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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Preoperative *u-PAR* Gene Expression in Bone Marrow Indicates the Potential Power of Recurrence in Breast Cancer Cases

Koshi Mimori, MD, PhD[1], Akemi Kataoka, MD, PhD[1], Hiroshi Yamaguchi, MD, PhD[2], Norikazu Masuda, MD, PhD[3], Yoshimasa Kosaka, MD, PhD[1], Hideshi Ishii, MD, PhD[1], Shinji Ohno, MD, PhD[2], and Masaki Mori, MD, PhD, FACS[1]

[1]Department of Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan
[2]Department of Breast Oncology, Kyushu Cancer Center, Fukuoka, Japan; [3]Osaka National Hospital, Osaka, Japan

ABSTRACT

Introduction. The clinical significance of isolated tumor cells (ITC) in peripheral blood (PB) and bone marrow (BM) as predictive markers in the recurrence or metastasis of breast cancer has not yet been determined. In the current study, we focused on the *urokinase plasminogen activator receptor* (*u-PAR*) gene as a powerful indicator of the potential to relapse after surgery.

Patients and Methods. We examined *CK-7* and *CK19* as an ITC marker and *u-PAR* as a candidate indicator for metastasis in PB and BM from 800 cases of breast cancer by quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). Serum tumor markers, carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA15-3), were compared with *u-PAR* or *CK* status.

Results. *CK7* in PB was positive in 262 cases that showed a poorer disease-free survival (DFS) than 478 *CK7(–)* cases ($P < 0.05$). The 153 cases of *u-PAR*(+) in BM showed significantly poorer DFS and overall survival (OS) than did the 579 cases of *u-PAR*(–) in BM ($P < 0001$ and $P < 0.0001$, respectively). In PB, a significant difference was also observed between 330 cases of *u-PAR*(+) and 437 cases of *u-PAR*(–) ($P < 0.0001$). The hazard ratio (HR) for prediction of recurrence was significantly higher in *u-PAR* ($P < 0.0001$; HR 0.0519) than the level of three serum tumor markers.

Discussion. *u-PAR* expresses in cancer cells during the dormant phase. The current findings revealed that the expression levels of *u-PAR* in PB and BM evaluated preoperatively indicate the potential to relapse or metastasize after surgery.

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M. Mori, MD, PhD, FACS
e-mail: mmori@gesurg.med.osaka-u.ac.jp

During the past two decades the presence of isolated tumor cells (ITC) has been determined; however, the clinical relevance to predict disease-free survival (DFS) or overall survival (OS) in patients of gastrointestinal tract cancer and breast cancer has not been determined.[1–6] Therefore, the current methodology to identify ITC does not apply to practical clinical usage at present. Why can we not use this method to predict DFS or OS more frequently than the conventional clinicopathological diagnosis? We concluded that there are problems with inadequate numbers of examined cases and a diversity of methodologies among institutes, such as target organs, target molecules, and assay systems.

Therefore, we collected both BM and PB from 800 cases of breast cancer in the Kyushu Cancer Center. This is the largest number of examined cases at a single institute in the world among published papers, except the immunohistochemical study by Braun et al.[2,7] As target genes, we chose genes that express specifically in epithelial cells in PB and BM in breast cancer. Among them, we examined *CK7* and *CK19* as candidate markers to detect ITC in breast cancer according to our previous work, which indicated that *CK7* had the best sensitivity as well as the best specificity.[8] Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) with primers and hybridization probe enabled us to achieve the highest specificity and fidelity.

Moreover, according to a previous study by Heiss et al., *u-PAR* expression on disseminated tumor cells detected by immunocytochemistry was significantly and clearly correlated with increasing tumor cell counts and clinical

prognosis.[9] Therefore, we decided to apply the *u-PAR* gene as another target gene in PB and BM from breast cancer cases to predict recurrence and metastasis. Recent studies have focused on dormant cancer cells in BM or PB as the prospective cause for the reemergence of cancer cells after several years.[10–12] With respect to tumor dormancy and recurrence, the *u-PAR* gene encodes one of the cell surface markers that is established as one of the tumor dormancy-related markers.[13] Therefore, we evaluated the role of *u-PAR* in PB and BM as a major indicator to predict recurrence and prognosis of breast cancer cases by measuring preoperatively.

