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# 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 $5 \times 10^6$ 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

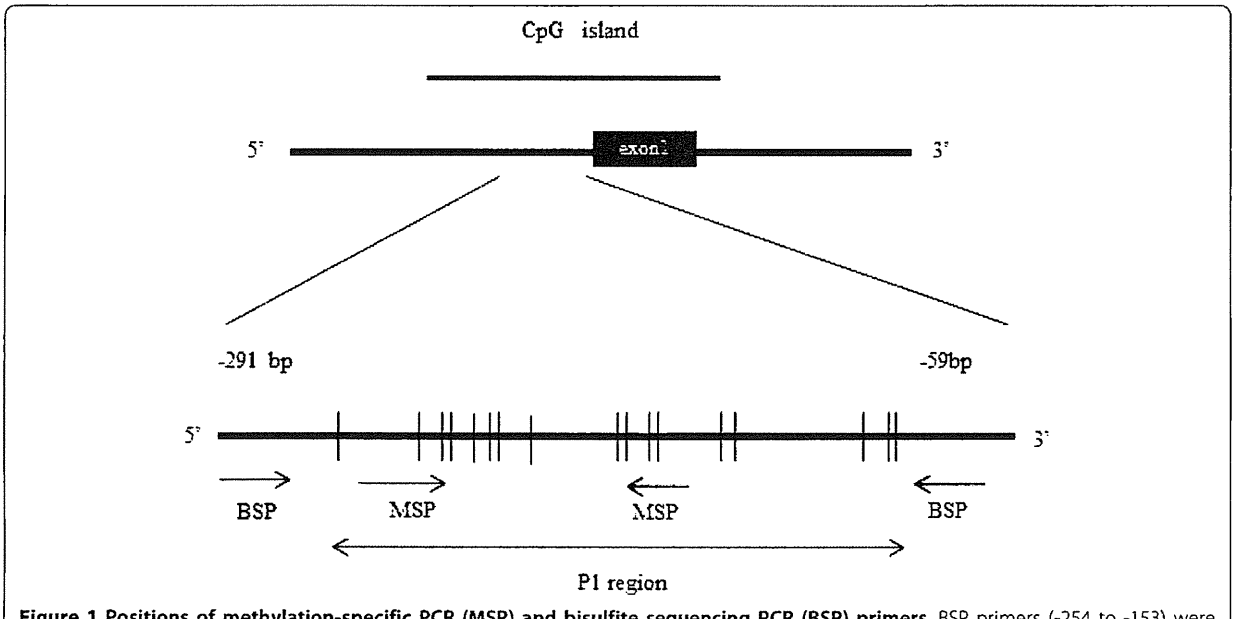


**Figure 1 Positions of methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) primers.** BSP primers (-254 to -153) were designed to include the 12 CpG sites. MSP forward and reverse primers contained two and three CpG sites, respectively.

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.

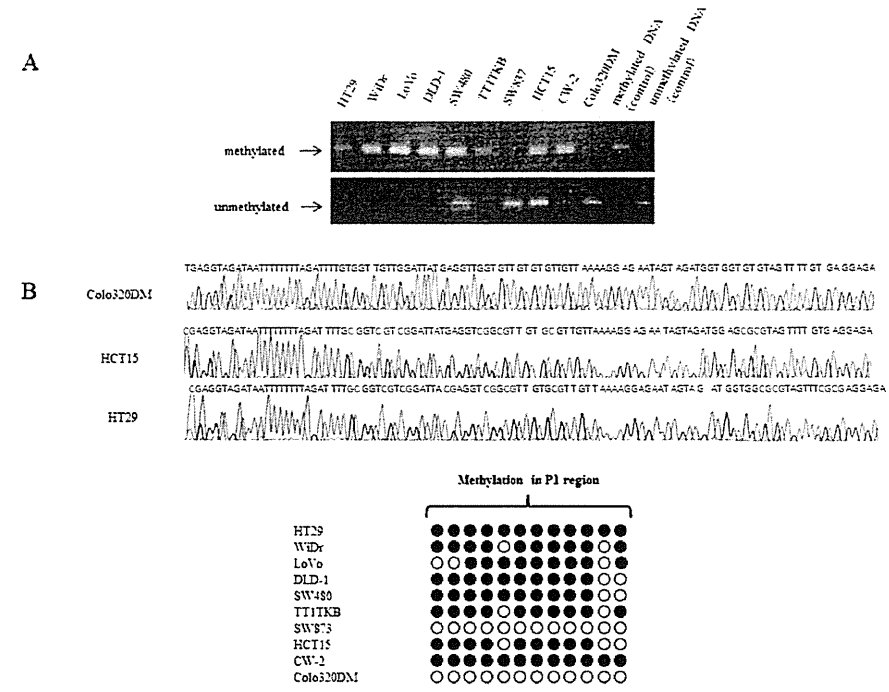


**Figure 2 Analysis of *KEAP1* promoter region methylation status in CRC cell lines.** (**A**) Results of MSP in 10 CRC cell lines. (**B**) Results of BSP. Representative methylation analysis of CpG islands at the promoter region of the *KEAP1* gene (upper). Methylation is indicated in red. Methylation 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).

