Overexpression of *SUGT1* in human colorectal cancer and its clinicopathological significance

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Abstract. As recent technological innovations make it possible to clarify the concordant relationship between genomic alterations and aberrant gene expression during the progression of colorectal cancer (CRC), we aimed at identifying new overexpressing genes with genomic amplification on the responsible loci in CRC. The candidate gene was found using cDNA microarray and array-based comparative genomic hybridization (CGH) analysis after laser microdissection (LMD) in 132 Japanese CRC. We focused on *SUGT1*, which is associated with the assembling of kinetochore proteins at the metaphase of the cell cycle, with significant association between genetic alterations and expression. *SUGT1* mRNA expression was evaluated in 98 CRC cases to determine the clinicopathological significance of *SUGT1* expression. The mean level of *SUGT1* mRNA expression in tumor tissue specimens was significantly higher than in non-tumor tissue. The high *SUGT1* expression group was characterized by a significantly elevated frequency of recurrence and a significantly poorer prognosis than the low expression group. There was a significant association between poor prognosis of CRC cases and the overexpression of *SUGT1* with genomic amplification of the loci concordantly. The amplification of SUGT1 might give rise to promote the transcription of the gene directly subsequent to the progression of CRC cases with worsening prognosis.

Introduction

The morbidity and mortality rates of colorectal cancer (CRC) in the United states and Europe have decreased recently (1,2), yet further studies are required to better understand this disease. Recent technological innovations now permit us to clarify DNA copy number alterations in CRC by using high resolution, array-based comparative genomic hybridization (array CGH), resulting in the definition of a specific pattern of DNA gains or losses (3-7). Tsafrir *et al* reported a correlation between gene expression and chromosomal aberrations in CRC using array CGH and cDNA microarray analysis (8). Their study focused on particular chromosomal regions and genes that are frequently gained and overexpressed (e.g., 7p, 8q, 13q and 20q) or lost and underexpressed (e.g., 1p, 4, 5q, 8p, 14q, 15q and 18). Based on these data, they identified genetic alterations which had prognostic significance.

From a technical point of view, it is easier to handle genomic DNA than mRNA, as the latter is fragile, and unstable. Therefore, we sought to identify genes whose impact could be determined by assessment of genomic copy number. We employed cDNA microarray analysis to identify candidate molecular markers that play a significant role in the progression of CRC by comparing resected cancer cells and the corresponding non-malignant cells extracted by means of laser microdissection (LMD) (9-11). Then, we performed cDNA microarray and array CGH analysis after LMD in a large scale study of Japanese CRC patients to identify new genomically amplified genes and determine the impact of their presence.

In the present study, we focused on a gene at 13q that is known to be frequently amplified (6,8,12) and shed light on the overexpression of *SUGT1* (suppressor of G2 allele of SKP1:13q14.3). We examined its expression and determined the correlation of gene with genomic amplification with CRC outcome. Originally, *SUGT1* was identified as a protein activating the kinetochore and SCF ubiquitin ligase complexes via interaction with Skp1 protein, which plays a crucial role in cell cycle regulation (13). The present study clarifies the clinical significance of the expression of SUGT1 in human CRC cases.

Materials and methods

Laser micro-dissection. The tissues from a series of 132 patients with CRC were collected for laser micro-dissection

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(LMD; Leica Laser Microdissection System, Leica Microsystems, Wetzlar, Germany) as previously described (14). For LMD, 5 μm frozen sections were fixed in 70% ethanol for 30 sec, stained with hematoxylin and eosin and dehydrated as follows: 5 sec each in 70, 95 and 100% ethanol and a final 5 min in xylene. Sections were air-dried, then micro-dissected by LMD. Target cells were excised, ≥100 cells per section, and bound to the transfer film, and total DNA extracted.

cDNA microarray. We used the commercially available Human Whole Genome Oligo DNA Microarray Kit (Agilent Technologies, Santa Clara, CA, USA). A list of genes on this cDNA microarray is available from http://www.chem.agilent.com/scripts/generic.asp?lpage=5175&indcol=Y&prodcol=Y&prodcol=N&indcol=Y&prodcol=N. Cyanine (Cy)-labeled cRNA was prepared using T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Manual (Agilent Technologies). Labeled cRNA was fragmented and hybridized to an oligonucleotide microarray (whole human genome 4x44K Agilent G4112F). Fluorescence intensities were determined with an Agilent DNA microarray scanner and were analyzed using G2567AA Feature Extraction Software Version A.7.5.1 (Agilent Technologies), which used the LOWESS (locally weighted linear regression curve fit) normalization method (15). This microarray study followed MIAME guidelines issued by the Microarray Gene Expression Data group (16). Further analyses were performed using GeneSpring version 7.3 (Silicon Genetics, San Carlos, CA, USA).