Furthermore, the large number of examined cases allowed us to evaluate the controversial conclusion of whether ITC in PB and BM can be a powerful clinical indicator to predict recurrence and prognosis. In addition, we reveal that *u-PAR* in PB and BM was a correlative marker of recurrence of breast cancer cases beyond the ITC markers as well as existing serum tumor markers.

MATERIALS AND METHODS

Bone Marrow and Peripheral Blood from Breast Cancer Cases

We examined 800 cases of breast cancer from the National Kyushu Cancer Center. Clinicopathologic variables in all cases are presented in Table 1 to determine the

TABLE 1 Clinicopathologic significance of u-PAR status in bone marrow and peripheral blood from breast cancer cases

	n	Bone marrow		*P* value	*n*	Peripheral blood		*P* value
		Positive	Negative			Positive	Negative	
	732	153	579		767	330	437	
Tumor				<0.0001				0.0006
Small	342	49	293		362	132	230	
Large	390	104	286		405	198	207	
Lymph node metastasis				ns				Ns
Positive	266	58	208		282	121	161	
Negative	466	95	371		485	209	276	
Metastasis				ns				ns
Positive	13	3	10		13	4	9	
Negative	719	150	569		754	326	428	
Histology				ns				ns
DCIS	45	10	35		47	23	24	
IDC	636	132	504		664	277	387	
Others	51	11	40		56	30	26	
Stage				ns				ns
0	14	1	13		14	6	8	
1	250	40	210		255	95	159	
2A	256	54	202		285	127	158	
2B	152	40	112		153	75	78	
3A	39	11	28		41	19	22	
3B	10	4	6		9	5	4	
4	11	3	8		10	2	8	
ER or PgR				ns				ns
Positive	556	110	446		579	244	335	
Negative	175	43	132		187	85	102	
Unknown	1	1	0		1	1	0	
HER2/Neu				ns				0.0047[a]
Positive	175	29	146		194	69	125	
Negative	360	66	294		392	188	204	
unknown	197	58	139		181	73	108	

ER estrogen receptor, *PgR* progesterone receptor, *DCIS* ductal carcinoma in situ, *IDC* invasive ductal carcinoma, ns nonsignficant

[a] HER2/neu positive cases indicated significantly higher incidence of u-PAR negative in peripheral blood

relationship between those factors and *u-PAR* status in bone marrow as well as that in peripheral blood. In brief, for analysis of bone marrow, 732 cases were eligible for further study, while 68 cases were excluded because of insufficient amounts of RNA and/or inadequate follow-up data. In 767 cases *u-PAR* was examined in peripheral blood, while 33 cases were excluded by the quality of RNA and inadequate follow-up data.

Both PB and BM were also collected from 29 cases of no malignancy that consisted of 20 cases of cholecystolithiasis, 3 cases of common bile duct stone, and 6 cases of incisional hernia to be used as the negative control from April 2000 to March 2003. After analysis of those 29 nonmalignant cases, no case was affected by cancer. Ethical committee approval for this project from Kyushu University and the National Cancer Center was obtained, and documented informed consent was obtained from all patients and control cases.

RNA Extraction

Total RNA was extracted from bone marrow and peripheral blood from the above clinical samples, for a total of PB and BM from 800 cases. Detailed procedures were described elsewhere.[14] In brief, BM was aspirated from the sternum of patients before the operation under general anesthesia. We discarded the first 1.0 ml of BM and PB to avoid contamination from the epithelial tissue of skin at the site, and collected a second 1.0 ml of BM and PB into 4.0 ml Isogen-LS (Nippon Gene, Toyama, Japan), and total RNA was extracted according to the manufacturer's protocol.

Primers and Probes for Quantitative Real-Time RT-PCR

The reverse-transcriptase reaction was performed as in our previous study.[8] In brief, first-strand cDNA was synthesized from 2.7 μg total RNA in 30 μl reaction mixture containing 5 μl 5 × RT buffer (GIBCO BRL, Gaithersburg, MD), 200 μM deoxyribonucleotide triphosphate (dNTP), 100 μM solution of random hexa-deoxynucleotide mixture, 50 units Rnasin (Promega, Madison, WI), 2 μl 0.1 M dithiothreitol, and 100 units Moloney leukemia virus RT (GIBCO BRL, Gaithersburg, MD). The mixture was incubated at 37°C for 60 min, heated to 95°C for 10 min, and then chilled on ice.