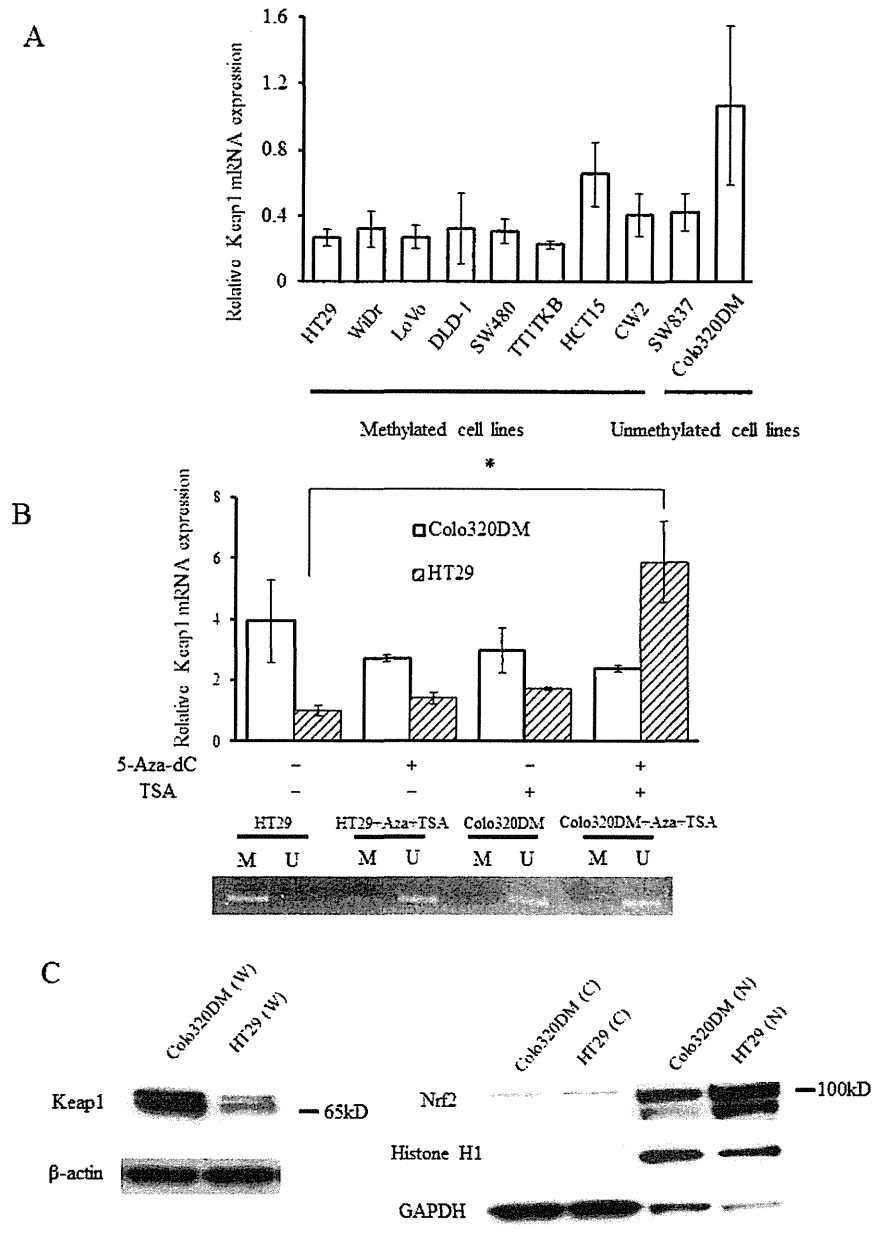


**Figure 3 *KEAP1* and *Nrf2* expression.** (**A**) *KEAP1* mRNA expression in 10 CRC cell lines was evaluated by real-time PCR. The expression level in Colo320DM cells was arbitrarily designated as 1. Columns, mean ($n$ = 3); bars, standard deviation (SD). (**B**, upper) *KEAP1* mRNA levels in HT29 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

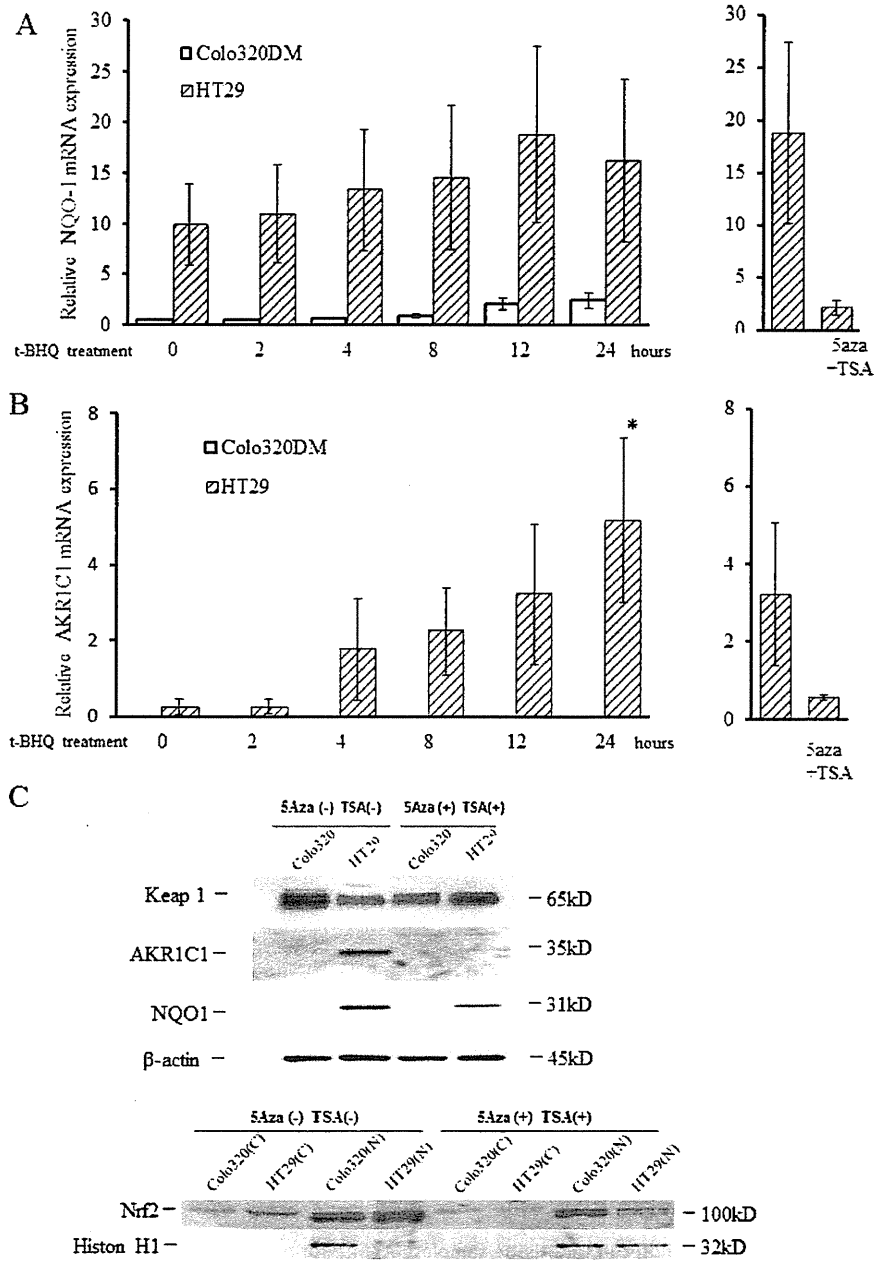


**Figure 4 Expression of the *Nrf2* target genes *NQO-1* and *AKR1C1* after t-BHQ treatment.** Real-time PCR analysis of the *Nrf2* target genes *NQO-1* (**A**, left) and *AKRC1* (**B**, left) in HT29 cells (methylated) and Colo320DM cells (unmethylated). Cells were treated with the Keap1 stimulator 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