Array-CGH. Array-CGH was performed using the Agilent Human Genome Microarray Kit 244K (Agilent Technologies). The array-CGH platform is a high resolution 60-mer oligonucleotide-based microarray containing ~244,400 probes spanning coding and non-coding genomic sequences with median spacing of 7.4 and 16.5 kb, respectively. Labeling and hybridization were performed according to the protocol provided by Agilent (Protocol v4.0, June 2006). Arrays were analyzed using Agilent DNA microarray scanner.

Array-CGH data analysis. The raw signal intensities of tumor DNAs were measured with Human Genome CGH Microarray 244K (Agilent Technologies) which were then transformed into log ratios to reference DNA with 'Feature Extraction' software (v9.1) of Agilent Technologies. The log ratio was thereafter used as the signal intensity of each probe. One hundred and thirty samples from different patients were subjected to circular binary segmentation (CBS) after median normalization (17). An R script written by us was used for the median normalization, whereas an R program implemented in the 'DNA copy' package of the Bioconductor project (http://www.bioconductor.org) was used for the CBS analysis. Instead of all of the CGH probes, 13,403 probes from chromosome 4 (NCBI Build 35) were analyzed in this study. An absolute log2 ratio >0.263 was used as the threshold for the gain or loss in DNA copy number for each probe.

Clinical samples. The tissues from another series of 98 CRC patients with information about clinicopathological features including prognosis were collected at Kyushu University at Beppu and affiliated hospitals between 1993 and 1999. Resected tumor and paired non-tumor tissue specimens were immediately cut from resected colon and placed in RNA Later (Takara, Shiga, Japan) or embedded in Tissue Tek OCT medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen and kept at -80˚C until RNA extraction. The median follow-up period was 3.0 years. Written informed consent was obtained from all patients and the study protocol was approved by the local ethics committee.

Total RNA extraction and first-strand cDNA synthesis. Frozen tissue specimens were homogenized, and the total RNA was extracted using the modified acid-guanidine-phenol-chloroform method as described previously (18,19). Total RNA (8.0 μg) was reverse transcribed to cDNA using M-MLV RT (Invitrogen Corp. Carlsbad, CA, USA).

Quantitative RT-PCR. The primer sequences for *SUGT1* mRNA were as follows: sense, 5'-CTG ACT AAG GCT TTG GAA CAG AA-3'; antisense, 5'-CTG TAA AAG TTT CTA GGG CAG CA-3'. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control and *GAPDH* primers were as follows: sense, 5'-TTG GTA TCG TGG AAG GAC TCT A-3'; and antisense, 5'-TGT CAT ATT TGG CAG GTT-3'. Real-time monitoring of PCR reactions was performed using the LightCycler™ system (Roche Applied Science, Indianapolis, IN, USA) and SYBER-Green I dye (Roche). Monitoring was performed according to the manufacturer's instructions, as described previously (20). In brief, a master mixture was prepared on ice, containing 1 μl of cDNA, 2 μl of DNA Master SYBER-Green I mix, 50 ng of primers and 2.4 μl of 25 mM $MgCl_2$. The final volume was adjusted to 20 μl with water. After the reaction mixture was loaded into glass capillary tubes, quantitative RT-PCR was performed with the following cycling conditions: initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec, annealing at 62˚C for 10 sec and extension 72˚C for 10 sec. After amplification, products were subjected to a temperature gradient from 67 to 95˚C at 0.2˚C/sec, under continuous fluorescence monitoring, to produce a melting curve of products.

