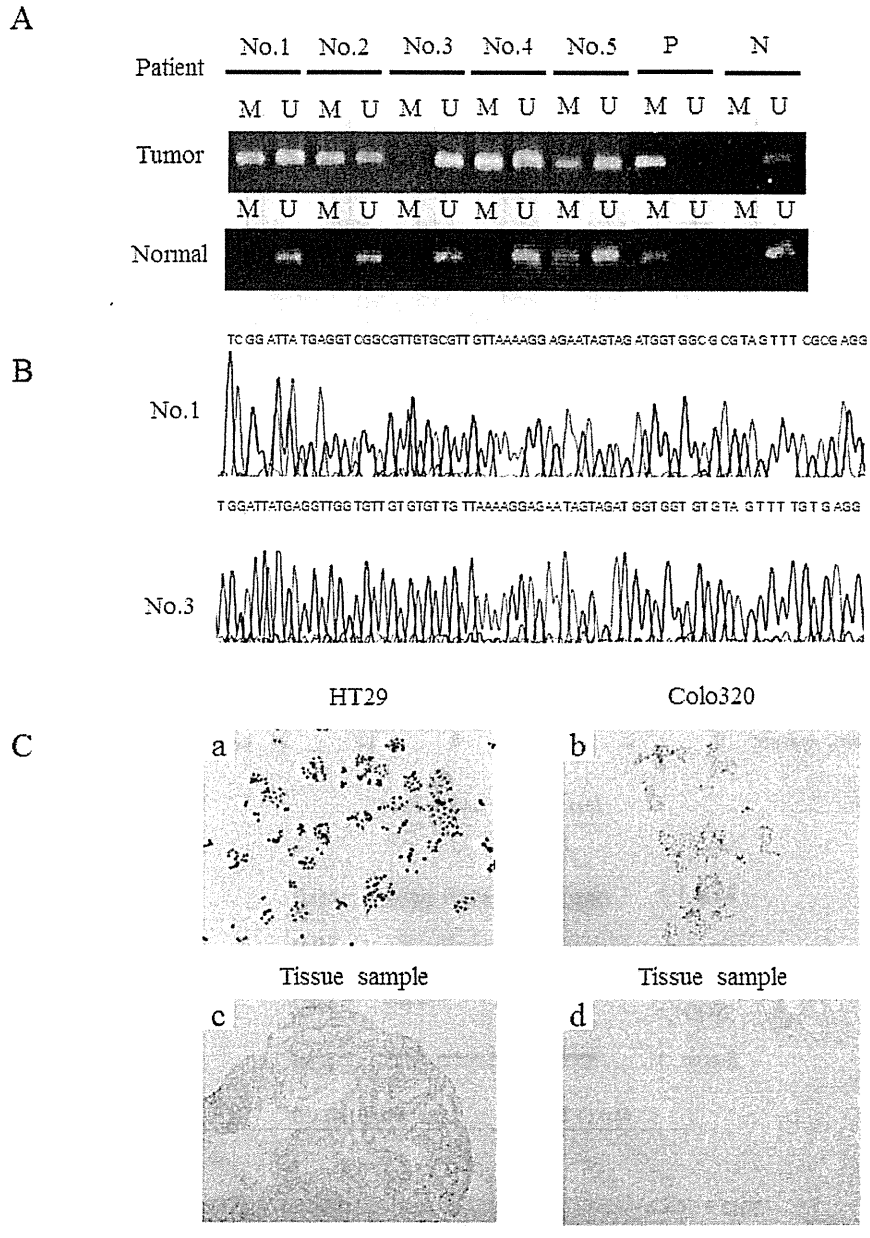

Figure 5 Methylation of the *KEAP1* promoter in CRC tissue samples. (**A**) MSP for the *KEAP1* promoter was performed using bisulfite-modified DNA from 40 CRC tissues and adjacent normal colorectal tissues. MSP results from 10 patients are shown. M: MSP of methylation-specific primers; U: MSP of non-methylation-specific primers; P: positive methylated DNA control; N: negative unmethylated DNA control. (**B**) Representative results of MSP sequence analysis of tumor tissues. The methylation status of the *KEAP1* promoter region in patient tumor tissues was determined using methylation-specific primers (upper), and non-methylation-specific primers (lower). Methylation is indicated in red. (**C**) Expression and subcellular localization of Nrf2. Nrf2 was more highly expressed in HT29 cells (**a**) and a methylated tissue sample (**c**) compared with Colo320 cells (**b**) and a non-methylated tissue sample (**d**). Expression and localization of Nrf2 was studied using an anti-human Nrf2 antibody.

metastasis, and serum concentration of carcinoembryonic antigen (CEA) (data not shown). Additionally, we analyzed methylated HT29 cells and tumor samples by immunohistochemistry using an anti-human Nrf2 antibody. As shown in Figure 5C, strong expression of Nrf2 protein was detected in the nuclei of HT29 cells and in a methylated tissue sample. This observation indicates that promoter methylation of the *KEAP1*

Table 2 *KEAP1* promoter methylation frequency in colorectal cancer and adjacent normal mucosa

		Methylation status	
Variable	N	Present	Absent
Tumor tissue	40	21 (53%)	19 (47%)
Adjacent normal mucosa	40	10 (25%)	30 (75%)
			$P < 0.05$

gene enables Nrf2 to translocate from the cytoplasm to the nucleus.

Discussion and conclusions

We found frequent hypermethylation of the *KEAP1* promoter region in human CRC cell lines. This hypermethylation of *KEAP1* resulted in reductions in *KEAP1* mRNA and protein expression, upregulation of Nrf2 activity, and thus overexpression of downstream genes, such as *NQO-1* and *AKR1C1*. We also observed aberrant methylation of *KEAP1* in human CRC tissues. This is the first report discussing activation of Keap1/Nrf2 signaling by *KEAP1* hypermethylation in CRC.