We performed real-time quantitative RT-PCR using a LightCycler instrument (Roche Diagnostics, Manheim, Germany) with the following target genes to detect ITC in PB and/or BM: *CK7*, primers: sense; 5′-ACA TCA AGA ACC AGC GTG CC-3′, antisense; 5′-TCA CGG CTC CCA CTC CAT CT-3′ and probes: donor; 5′-TGA GCG TGA AGC TGG CCC TGG ACA TCG A-fluorescein-3′ and acceptor; 5′-LCRed640- ATC GCC ACC TAC CGC AAG CTG CTG GAG G-3′-phosphorylated. *CK19,* primers: sense; 5′-AAG GTG GAT TCC GCT CCG GGC A-3′, antisense; 5′-ATC TTC CTG TCC CTC GAG CA-3′ and probes: donor; 5′-TTC AAT TCT TCA GTC CGG CTG G-fluorescein-3′ and acceptor; 5′-LCRed640- GAA CCA GGC TTC AGC ATC CTT C 3′-phosphorylated; *urokinase plasminogen activator receptor* (*u-PAR*), primers: sense; 5′-TGA ATC AAT GTC TGG TAG C-3′, antisense; 5′-TGG TTA CAG CCA CTT TTA GT-3′, and probes: donor; 5′-GCT ATA TGG TAA GAG GCT GTG CAA CCG CCT-3′-fluorescein and acceptor; 5′-LCRed640-AAT GTG CCA ACA TGC CCA CCT GGG T-3′-phosphorylated. We utilized glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as an internal control; primer: sense; 5′-TGA ACG GGA AGC TCA CTG G-3′, antisense; 5′-TCC ACC ACC CTG TTG CTG TA-3′, and probe: donor; 5′-GAG TGG GTG TCG CTG TTG AAG TCA-3′-fluorescein, acceptor; 5′-LCRed640-AGG AGA CCA CCT GGT GCT CAG TGT A-3′-phosphorylated. All primers and probes were synthesized and purified by reverse-phase high-performance liquid chromatography and the optimal reagent concentrations and PCR cycling conditions were established. Each run of RT-PCR reaction included positive controls synthesized from plasmids by the Nippon Gene Research Laboratories (Sendai, Japan).

Quatitated RT-PCR Condition

The amplification of the *u-PAR* profile consisted of one cycle at 95°C for 10 min (denaturation) followed by 40 cycles of 95°C for 10 s, 62°C for 15 s, and 72°C for 8 s. For amplification of *GAPDH*, an initial denaturation at 95°C for 10 min was followed by 15 s at 95°C, 15 s at 60°C, and 13 s at 72°C. For *CEA* amplification, an initial denaturation was also followed by 15 s at 95°C, 15 s at 56°C, and 11 s at 72°C. All experiments were performed two times to confirm reproducibility. If the second result was greater than two times or less than 50% of the first one, we performed a third experiment. Then, we calculated the average using two accepted data.

Statistical Analysis

Clinicopathologic significance of *u-PAR* expression was evaluated using Student's *t*-test. To analyze disease-free and overall survival, log-rank (Mantel–Cox) analysis was performed on *CK7*, *CK19*, and *u-PAR* in BM and PB, as well as on serum tumor markers. All tests were analyzed using JMP software (SAS Institute Inc., Cary, NC, USA). Statistical significance was determined as *P*-value from two-sided tests of less than 0.05.

RESULTS

Clinicopathologic Significance of u-PAR in Bone Marrow and Peripheral Blood

In the BM analysis in Table 1 (left side), there was a significant association between tumor size and *u-PAR* expression. We divided the tumors into two subgroups based on size: 342 cases were small tumors (Tis and T1) and 390 cases were large (T2, T3, and T4). Incidence of *u-PAR*-positive expression in bone marrow was significantly higher in cases with large tumor size ($P < 0.0001$).

In the PB analysis in Table 1 (right side), a statistically significantly higher incidence of *u-PAR*-positive expression was observed in 405 cases of large tumor size ($P = 0.0006$). It is intriguing that the human epidermal growth receptor 2 (HER2)/neu-negative cases showed a higher incidence of *u-PAR*-positive cases ($P = 0.0047$); however, there was no relationship between *u-PAR* expression and any clinicopathologic factor.

Prognostic Value of CK7 and u-PAR to Predict Disease-Free Survival

In Fig. 1, the 262 cases of *CK7*(+) in PB showed significantly worse prognosis than the 478 cases of *CK7*(–). However, a significant difference between *CK7*(+) and *CK7*(–) was observed only in the cases free of lymph node metastasis after 1 year ($P < 0.05$). On the other hand, *CK19*(+) in BM was found in 213 out of 750 cases, while *CK19*(+) in PB was found in 135 out of 750 cases; however, there was no clinical relevance of *CK19* expression in PB or in BM (data not shown).