Data analysis for quantitative RT-PCR. We used LightCycler Software version 3.5 program (Roche Molecular Biochemicals) to calculate the cycle numbers. After proportional baseline adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was first distinguishable from the baseline. This cycle number was used as the crossing point value. A standard curve was produced by measuring the crossing point of each standard value and plotting it against the logarithmic value of concentration. Concentrations of unknown samples were calculated by plotting their crossing points against the standard curve and dividing by *GAPDH* content. *GAPDH* expression confirmed no differences between tumor and normal tissue.

Immunohistochemistry. Immunohistochemistry studies of SUGT1 were performed on formalin-fixed, paraffin-embedded surgical sections obtained from patients with CRC. Tissue

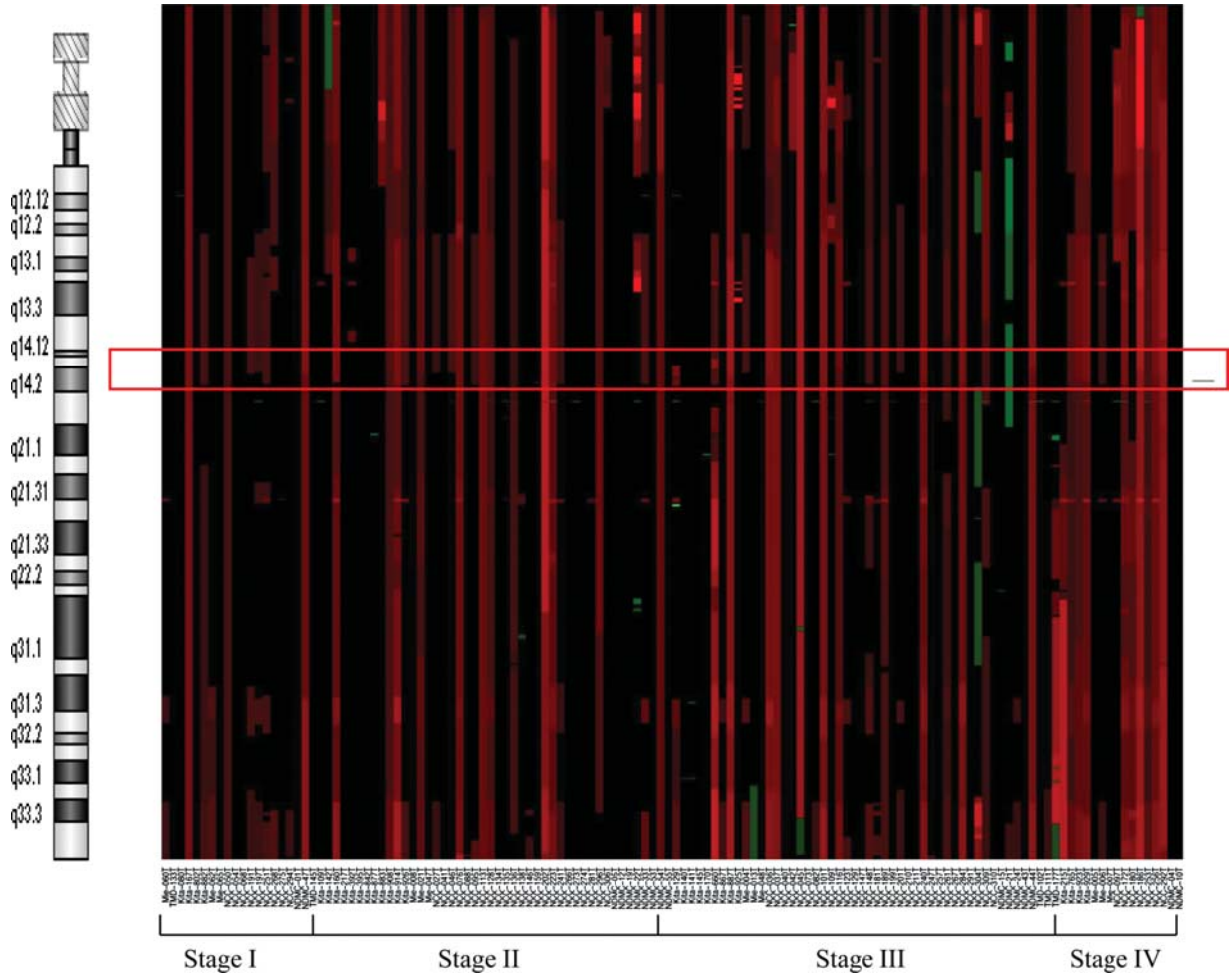

Figure 1. Heat-map representation of *SUGT1* copy number aberrations in CRC specimens determined by CGH array. Each column represents CRC cases arranged according to TNM staging. *SUGT1* location is enclosed in lines. Copy number loss is indicated in green, gain in red.