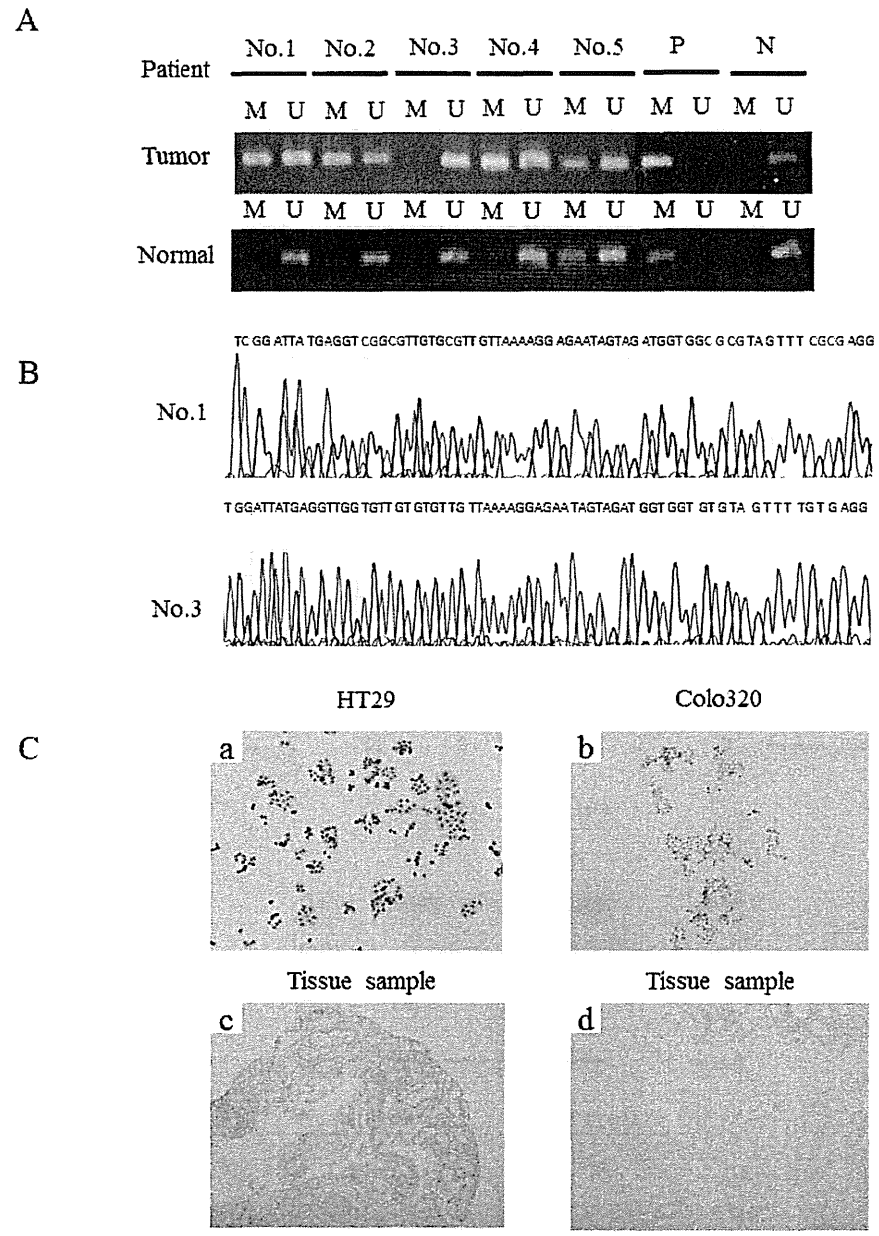


**Figure 5 Methylation of the *KEAP1* promoter in CRC tissue samples.** (**A**) MSP for the *KEAP1* promoter was performed using bisulfite-modified DNA from 40 CRC tissues and adjacent normal colorectal tissues. MSP results from 10 patients are shown. M: MSP of methylation-specific primers; U: MSP of 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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# Sequential irinotecan hydrochloride/S-1 for S-1-resistant inoperable gastric cancer: A feasibility study

TAKENORI TAKAHATA[1], JUGOH ITOH[1], TAROH SATOH[5], ATSUSHI ISHIGURO[1], YOSHIFUMI MATSUMOTO[1], SATOSHI TANAKA[1], SOH SAITOH[3], HIROSHI TOHNO[4], SHINSAKU FUKUDA[2], YASUO SAIJO[1] and YUH SAKATA[6]

Departments of [1]Medical Oncology, and [2]Gastroenterology and Hematology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562; [3]Department of Gastroenterology and Medical Oncology, Aomori Prefectural Hospital, 2-1-1 Higashitsukurimichi, Aomori 030-8553; [4]Department of Internal Medicine, Hirosaki City Hospital, 3-8-1 Omachi, Hirosaki 036-8004; [5]Department of Medical Oncology, Kinki University School of Medicine, 377-2 Ohnohigashi, Osakasayama 589-8511; [6]Department of Medical Oncology, Misawa City Hospital, 164-65 Horiguchi, Misawa 033-0022, Japan

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**Abstract.** Irinotecan hydrochloride (CPT-11) is reported to be involved in the downregulation of thymidylate synthase (TS), a target molecule of 5-fluorouracil (5-FU) and oral fluoropyrimidine S-1. Therefore, we hypothesized that a preceding administration of CPT-11 against S-1-resistant tumors may recover sensitivity to S-1. To this end, we planned a S-1/CPT-11 sequential therapy as a feasibility study in S-1-refractory gastric cancer patients. In the first course, CPT-11 was administered intravenously at 150 mg/m$^2$ on days 1 and 15. Subsequently, S-1 was administered orally for 4 weeks from day 29 to 57, followed by a 2-week interval (sequential S-1/CPT-11). When the tumor showed a complete response (CR) or partial response (PR), the same dose of S-1 monotherapy was continued unless progressive disease (PD) was observed. When the response was stable disease (SD), S-1 was administered at the same dose for just 2 weeks (days 1-15), no drug was administered for the following 2 weeks (4-week cycle) and CPT-11 was administered intravenously at 100 mg/m$^2$ on days 1 and 15 (concurrent S-1/CPT-11) unless PD was observed. In the case of PD, the study was terminated. The primary endpoint was an antitumor effect and secondary endpoints were median survival time (MST), progression-free survival (PFS), time-to-treatment failure (TTF) and safety. The response rate (RR) following the first course was only 5.9% and the most positive RR was 11.8%. The MST, median TTF and PFS were 381, 69 and 71 days, respectively. Leukocytopenia was observed in more than half of the patients. Since the RR was lower than estimated in an interim analysis, the trial was terminated and the protocol was concluded to be unfeasible.

*Correspondence to:* Dr Takenori Takahata, Department of Medical Oncology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan
E-mail: takatake@cc.hirosaki-u.ac.jp

*Key words:* CPT-11, S-1, gastric cancer

## Introduction

Gastric cancer treated by surgical resection is radical and shows a favorable prognosis; however, when cases are inoperable due to the advanced stage, the prognosis is poor, with a 10% 5-year survival rate. Chemotherapies against gastric cancer have been developed as combination chemotherapies since the 1980s. In the 1990s, phase III randomized comparative studies between best supportive care (BSC) and chemotherapies revealed a significant improvement in overall survival rates (1-3).