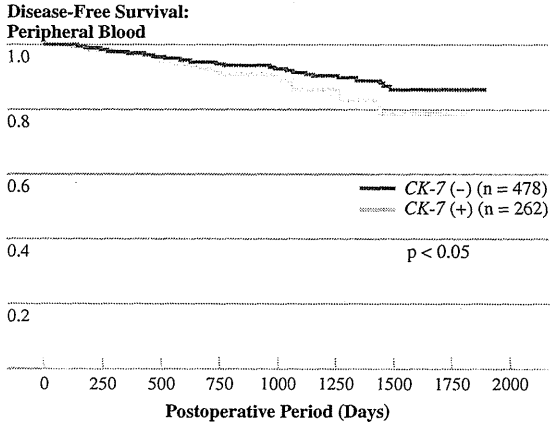

FIG. 1 Clinical relevance of cytokeratin 7 (*CK7*) gene expression and disease- free survival rate in breast cancer cases. There were significant differences between the 262 cases of *CK7*(+) and the 478 cases of *CK7*(–); however, this was observed only if restricted to cases free of lymph node metastasis after 1 year has passed ($P < 0.05$)

Disease-free survival (DFS) rate was remarkably different between *u-PAR*(+) and *u-PAR*(–) in BM and PB. In BM, disease-free survival rate was significantly worse in 153 cases of *u-PAR*(+) than in 557 cases of *u-PAR*(–) (Fig. 2a; $P < 0.0001$). In PB, 327 cases of *u-PAR*(+) showed significantly worse disease-free survival rate compared with 418 cases of *u-PAR*(–) (Fig. 2b). However, overall survival (OS) rate in BM was significantly poorer in 153 cases of *u-PAR*(+) than it was in 557 cases of *u-PAR* (–) (Fig. 3a; $P < 0.001$). In PB, *u-PAR*(+) had poorer OS than 418 cases of *u-PAR*(–); however, there was no significant difference between them (Fig. 3b).

Additionally, we combined the data of *u-PAR* and *CK* gene expression. As a result, in BM, *u-PAR*(+)/*CK*(+) showed the highest recurrence rate; however, *u-PAR* status alone was adequate to predict recurrence compared with the combined data.

Comparison with Serum Tumor Markers

We examined preoperative serum tumor markers, carcinoembryonic antigen (CEA) and cancer antigen (CA15-3), just prior to surgery in 695 and 682 cases of breast cancer, respectively (Fig. 4). We divided the value of serum tumor marker into high and low by the standard value at our hospital in each marker, i.e., 5.0 ng/ml for CEA and 7.0 U/ml for CA15-3. As shown in Fig. 4, 39 cases of high serum CEA level had much poorer DFS rate than 656 cases of low CEA level; however, there was no statistical significance between them. We could not find any clinical usefulness in serum CA15-3 levels measured just prior to surgery in breast cancer cases.

DISCUSSION

The initial purpose of the current study was to attempt to answer the controversial issue of whether or not isolated tumor cells in BM and PB have clinical significance in predicting OS and DFS. Therefore, we collected them from large numbers of breast cancer cases. We then performed quantitative RT-PCR with primers and probes to evaluate gene expression objectively and precisely compared with previous studies using immunocytological analysis. As a result, we observed a significantly higher incidence of *CK7* in PB from 740 cases of breast cancer; however, the difference of expression levels between *CK7*(+) and *CK7*(–) was inadequate to apply the evaluation of ITC (*CK7*) expression in PB to predict DFS. Additionally, we could not find any clinical relevance for predicting OS by the evaluation of ITC in PB, and ITC in BM was not associated with clinicopathologic significance in breast cancer cases. Therefore, we urgently have to identify new indicators to