sections were deparaffinized, soaked in 0.01 M sodium citrate buffer and boiled in a microwave for 5 min at 500 W to retrieve cell antigens. The primary rabbit polyclonal antibodies against SUGT1 (Protein Tech Group Inc., Chicago, IL, USA) were used at a dilution of 1:150. Tissue sections were immunohistochemically stained using EnVision reagents (EnVision+ Dual Link System-HRP, Dako Cytomation, Glostrup, Denmark). All sections were counterstained with hematoxylin.

Statistical analysis. For continuous variables, data were expressed as the means ± SD. The relationship between *SUGT1* mRNA expression and clinicopathological factors was analyzed using a χ^2 test and Student's t-test. Overall survival curves were plotted according to the Kaplan-Meier method and the generalized log-rank test was applied to compare the survival curves. All tests were analyzed using JMP software (SAS Institute Inc., Cary, NC) and the findings were considered significant when p-value was <0.05.

Results

Aberrations in SUGT1 copy number in CRC specimens. We investigated copy number aberrations in 132 CRC specimens using laser micro-dissection and CGH array. We focused on chromosome 13q which is reportedly amplified in CRC. Fig. 1 shows the heat map representation of copy number aberrations in 13q according to TNM staging classification. As shown in Fig. 2, there is a significant association between genetic alterations and expression of *SUGT1* (correlation coefficient: 0.665, p<0.0001). Therefore, gain of *SUGT1* expression was caused by genetic alteration in the flanking region of *SUGT1*. We noted copy number amplification in 44 cases (33.3%). The frequency of copy number amplification of *SUGT1* increased along with the progression of TNM stage.

Expression of SUGT1 mRNA in CRC cases. *SUGT1* mRNA expression in 98 clinical tissue specimens was examined by reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR, with quantified values used to calculate *SUGT1/GAPDH* ratios. Results indicated that the mean level of expression of *SUGT1* mRNA in tumor tissue specimens was significantly higher than that in non-tumor tissue (p=0.0009) (Fig. 3A).

Immunohistochemistry of SUGT1 protein expression in CRC cases. Expression of SUGT1 protein was evaluated by

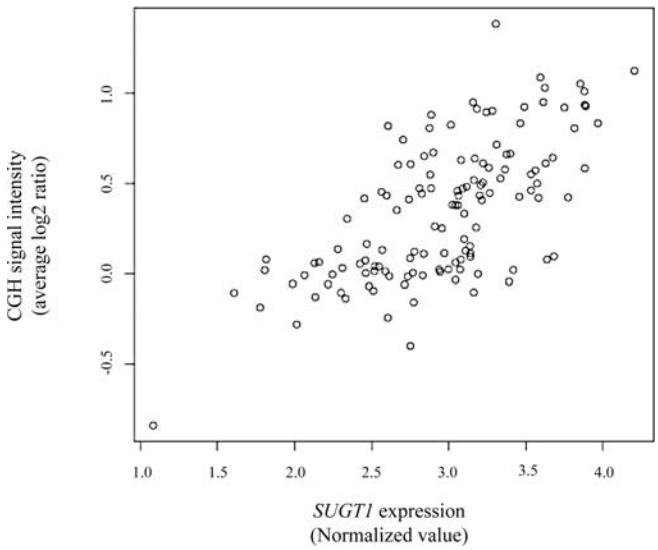

Figure 2. Concordant amplification of *SUGT1* expression and copy number alteration in the flanking regions of *SUGT1*. There is a significant association between genetic alterations and expression of *SUGT1* (correlation coefficient: 0.665, p<0.0001). The x-axis indicates the normalized SUGT1 expression value of cDNA microarray and y-axis CGH signal intensity (average log2 ratio) in the flanking regions of *SUGT1*.

immunohistochemistry of resected colon cancer specimens using an anti-SUGT1 antibody. SUGT1 staining was remarkably stronger in colon cancer tissues than in corresponding normal tissues. SUGT1 expression was localized to the cell cytoplasm (Fig. 3B and C).