Irinotecan hydrochloride (CPT-11), synthesized from camptothecin contained in the Chinese tree *Camptotheca acuminate*, inhibits type I topoisomerase and DNA synthesis, and thus demonstrates antitumor effects. In Japan, CPT-11 has been approved for various types of cancer, including small and non-small cell lung cancer, uterine cervical cancer, ovarian cancer, gastric cancer, colorectal cancer, breast cancer, squamous cell carcinoma of the skin, and malignant lymphoma. The overall response rate (RR) to CPT-11 monotherapy is reported to be 23.3% in late phase II trials for advanced gastric cancer (4).

S-1 is an oral anticancer drug containing a combination of tegafur (FT), a prodrug of 5-fluorouracil (5-FU), 5-chloro-2,4-dihydroxypyrimidine (CDHP) that inhibits the activity of dihydropyrimidine dehydrogenase (DPD), and potassium oxonate (Oxo), which reduces the gastrointestinal toxicity of 5-FU. The S-1 monotherapy for advanced gastric cancer revealed non-inferiority to 5-FU infusion in the JCOG9912 study. A subsequent study revealed that a combination of S-1 and cisplatin (CDDP) is superior to the S-1 monotherapy (5). Since these trials, S-1 plus CDDP has been one of the standard chemotherapies against advanced gastric cancer in Japan.

S-1 or S-1-containing regimens are used in adjuvant chemotherapy following surgery or in first-line chemotherapy

for inoperable gastric cancer. One of the mechanisms of resistance against S-1 is thought to be an increase of thymidylate synthase (TS) activity, which is a target of 5-FU in tumor cells. A study revealing that irinotecan downregulates intratumoral TS and makes 5-FU more effective in human colon cancer xenografts suggests the possibility of overcoming S-1 resistance by adding irinotecan (6). In this context, we planned a feasibility study in which S-1-pretreated gastric cancer patients were treated with a combination chemotherapy of S-1 and CPT-11 as a feasibility test.

## Materials and methods

*Patient selection.* Patients with histologically confirmed gastric cancer with measureable or evaluable lesions were eligible for this study. Patients were required to have been previously treated with a first-line chemotherapy containing S-1, but not CPT-11. Other eligibility criteria were: 20-75 years old, Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0-2, capable of oral intake, white blood cell count (WBC) of 3,500-12,000/$\mu$l, neutrocyte count (Neu) >2,000/$\mu$l, platelet count (PLT) >100,000/$\mu$l, hemoglobin (Hb) level >9.0 g/dl, serum total bilirubin (T-bil) <1.5 mg/dl, serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) <2 times the normal limit, serum creatinine within the normal limit, creatinine clearance calculated with the Cockcroft-Gault equation >50 ml/min, survival expectancy of at least 3 months, and written informed consent for this study.

*Study design.* This study is a multicenter, non-randomized, open-label feasibility study. An overview of the study is shown in Fig. 1. In the first course, CPT-11 was administered intravenously at 150 mg/m$^2$ on days 1 and 15. Subsequently, S-1 was administered orally for 4 weeks from day 29 to 57, followed by a 2-week interval (sequential S-1/CPT-11). The dosage of S-1 was based on body surface area (BSA): 40 mg (BSA <1.25 m$^2$), 50 mg (BSA ≥1.25 and <1.5 m$^2$) or 60 mg b.i.d. (BSA ≥1.5 m$^2$). A CT scan was performed during this 2-week interval to evaluate the tumor response according to the Response Evaluation Criteria in Solid Tumors (RECIST). When the tumor showed a complete response (CR) or partial response (PR) in the first course, the same dose of S-1 monotherapy was continued unless progressive disease (PD) was observed. When the response was stable disease (SD), S-1 was administered at the same dose for only 2 weeks (days 1-15), no drug was administered for the following 2 weeks (4-week cycle) and CPT-11 was administered intravenously at 100 mg/m$^2$ on days 1 and 15 (concurrent S-1/CPT-11) unless PD was observed. In the case of PD, the study was terminated. There was no restriction in third-line chemotherapy. Adverse events were evaluated using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE), version 3.0. The protocol was approved by the Institutional Review Board of Hirosaki University School of Medicine and other institutes. The primary endpoint of this study was an antitumor effect and secondary endpoints were median survival time (MST), progression-free survival (PFS), time-to-treatment failure (TTF) and safety.

*Statistical analysis.* For prognostic values, the MST, PFS and TTF were calculated using the Kaplan-Meier method from the date of registration. The estimated RR and threshold RR were set at 15 and 5%, respectively, and the calculated minimum sample size was estimated to be 43, with an $\alpha$ value of 0.05 and a $\beta$ value of 0.20. We estimated that the number of possible patient exclusions or dropouts would be 2, and the sample size was increased to 45. In the planned interim analysis, when the number of response cases was ≤1 out of 12 enrolled cases, the study would be terminated since the estimated RR would be <5%.

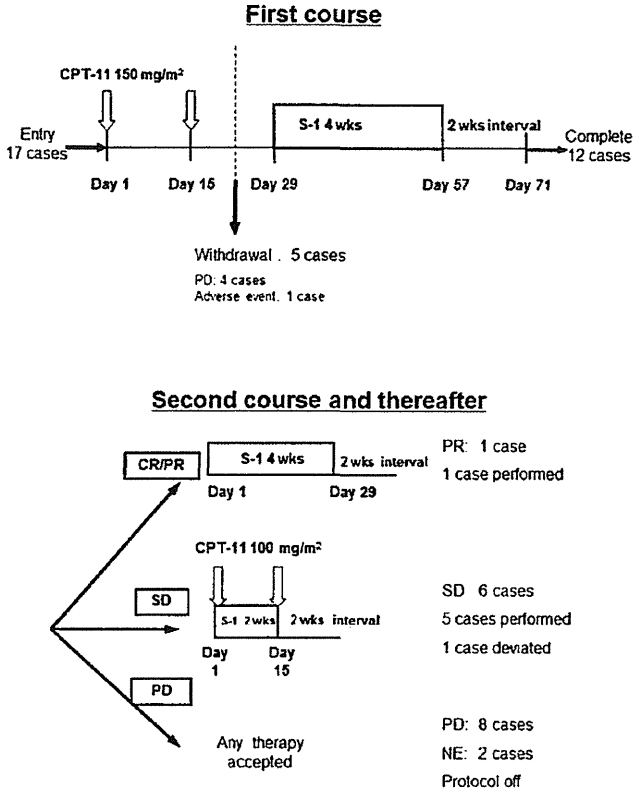


Figure 1. Protocol design of the CPT-11/S-1 sequential study. In the first course, 150 mg/m$^2$ CPT-11 (days 1 and 15) and 4-week S-1 (day 29 to 57) was administered followed by a 2-week interval (sequential S-1/CPT-11). In case of CR or PR, the same dose of S-1 monotherapy was continued unless PD was observed. For SD, S-1 (days 1-15), and 100 mg/m$^2$ CPT-11 (days 1 and 15) was administered in a 4-week cycle (concurrent S-1/CPT-11) unless PD was observed. In the case of PD, the study was terminated.