Loss of Keap1 function has been reported associated with *KEAP1* gene mutations in tumor tissue samples from lung, gall bladder, breast, and prostate cancer [15,18,19,21]. We found only synonymous mutations consisting of a C-to-T transition with G157G in exon 2, a T-to-C transition of L471L in the DGR4 domain, and a C-to-T transition with Y537Y in the DGR5 domain in CRC cell lines. However, these mutations were single-nucleotide polymorphisms. Frequent *KEAP1* gene mutations were reported in human non-small cell lung cancer (NSCLC) [15]. All mutations were within highly conserved amino acid residues located in the Kelch or intervening region domain of the Keap1 protein, suggesting that these mutations were likely to abolish Keap1 repressor activity against Nrf2. In addition, C23Y mutation in the N-terminal domain of Keap1 has been reported to have impaired ability to repress Nrf2 activity due to its inability to stimulate the ubiquitylation and degradation of Nrf2 in breast cancer [19]. A C-to-T transition with T314M and a T-to-C transition with Y255H were detected in six prostatic cancer cell lines [21]. Shibata *et al.* also reported mutations of *KEAP1* in biliary tract cancer tissue [18]. These changes are in the central intervening region of Keap1 and alter highly conserved amino acids.

Another mechanism of impaired Keap1 activity is hypermethylation of *KEAP1*. We found that 8 of 10 CRC cell lines had methylated CpG islands in the promoter region of the *KEAP1* gene where methylation was found in other types of cancer [20,22,23]. Hypermethylation of *KEAP1* resulted in decreased mRNA expression, which was confirmed by the increase in *KEAP1* mRNA expression by combined treatment with the DNA methyltransferase inhibitor 5-Aza-dC and the histone deacetylase inhibitor TSA (Figure 4C). Hypermethylation of *KEAP1* caused final stimulation of Nrf2 target genes. However, the reason for the expression of *KEAP1* mRNA being lower in unmethylated SW837 cells than in methylated HCT15 cells is unknown. Wang *et al.* investigated three lung cancer cell lines and five tumor samples, and found frequent hypermethylation of the CpG islands in the promoter region of *KEAP1* and reduced levels of *KEAP1* mRNA expression. In contrast, a normal bronchial cell line had clearly less methylation of the *KEAP1* promoter region and elevated mRNA expression [20]. Hypermethylation of *KEAP1* found in prostate cancer also stimulated the Nrf2 signal [21].