Clinicopathological significance of SUGT1 mRNA in CRC cases. We divided the 98 CRC cases into two groups according to the median tumor (T)/normal (N) ratio of *SUGT1* mRNA expression level as determined above. Thus, 49 cases were placed in the high *SUGT1* expression group and 49 cases in the low *SUGT1* expression group. The association between clinicopathological features and *SUGT1* mRNA expression is summarized in Table I. In the high *SUGT1* expression group, the frequency of recurrence was elevated compared to the low *SUGT1* expression group (p=0.03). Univariate analysis identified *SUGT1* expression, tumor size, depth of tumor invasion, lymph node metastasis, lymphatic invasion and venous invasion as prognostic factors for 5-year overall survival following surgery. Variables with p-values <0.05 by univariate analysis were selected for multivariate analysis using Cox's proportional hazards model. *SUGT1* expression [relative risk (RR): 1.55, confidence interval (CI) 1.00-2.47, p=0.04] was found to be a significant factor affecting 5-year overall survival following surgery in multivariate analysis (Table II). Analysis of 5-year overall survival curves showed that patients in the high *SUGT1* expression group had a significantly poorer prognosis than those in the low expression group (p=0.04) (Fig. 4).

Discussion

In the present study, we performed cDNA microarray and array CGH analysis after LMD in a large scale study of 132 Japanese CRC cases. Because chromosome 13q is reportedly amplified in CRC, we focused on the gene *SUGT1* that had