## Results

*Patient characteristics.* Patient characteristics are shown in Table I. A total of 17 patients (14 males and 3 females), were enrolled in this study between November 2004 and June 2006. The median age was 63 years, and a PS of 0/1 was observed in 14/3 patients, respectively. Histological results revealed that 8 patients had intestinal and 9 had diffuse cancer types. Eleven patients had primary lesions and 5 did not, and no information was available in 1 case. Prior to enrollment in this study, 7 patients underwent surgical treatment and 10 patients did not. Previous chemotherapy regimens were S-1 monotherapy (n=9), S-1/CDDP (n=7) and S-1/taxotere (TXT) (n=2). None of the patients had received radiotherapy.

Table I. Patient characteristics.

| Characteristics | Patient no. |
|---|---|
| Gender (M/F) | 14/3 |
| Age (mean/median) | 59.6/63 |
| Performance status (0/1) | 14/3 |
| Untreated/recurrence | 10/7 |
| Histology (intestinal/diffuse) | 8/9 |
| Primary lesion (+/-/unknown) | 11/5/1 |
| Metastasis | |
| Lung | 2 |
| Liver | 8 |
| Bone | 1 |
| Abdominal lymph node | 11 |
| Other | 5 |
| Radiotherapy (+/-) | 0/17 |
| Surgical operation (+/-) | 7/10 |
| Prior chemotherapy regimen | |
| S-1 | 8 |
| S-1/CDDP | 7 |
| S-1/taxotere | 2 |
| Detail of S-1 resistance | |
| Unresectable and formerly effective | 5 |
| Unresectable and formerly resistant | 5 |
| Recurrence during adjuvant chemotherapy | 5 |
| Recurrence after adjuvant chemotherapy | 2 |

Table II. Tumor response.

A, After the first course.

| n | CR | PR | SD | PD | NE | RR |
|---|---|---|---|---|---|---|
| 17 | 0 | 1 | 6 | 8 | 2 | 5.9% |

B, Overall.

| n | CR | PR | SD | PD | NE | RR |
|---|---|---|---|---|---|---|
| 17 | 0 | 2 | 5 | 8 | 2 | 11.8% |

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; RR, response rate.

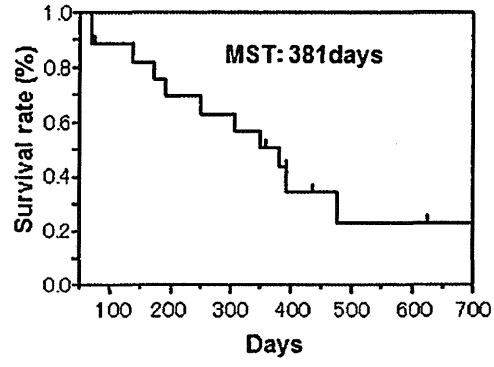


Figure 2. Survival curve of the CPT-11/S-1 sequential study. The MST was calculated using the Kaplan-Meier method from the date of registration and was 381 days.

*Tumor response and survival rate.* Five out of 17 patients ceased chemotherapy halfway through the first course of treatment due to PD in 4 patients and an adverse event in 1 patient. Twelve patients completed the first course. However, 5 patients out of 12 dropped out due to PD following completion of the first course. The tumor response following the first cycle is shown in Table II. Only 1 patient showed PR following the first course (RR, 5.9%; 95% CI, 0.1-28.7). The PR patient received 2 cycles of S-1 monotherapy and the 5 SD patients received a median of 2 cycles (mean 3.6 cycles; range 1-6) of concurrent S-1/CPT-11. One of the 5 SD patients showed PR during the S-1/CPT-11 chemotherapy. Therefore, the most positive tumor response rate was 2 PR, 5 SD, 8 PD and 2 not evaluable (NE) (RR, 11.8%; 95% CI, 1.5-31.4) (Table II). An overall survival curve is shown in Fig. 2. Regarding the prognostic analysis by the log-rank test, the MST, median TTF and PFS were 381, 69 and 71 days, respectively.

Retrospectively, we performed a subset analysis using stratification by different patient background of S-1 resistance. We compared the S-1-sensitive group (for which the first-line chemotherapy was effective or 6-month adjuvant chemotherapy was completed) and the S-1-resistant group (for which the first-line chemotherapy was not effective or adjuvant chemotherapy was not completed). However, no significant difference in MST, TTF or PFS was observed in the subset analysis.

After 17 cases had enrolled in this study, an interim analysis was performed according to the initial schedule. Since the RR following the first course was only 5.9% and 10 cases received only the first course of chemotherapy, this protocol was evaluated as being unfeasible.

*Toxicity.* Observed toxicities associated with this chemotherapy protocol are shown in Table III. The most common toxicities were leukocytopenia and neutropenia in both the first and subsequent courses (58.8 and 66.7%, respectively). Nausea and vomiting were also common. One patient had G4 neutropenia and a further patient had G4 anorexia. Diarrhea, a common adverse event of CPT-11 and S-1, was observed more frequently in concurrent S-1/CPT-11 chemotherapy, but not in the sequential regimen. Chemotherapy-related mortality was not observed.

## Discussion

This trial tested the feasibility of sequential CPT-11 and S-1 in S-1-refractory gastric cancer patients. The RR following the first course was only 5.9% and the most positive RR was 11.8% (Table II). The MST was 381 days (Fig. 2) and the median TTF and PFS were 69 and 71 days, respectively. Therapy-related mortality was not observed, although leukocytopenia and neutropenia were observed in over half of the patients (Table III), indicating moderate toxicity of this protocol. Since the RR following the first course was lower than that estimated in an interim analysis, the trial was terminated.

Table III. Adverse events during the course of the study.