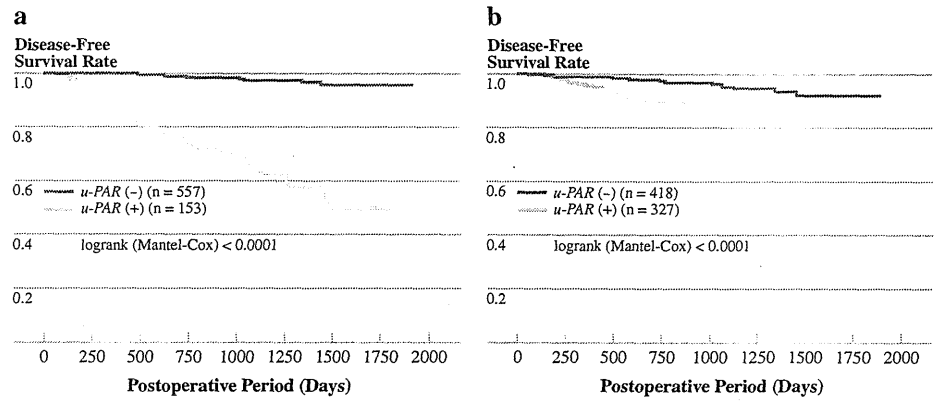

FIG. 2 Clinical significance of u-PAR gene expression in breast cancer cases. **a** Disease-free survival rate was significantly poorer in 153 cases of *u-PAR*(+) than in 557 cases of *u-PAR*(–) in bone marrow ($P < 0.0001$). **b** In peripheral blood, 327 cases of *u-PAR*(+) expression also showed significantly worse disease-free survival rate than 418 cases of *u-PAR*(–) expression ($P < 0.0001$)

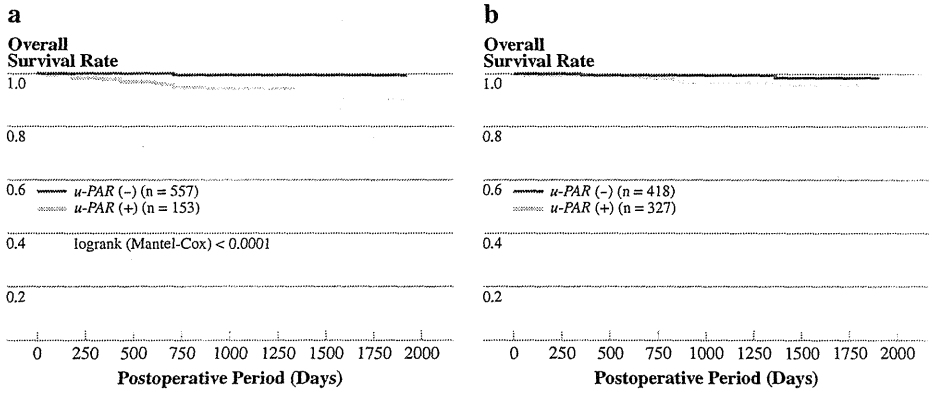

FIG. 3 Clinical significance of *u-PAR* gene expression in predicting overall survival rate (OS). **a** Significantly poorer prognosis was observed in 153 cases of *u-PAR*-positive expression than in 557 cases of *u-PAR*-negative expression in bone marrow ($P < 0.001$). **b** There was no significant difference in *u-PAR* gene status expression in peripheral blood from breast cancer cases

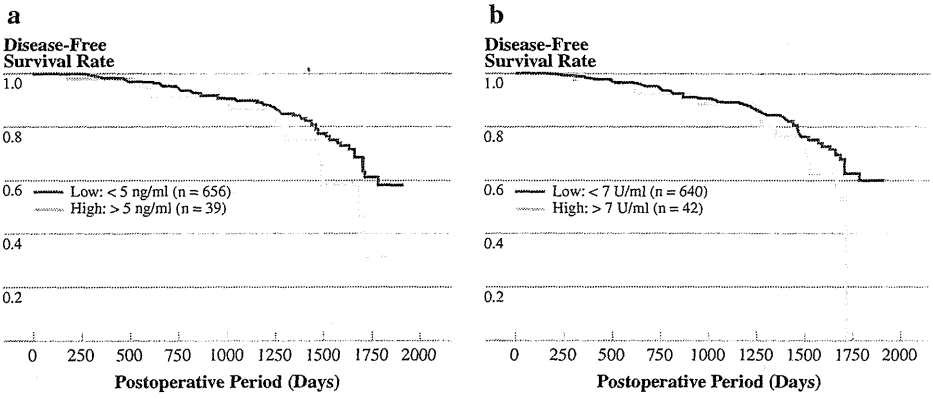

FIG. 4 Comparison of disease-free survival rate between serum tumor marker positive and negative cases by preoperative examination. **a** The cutoff value of preoperative serum CEA is 5.0 ng/ml; 656 cases of low CEA showed lower incidence of recurrence than 39 cases of high CEA; however, this difference was not significant. **b** Serum levels of preoperative CA15-3 were divided into two groups by 7.0 U/ml, yielding 42 cases of high level and 640 cases of low level; however, there was no significant difference in CA15-3 levels

predict DFS and OS in PB and BM from breast cancer cases instead of the identification of ITC.