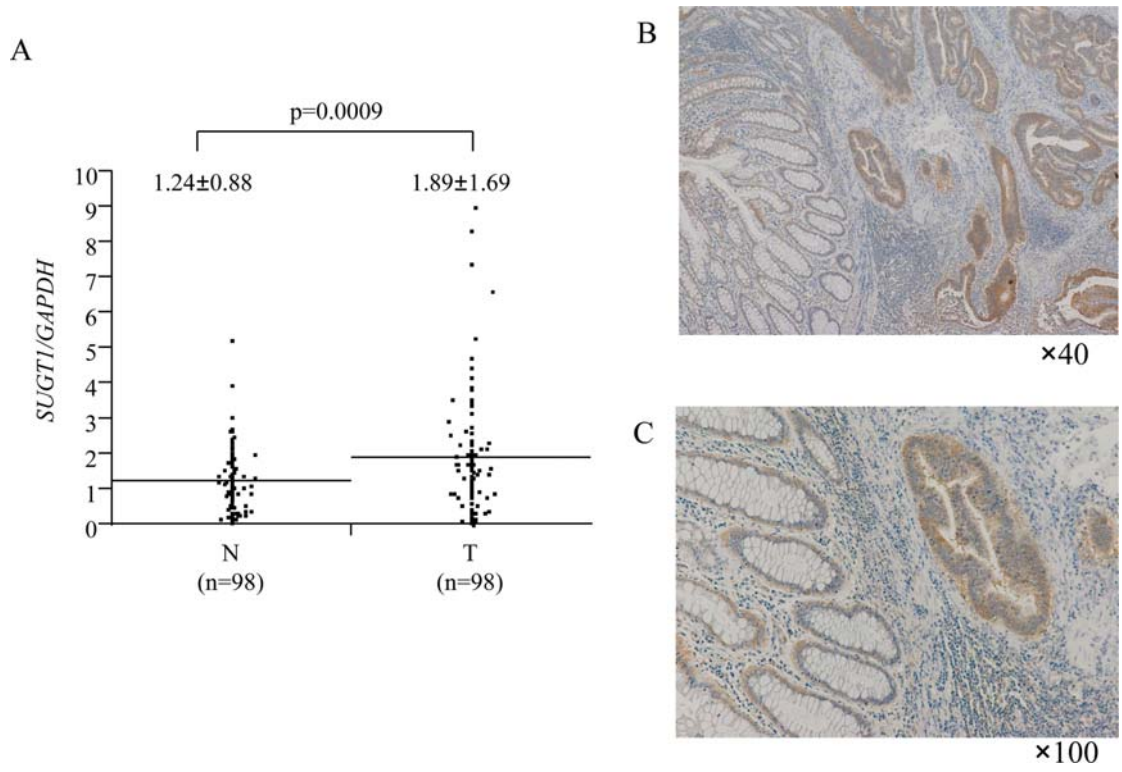

Figure 3. *SUGT1* expression in CRC. (A) *SUGT1* mRNA expression in cancer and non-cancer tissues from CRC patients as assessed by real-time quantitative PCR (n=98). Horizontal lines indicate mean value in each group (T, cancer tissue; N, non-cancerous tissue) (B and C). Immunohistochemical analysis of CRC patient samples with SUGT1 antibody. The majority of staining occurred in cancer cells (original magnification: B, x40; C, x100).

Table I. *SUGT1* mRNA expression and clinocopathological factors.

Factors	Tumor low expression (n=49)		Tumor high expression (n=49)		p-value
	No.	%	No.	%	
Age (mean ± SD)	65.7 ± 1.8		67.1 ± 1.8		0.58
Sex					
Male	31	63.3	28	57.1	0.53
Female	18	36.7	21	42.9	
Histological grade					
Well	14	28.6	23	46.9	0.08
Moderately, poorly	35	71.4	26	53.1	
Size					
<50 mm (small)	23	46.9	17	34.7	0.21
>51 mm (large)	26	53.1	32	65.3	
Depth of tumor inavasion[a]					
m, sm, mp,ss	17	34.7	14	28.6	0.51
se, si	32	65.3	35	71.4	
Lymph node metastasis					
Absent	31	63.3	24	49.0	0.15
Present	18	36.7	25	51.0	
Lymphatic invasion					
Absent	32	65.3	28	57.1	0.41
Present	17	34.7	21	42.9	
Venous invasion					
Absent	42	85.7	42	85.7	1.00
Present	7	14.3	7	14.3	
Liver metastasis					
Absent	45	91.8	47	95.9	0.39
Present	4	8.2	2	4.1	
Peritoneal dissemination					
Absent	48	98.0	46	93.9	0.29
Present	1	2.0	3	6.1	
Distant metastasis					
Absent	44	89.8	44	89.8	1.00
Present	5	10.2	5	10.2	
Recurrence					
Absent	42	85.7	33	67.4	0.03
Present	7	14.3	16	32.6	
TNM staging					
I, II	31	63.3	23	46.9	0.10
III, IV	18	36.7	26	53.1	

[a]Tumor invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se), and invasion of adjacent strucures (si).

Table II. Results of univariate and multivariate analysis of clinicopathlogical factors for 5-year overall survival.

Factors	Univariate analysis			Multivariate analysis		
	RR[a]	95% CI[b]	p-value	RR	95% CI	p-value
Age (<65/>66)	0.81	0.54-1.18	0.26	-	-	-
Sex (male/female)	0.98	0.65-1.43	0.92	-	-	-
Histology grade (well/mod and poor)	1.23	0.83-1.91	0.33	-	-	-
Tumor size (<50 mm/>51 mm)	1.57	1.06-2.33	0.02	1.37	1.06-2.42	0.02
Depth of tumor invasion (m, sm, mp,ss/se, si)[c]	1.92	1.30-2.82	0.001	1.56	1.03-2.39	0.04
Lymph node metastasis (negative/positive)	2.30	1.51-3.80	<0.001	2.02	1.29-3.40	0.002
Venous invasion (negative/positive)	1.91	1.25-2.84	0.004	1.77	1.12-2.71	0.02
SUGT1 mRNA expression (low/high)	1.47	1.00-2.21	0.04	1.55	1.00-2.47	0.04

[a]RR, relative risk; [b]CI, confidence interval. [c]Tumor invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se), and invasion of adjacent strucures (si).