A, First course.

| n=17 | G1 | G2 | G3 | G4 | All grades (%) | >G3 (%) |
|---|---|---|---|---|---|---|
| Leukocytopenia | 3 | 5 | 2 | 0 | 58.8 | 11.8 |
| Neutropenia | 1 | 3 | 5 | 1 | 58.8 | 35.3 |
| Anemia | 1 | 3 | 2 | 0 | 35.3 | 11.8 |
| Thrombocytopenia | 1 | 0 | 0 | 0 | 5.9 | 0.0 |
| Nausea/vomiting | 4 | 1 | 3 | 0 | 47.1 | 17.6 |
| Stomatitis | 0 | 1 | 0 | 0 | 5.9 | 0.0 |
| Diarrhea | 1 | 2 | 1 | 0 | 23.5 | 5.9 |
| Exanthema | 0 | 0 | 1 | 0 | 5.9 | 5.9 |
| Alopecia | 3 | 3 | 0 | 0 | 35.3 | 0.0 |
| Anorexia | 1 | 0 | 2 | 1 | 23.5 | 17.6 |
| General malaise | 2 | 1 | 3 | 0 | 35.3 | 17.6 |
| Abdominal pain | 0 | 1 | 0 | 0 | 5.9 | 0.0 |
| Abdominal distension | 0 | 1 | 0 | 0 | 5.9 | 0.1 |

B, After the first course.

| n=6 | G1 | G2 | G3 | G4 | All grades (%) | >G3 (%) |
|---|---|---|---|---|---|---|
| Leukocytopenia | 1 | 3 | 0 | 0 | 66.7 | 0.0 |
| Neutropenia | 1 | 0 | 2 | 1 | 66.7 | 50.0 |
| Anemia | 0 | 0 | 1 | 0 | 16.7 | 11.8 |
| Nausea/vomiting | 3 | 0 | 0 | 0 | 50.0 | 17.6 |
| Stomatitis | 1 | 0 | 0 | 0 | 16.7 | 0.0 |
| Diarrhea | 0 | 2 | 1 | 0 | 50.0 | 5.9 |
| Alopecia | 1 | 1 | 0 | 0 | 33.3 | 0.0 |
| Anorexia | 2 | 1 | 1 | 0 | 66.6 | 17.6 |
| General malaise | 1 | 1 | 0 | 0 | 33.3 | 17.6 |
| Skin pigmentation | 1 | 0 | 0 | 0 | 16.7 | 0.1 |

The rationale of this study was that a preceding administration of CPT-11 against S-1-resistant tumors may affect the downregulation of TS, resulting in recovered sensitivity to S-1 (6). We chose the sequential, rather than concurrent, administration of CPT-11 and S-1, for two reasons. The first was that the downregulation of TS requires a certain period of time to take effect following CPT-11 administration. The second was that avoidance of concurrent administration of these drugs may alleviate adverse effects and achieve a longer continuity period of chemotherapy.

Practical uses of the S-1/CPT-11 combination have been reported (7) as a second-line therapy. It was reported that the S-1 monotherapy, following failure of preceding S-1-containing regimens, was worthy of testing in larger-scale clinical trials. However, a recent study negatively evaluated this re-use of S-1 (8). This strongly suggests that continuation of S-1 administration following failure is pointless.

Consequently, the reversal of S-1 resistance indicates a potential way to reuse this drug. DPD, TS and CYP2A6 are involved in fluorouracil drug resistance, and modulators of these enzymes are candidates for concurrent or sequential usage. It is reported that inter-individual deviation in the gene expression and activity of these enzymes are associated with the ability to predict the effects of the chemotherapy (9-12). This suggests that tailor-made chemotherapy using intratumoral TS activity is possible. Using a xenograft model Fukushima *et al* reported that CPT-11 reversed chemo-resistance against 5-FU (6). The concept of our study originated from this application to clinical use.

As a result, the overall RR was 11.8% and the TTF, PFS and MST were 69, 71 and 381 days, respectively. The TTF and PFS were relatively short but the MST was long. However, with regard to our main aim of testing, the reversal of S-1 resistance by a preceding treatment of CPT-11 was unfeasible since the RR following the first cycle was lower than estimated.

We investigated whether the time point of S-1 resistance acquisition affected the RR of this study by additional subset analyses. The previously resistant group tended to demonstrate a poor response, although the difference was not significant. This may be due to the small sample size.

Three patients withdrew from the study due to adverse events, rather than PD. A number of ≥G3 non-hematological adverse events were observed including 3 cases of nausea and vomiting, 1 of diarrhea, 1 of exanthema, 2 of anorexia, 3 of

general malaise, and one ≥G4 hematological adverse event in the first course. Nine G3 hematological adverse events also occurred. Intensity of CPT-11 administration was found to be over the recommended dose, although we did not determine the uridine-diphosphate glucuronosyltransferase (UGT) 1A1 polymorphism (13).

A randomized, multicenter phase II/III study, JACCRO GC-05, comparing CPT-11/S-1 and CPT-11 monotherapy as a second-line therapy in S-1-resistant patients with advanced gastric cancer is currently under way. Future findings may thus reveal whether CPT-11 provides any favorable effects on S-1 resistance, which was not shown in the present study.

In conclusion, S-1/CPT-11 sequential therapy based on the reversal of S-1 drug resistance by CPT-11 was tested, although the interim analysis revealed a lower response rate than expected. This protocol was concluded as being unfeasible.

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# ≪小細胞肺癌治療の考え方と実践≫
# 二次治療のエビデンス

石黒　敦　西條康夫*


## 要旨

- 小細胞肺癌に対する初回導入化学療法としてcisplatin＋irinotecan，あるいはcisplatin＋etoposideによる併用療法4コースが標準であり，維持化学療法は行われない．
- 再発小細胞肺癌の二次治療に際しては初回治療終了後から再発までの期間が重要であり，再発部位，performance status(PS)などを考慮する必要がある．
- 二次治療における標準的な化学療法は確立していない．
- 肺癌診療ガイドライン(2011年3月)によれば，sensitive relapseでは再発時の化学療法の効果が高く，re-challengeをはじめ化学療法を行うように勧められているが，refractory relapseに対する化学療法の意義は確立していない．


非小細胞肺癌に比べ，小細胞肺癌に有効な薬剤の開発は停滞している．その結果，近年では小細胞肺癌における化学療法の効果の改善も乏しい．小細胞肺癌の二次治療において，標準治療は決まっていない．しかしながら，臨床試験の結果をみながら，小細胞肺癌の二次治療について考えてみたい．

## 再発までの期間による二次治療効果の差異

再発小細胞肺癌は初回治療に比して化学療法や放射線療法に対する感受性が不良であるが，初回治療終了後から再発までの期間が長い症例のほうが，化学療法の有効性が高いことが報告されている[1,2]．したがって，初回化学療法が奏効し，初回治療終了後から再発までの期間が長い症例(60～90日以上)を“sensitive relapse(治療感受性の再発)”として，初回治療が奏効していない，または初回治療終了後から再発までの期間が短い症例を“refractory relapse(治療抵抗性の再発)”として治療が行われている．1984年から2011年にかけて21の臨床研究が対象となり，sensitive relapse(再発までの期間が90日以上)およびrefractory relapseに対する系統的解析が報告された[3]．再発小細胞肺癌1,692例(refractory relapse 780例/

キーワード：sensitive relapse, refractory relapse, nogitecan(NGT), amrubicin(AMR).