Biological effects of constitutive Nrf2 activation by Keap1 dysfunction due to mutations or low-level expression by hypermethylation have been reported previously [18,23,24]. Constitutive expression of the cytoprotective gene by Nrf2 activation in lung cancer cells led to chemotherapy resistance [23]. Nrf2 activation also stimulated growth of lung cancer cells. Nrf2 activation by *KEAP1* mutation or hypermethylation of promoter CpG islands causes radioresistance and promotes tumor growth in prostatic cancer [21]. In the present study, we observed accumulation of Nrf2 protein in the nuclei in methylated HT29 cells, and overexpression of phase II detoxifying enzymes NQO-1 and AKR1C1 both at baseline and after t-BHQ stimulation. These reports indicate that KEAP1 functions as a tumor suppressor gene in human tumors. Although we did not evaluate the biological effects of activated Nrf2, we assume that CRC cells with *KEAP1* gene hypermethylation may be resistant to chemotherapeutic agents and show upregulated cell growth, as reported in other types of cancer.

There have been only two previous reports regarding Keap1/Nrf2 l in CRC cells [24,25]. Activation of the Keap1/Nrf2 signaling pathway mediates protective responses to mitigate nitric oxide (NO)-induced damage and may contribute to the resistance of CRC cells to NO-induced cytotoxicity [24]. Arlt *et al.* reported that Nrf2 activity is elevated in colon cancer, accounting for overexpression of the proteasome subunit proteins and thus for increased proteasome activity [25]. Conversely, small interfering RNA-mediated Nrf2 knockdown decreased their expression and reduced proteasome activity, thus indicating that Nrf2 is related to colorectal carcinogenesis. This Nrf2 activation may be due to the low level of Keap1 expression due to hypermethylation, as found in the present study.

Biological effects that activate Nrf2 signaling prompted us to study the relationship between the status of Keap1/Nrf2 signaling and clinicopathological features of the tumors. Type II endometrial cancer, which is mostly

malignant and is associated with a poor prognosis among gynecological malignancies, shows elevated Nrf2 protein expression, whereas benign tumors and type I endometrial cancer do not [26]. On immunohistochemical analysis of human NSCLC, increased Nrf2 expression and low or absent Keap1 expression were associated with worse survival [27]. In contrast, the prognosis of malignant glioma was better among patients with than among those without a methylated *KEAP1* promoter region [22]. Although we did not investigate the prognosis of patients with CRC, further studies are needed to understand the role of Keap1/Nrf2 signaling in human CRC.

In conclusion, the results of the present study revealed hypermethylation of the *KEAP1* promoter region in human CRC, leading to downregulation of *KEAP1* mRNA expression, thus activating Nrf2 and expression of its downstream target genes. Cancerous tissues exhibited more frequent methylation of *KEAP1* than normal tissue in surgically resected CRC specimens.

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Author details
[1]Department of Medical Oncology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. [2]Department of Gastroenterology and Hematology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. [3]Department of Pathology and Bioscience, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. [4]Department of Stress Response Science, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. [5]Department of Rheumatology and Immunology, Shengjing Hospital of China Medical University, Shenyang, China.

Authors' contributions
HN, TT, ZQ, YX, SR, and MJ performed experiments and summarized the data. IJ, IA, IK, FS, and SY designed the experiments. HN, TT, and SY wrote the paper; all authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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