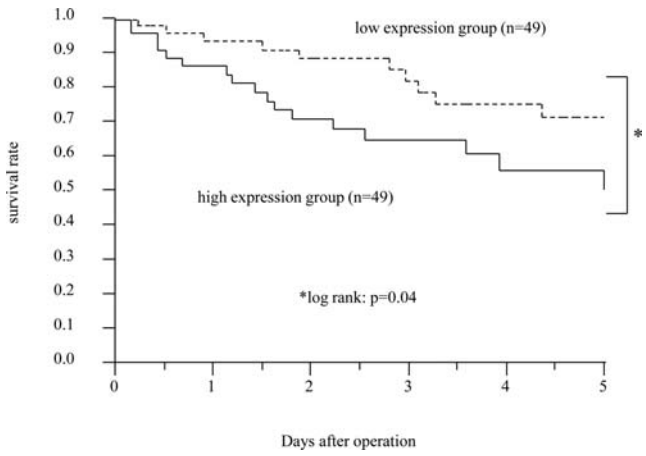

Figure 4. Kaplan-Meier 5-year overall survival curves for CRC patients according to the level of *SUGT1* mRNA expression. The overall survival rate for patients in the low expression group was significantly higher than that for patients in the high expression group (p=0.04, log-rank test). Low expression group (broken line, N=49). High expression group (unbroken line, N=49).

significant correlation between array CGH and cDNA microarray to determine its impact on progression of CRC. The ability to perform high-resolution genome-wide DNA copy number analysis on array CGH analysis allowed us to directly determine changes in DNA copy number in CRC. In a previous study, Platzer *et al* showed that chromosomal amplifications were observed frequently in CRC metastasis (7p, 8q, 13q and 20q), but increased expression of genes within these amplicons was rare (12). Tsafrir *et al* showed that particular chromosomal regions are frequently amplified and overexpressed (e.g., 7p, 8q, 13q and 20q) in the majority of metastatic samples (8). The methodologies used in those studies might have caused the discrepancy between their results. In addition, we recommend that the microdissected cells extracted from CRC tumors should be applied for array CGH analysis to avoid bias in the quantity and quality (5,6). It is possible that the reported discrepancies between the amplified genetic regions and the transcriptional levels are not only characteristic of the genes themselves, but also due to technical errors. Lassmann *et al* and Kim *et al* used microdissected samples and demonstrated a positive association between array CGH and cDNA microarray data for *BRCA2* at the 13q locus (6) and *CAMTA1* at the 1p locus (5) (p<0.0001 and p=0.009, respectively). In our large scale study using microdissected samples, we confirmed their findings of a significant relationship between gene dosage and expression level.

Our focus on the *SUGT1* gene demonstrated a strong correlation between array CGH and cDNA microarray. The expression of *SUGT1* in CRC was significantly higher in tumor tissues compared to normal tissues, as demonstrated by both quantitative RT-PCR and immunohistochemical analysis (Fig. 3). Furthermore, we found that the frequency of disease recurrence after surgery in the high *SUGT1* expression group was significantly elevated in comparison to that in the low expression group (p=0.03) (Table I). Thus, the high *SUGT1* expression group had a significantly poorer prognosis than those in the low expression group (p=0.04) (Fig. 4). Furthermore, *SUGT1* expression was an independent and significant prognostic factor for survival (Table II).

The association between poor clinical outcome and overexpression of SUGT1 may be explained as follows. Interaction of SUGT1 with HSP90 is required for the assembly of the human kinetochore from late S phase to G2/M phase (21).

Furthermore, depletion of SUGT1 sensitizes HeLa cells to the HSP90 inhibitor, 17-allylaminogeldanamycin (17-AAG), which is currently in clinical trials (22). 17-AAG is thought to exert antitumor activity by simultaneously targeting several oncogenic signaling pathways. The study also revealed that overexpression of *SUGT1* might restore the localization of kinetochore proteins and chromosome alignment in cells treated by 17-AAG. Thus, overexpression of SUGT1 reduced tumor cell susceptibility to chemotherapy. In this regard, we performed adjuvant chemotherapy in half of the CRC patients with lymph node metastasis after the curative operation. We speculate that differing susceptibility to chemotherapy may correlate with *SUGT1* expression levels. Our data will be reported at the conclusion of clinical follow-up.

In conclusion, in a large scale study of Japanese CRC, array CGH analysis and cDNA microarray after LMD showed that the novel gene *SUGT1* can be useful for determining prognosis in CRC. Our results indicate that this method of analysis might uncover additional genes indicative of patient prognosis.

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