* A. Ishiguro：弘前大学腫瘍内科学；Y. Saijo(教授)：新潟大学医学部総合医学教育センター腫瘍学分野．

Table 1. NGTによる再発小細胞肺癌に対する主な治療成績

| 著者 | 発表年 | phase | 投与量 (mg/m²) | 化学療法感受性 | 症例数 | 奏効率 (%) | MST (月) | grade 3/4(%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 好中球減少 | FN |
| Takeda et al[6] | 2003 | Ⅱ | 1.0 | sensitive | 50 | 26.0 | 9.3 | 92 | 24 |
| Inoue et al[12] | 2008 | Ⅱ | 1.0 | sensitive | 19 | 21.0 | 11.7 | 87 | 3 |
| | | | | refractory | 11 | 0.0 | 5.4 | | |
| Jotte et al[14] | 2011 | Ⅲ | 1.5(iv) | sensitive | 117 | 16.9 | 9.9 | 53.3 | 3.6 |
| | | | | refractory | 96 | | 5.7 | | |

FN：発熱性好中球減少症.

sensitive relapse 912例)における奏効率，中央生存期間(MST)はそれぞれ14.8%/27.7%($p$=0.0001)，5.45ヵ月/7.73ヵ月($p$=0.0035)であり，sensitive relapse群で良好な治療効果と生存期間の延長が有意に示された．また，refractory relapse群はさらなる新規治療の開発が必要であるものの，一定の治療効果が得られることが確認された．

## 二次治療におけるレジメン選択の考え方

二次治療においてbest supportive care(BSC)との唯一の無作為化比較第Ⅲ相試験が，初回治療終了後45日以上経過して再発を認めたsensitive relapseを対象に検討が行われた[4]．主要評価項目であるMSTは経口nogitecan(NGT，欧米ではtopotecan)群(2.3 mg/m²，day 1～5)25.9週に対して，BSC群では13.9週であり，また，NGT群の奏効率は7%と低かったが，44%がstable diseaseとなったことで化学療法群であるNGTによる生存期間の延長が認められた($p$=0.01)．

比較的全身状態が保たれたsensitive relapse症例に対して，初回化学療法と同じレジメンを再投与すること(re-challenge)の有効性が報告されているが，1980年代の報告であり，その意義は確立していない(日本肺癌学会：肺癌診療ガイドライン，2011年3月)．前述の系統的解析によれば有効性が期待できる化学療法レジメンは限られていることから，sensitive relapseへのre-challengeを推奨しているが，2012年の米国臨床腫瘍学会(ASCO)では再発までの期間が90日以上であったsensitive relapse症例65例において，re-challenge群(19例)と他剤治療群(46例)(46例中21例はamrubicin(AMR)投与)とを比較検討した成績が報告された．MSTでは両群間に有意差はなく，re-challengeを試みるよりもまずはAMRのような単剤での治療が推奨される結果であった[5]．欧米と本邦では一次治療から二次治療にわたり選択される化学療法レジメンが異なる背景などがあり，re-challengeについては前向き臨床試験による再検証を要するものと考えられる．

## 二次治療において推奨される化学療法レジメン

複数の比較試験や第Ⅱ相試験により，etoposide(VP-16)，irinotecan(CPT-11)，AMR，NGTなどの有効性が報告されているが，二次治療における標準的な化学療法は確立していない．NGTは米国食品医薬局(FDA)に認可されている唯一の薬剤であり，また，多くの国々での二次治療における第一選択薬となっている．一方，AMRは本邦において検討が多くなされ，その有効性が認められてきたが，現在のところ本邦のみの承認である．

NGTはDNAの複製や転写などの機能に関わるトポイソメラーゼⅠ阻害薬であり，欧米での承認用量は1.5 mg/m²であるが，本邦では開発段階で重篤な有害事象を認めたため，1.0 mg/m²の5日間連日点滴静注，3週ごとでの投与が行われている．その第Ⅱ相試験では奏効率26.0%，MSTは262日と報告された(Table 1)[6]．有害事象のうちgrade 3以上の好中球数減少は84.5%であった

Table 2. AMRによる再発小細胞肺癌に対する主な治療成績

| 著者 | 発表年 | phase | 投与量 (mg/m²) | 化学療法感受性 | 症例数 | 奏効率 (%) | MST (月) | grade 3/4(%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 好中球減少 | FN |
| Kato et al[9] | 2006 | II | 45 | sensitive | 24 | 50.0 | 10.4 | 97 | 35 |
| | | | | refractory | 10 | 60.0 | 6.8 | | |
| Onoda et al[10] | 2006 | II | 40 | sensitive | 44 | 52.0 | 11.6 | 83 | 5 |
| | | | | refractory | 16 | 50.0 | 10.3 | | |
| Inoue et al[12] | 2008 | II | 40 | sensitive | 17 | 53.0 | 9.9 | 93 | 14 |
| | | | | refractory | 12 | 17.0 | 5.3 | | |
| Ettinger et al[13] | 2010 | II | 40 | refractory | 75 | 21.0 | 6.0 | 67 | 12 |
| Jotte et al[14] | 2011 | III | 40 | sensitive | 225 | 31.0 | 9.2 | 41.2 | 9.3 |
| | | | | refractory | 199 | | 6.2 | | |
| Kaira et al[11] | 2010 | II | 35 | sensitive | 10 | 60.0 | 12.0 | 41.4 | 3.4 |
| | | | | refractory | 19 | 36.8 | 11.0 | | |

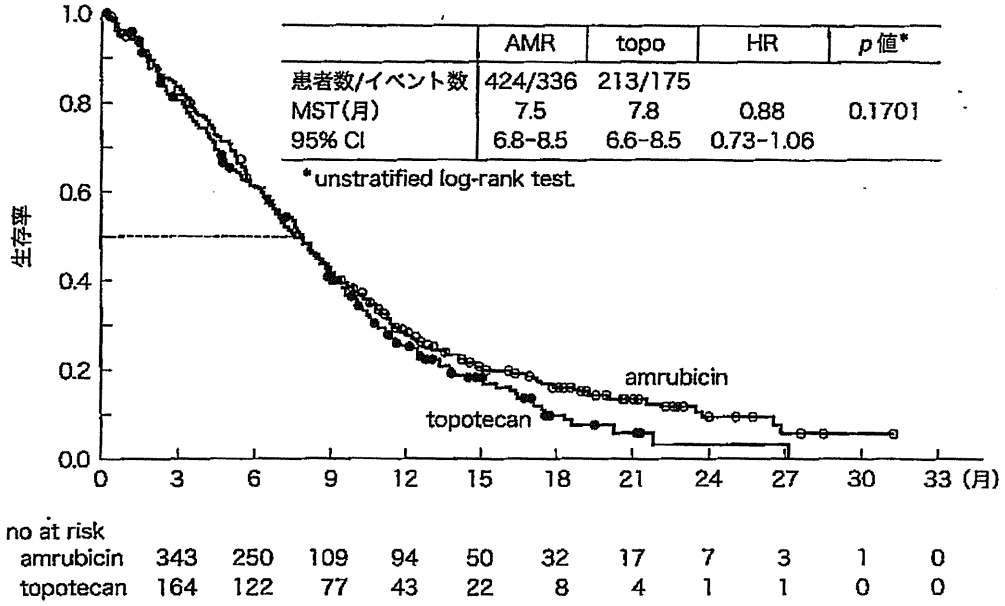
が，これらはG-CSF投与の有無にかかわらず，大部分の症例において，投与開始から14～21日間(中央値)で回復を認め，コース数を重ねることによる悪化傾向は示さなかった[7]．また，NGTは未変化体自体が活性体であり腸肝循環しないことから，CPT-11に比して重篤な下痢をきたすことは少ない(grade 3以上，1.0%)．

経口NGT 2.3 mg/m²と静注NGT 1.5 mg/m²についてのランダム化比較第III相試験では，生存をはじめ有効性，毒性や三次治療への移行率に関して有意差は認められなかった．利便性に優れることから米国では経口薬が多く用いられるようになったが，本邦では注射製剤のみの承認となっている[8]．

AMRは本邦で開発されたアントラサイクリン系のトポイソメラーゼII阻害薬であり，主な有害事象は血液毒性で，心毒性をきたしにくいといわれている．本邦からは再発小細胞肺癌に対して用量設定が異なった4つの第II相試験が報告されている(Table 2)．AMR 45 mg/m²を投与した試験では34例(refractory relapse 10例，sensitive relapse 24例)において奏効率，MSTが，refractory relapseで60%，6.8ヵ月，sensitive relapseで50%，10.4ヵ月であった[9]．AMR 40 mg/m²を投与した試験では60例(refractory relapse 16例，sensitive relapse[60日以上]44例)において奏効率，PFS，MSTはrefractory relapseで50%，2.6ヵ月，10.3ヵ月，sensitive relapseで52%，4.2ヵ月，11.6ヵ月であった[10]．AMR 35 mg/m²による試験では29例(refractory relapse 19例，sensitive relapse[90日以上]10例)において奏効率，PFS，MSTはrefractory relapseで36.8%，4.0ヵ月，11.0ヵ月，sensitive relapseで60.0%，4.0ヵ月，12.0ヵ月であった[11]．

有害事象としてgrade 3/4の好中球減少(45 mg/m²：97% vs. 35 mg/m²：41.4%)や発熱性好中球減少(45 mg/m²：35% vs. 35 mg/m²：3.4%)は用量依存性に頻度が高く，投与量決定に際しては年齢，PSを含めた全身状態の再評価を要するものと考えられる[2]．また，AMRはrefractory relapseに対してもsensitive relapseに劣らない有効性が示唆されたが，AMR(40 mg/m²，day 1～3)とNGT(1.0 mg/m²，day 1～5)とのランダム化比較第II相試験の結果は異なるものであった[12]．

この試験には60例が登録され，59例が評価可能であった(refractory relapse 23例，sensitive




| | AMR | topo | HR | p値* |
|---|---|---|---|---|
| 患者数/イベント数 | 424/336 | 213/175 | | |
| MST(月) | 7.5 | 7.8 | 0.88 | 0.1701 |
| 95% CI | 6.8-8.5 | 6.6-8.5 | 0.73-1.06 | |

*unstratified log-rank test.

| no at risk | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| amrubicin | 343 | 250 | 109 | 94 | 50 | 32 | 17 | 7 | 3 | 1 | 0 |
| topotecan | 164 | 122 | 77 | 43 | 22 | 8 | 4 | 1 | 1 | 0 | 0 |

**Fig. 1. AMR 群と NGT 群の無作為化第III相試験(ACT-1 試験)**

relapse[90 日以上]36 例)．奏効率，PFS，MST は AMR 群で 38%，3.5ヵ月，8.1ヵ月(refractory relapse：17.0%，2.6ヵ月，5.3ヵ月，sensitive relapse：53.0%，3.9ヵ月，9.9ヵ月)，NGT 群では 13%，2.2ヵ月，8.4ヵ月であった(refractory relapse：0.0%，1.5ヵ月，5.4ヵ月，sensitive relapse：21.0%，3.0ヵ月，11.7ヵ月)．NGT 群において三次治療として大部分に AMR が選択され，多変量解析の結果から AMR は NGT に比して生存期間延長に寄与する可能性があり，再発小細胞肺癌に対しては AMR の投与機会を逸することなく治療を行うべきであることが示唆された．

欧米では AMR の効果と安全性を評価するため，AMR(40 mg/m$^2$, day 1〜3)と NGT(1.5 mg/m$^2$, day 1〜5)を比較する無作為化第Ⅲ相試験が実施され，2011 年の ASCO において報告された[14]．637 例が登録され，AMR 群(424 例)と NGT 群(213 例)に 2：1 の割合で無作為に割り付けられた(sensitive relapse[90 日以上])．主要評価項目である MST では AMR 群 7.5ヵ月，NGT 群 7.8ヵ月であり，有意差を認めなかったが($p$= 0.1701，ハザード比 0.880[0.733-1.057])(Fig. 1)，副次評価項目である奏効率は 31.1% vs. 16.9% ($p$= 0.0001)，PFS は 4.1ヵ月 vs. 3.5ヵ月($p$= 0.0182)であり，いずれも AMR 群のほうが有意に優れていた．食欲，咳，呼吸困難といった臨床症状の改善効果に関しても，AMR 群で有意な改善が認められた．さらにサブセット解析では，refractory relapse に限れば，AMR 群での有意な改善が認められた(6.2ヵ月 vs. 5.7ヵ月，$p$= 0.0469，ハザード比 0.766[0.589-0.997])(Fig. 2)．以上より，AMR は NGT と同等の有用性をもつことが欧米においても確認された．

sensitive relapse の場合，re-challenge は一つの治療選択肢である．二次治療では etoposide と irinotecan 両薬剤ともに単剤での効果は不十分なため，一次治療と同様にプラチナ製剤を併用すべきであるとされる[15]．再発まで 8 週以上経過した sensitive relapse を対象に G-CSF を併用しながら cisplatin＋VP-16 と cisplatin＋CPT-11 による毎週交替投与を行う PEI 療法の第Ⅱ相試験が実施された[16]．奏効率 78%，MST 11.8ヵ月との良好な成績であり，NGT に対する PEI 療法の優越性を検証する第Ⅲ相試験が進行している．分子標的治療薬としてチロシンキナーゼ阻害薬(imatinib, gefitinib)，血管新生阻害薬(bevacizumab)，farnesyl