We disclosed the distinctive clinical significance of *u-PAR* as an important recurrent marker of breast cancer cases, and also found an intriguing inverse relationship between *u-PAR* and HER2/neu expression in peripheral blood just prior to surgery. Considering the intimate relationship between u-PAR and tumor dormancy as in

previous studies, we believed that *u-PAR*-overexpressing cells in the current study were originally from abundant cancer cells at the dormant phase without upregulated cellular activity as characterized by HER2/neu protein expression.[13,15–18] Besides, several studies recently described the role of *u-PAR* gene as a tumor dormancy marker.[19,20] Allgayer et al. mentioned that u-PAR is a key player in regulating the shift between single-cell tumor dormancy and proliferation, and they concluded that u-PAR might be an essential molecule in bone marrow disseminated tumor cells for long-term survival during dormancy, and/or reactivation of their proliferation years after primary treatment.[21] Therefore, the current study can be an initial one to show the role of u-PAR as an indicator of disseminated tumor cells for long-term survival during dormancy on a large scale for breast cancer cases. Besides, larger size of tumor showed positive expression of *u-PAR* in peripheral blood and bone marrow. We assumed that higher population of *u-PAR*-expressing cells must be present in larger-size tumors, worsening the relapse-free survival rate (Fig. 2) and overall survival rate (Fig. 3).

Furthermore, regarding the origins of the *u-PAR* gene, we supposed the following two possibilities: from cancer cells in the dormant phase as described above, or originated from the tumor-bearing host. In addition to our recent study, several previous studies disclosed that metastasis and/or recurrence in solid cancer was generated by the presence of host-side factors, such as cytokines, chemokines, and "niche cells" working with possible cancer stem cells, hematopoietic progenitor cells (HPC), circulating endothelial cells (CEC), mature endothelial cells, tumor vessels, endothelial progenitor cells, and inflammatory cytokines, such as interleukin (IL)-10 and IL-12 *R*.[22–27] In the current study, an RT-PCR assay should have detected *u-PAR*-expressing cells among the highly populated and enriched cells in blood; however, further study is required to determine the origin of *u-PAR*-gene-expressing cells in PB and BM.

Considering clinical application of *u-PAR*, we found the significant magnitude of *u-PAR* gene in the prediction of DFS as well as OS from peripheral blood much easier without invasion to patients rather than bone marrow. Therefore, the evaluation of expression of *u-PAR* gene status will be available at the outpatient clinic.

In conclusion, we predicted cancer recurrence by evaluating *u-PAR* expression rather than looking for the existence of cancer cells (*CK7*) in the circulating system. The origin of *u-PAR* expression remains unknown; however, the most intriguing matter relevant to patients is that measurement of *u-PAR* gene by RT-PCR can be applied clinically to predict recurrence and overall survival instead of those serum tumor markers preoperatively. The current finding enabled us to select patients to be treated with adjuvant chemotherapy in addition to the criteria for the standard breast cancer treatment.

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Clinicopathological significance of *stanniocalcin 2* gene expression in colorectal cancer

Keisuke Ieta[1,2], Fumiaki Tanaka[1], Takehiko Yokobori[1,2], Yoshiaki Kita[1], Naotsugu Haraguchi[1], Koshi Mimori[1], Hiroyuki Kato[2], Takayuki Asao[2], Hiroshi Inoue[1], Hiroyuki Kuwano[2] and Masaki Mori[1]*

[1]*Department of Surgery and Molecular Oncology, Medical Institute of Bioregulation, Kyushu University, Beppu, Oita, Japan*
[2]*Department of General Surgical Science, Graduate School of Medicine, Gunma University, Maebashi, Gunma, Japan*

Laser microdissection (LMD) and microarray were used to identify genes associated with colorectal cancer. *Stanniocalcin 2* (*STC2*) expression and clinicopathological significance in 139 clinical colorectal cancer samples were specifically investigated using real-time quantitative reverse transcription-polymerase chain reaction. A number of genes upregulated in colorectal cancer cells compared to normal colorectal epithelial cells were identified including *STC2*. *STC2* gene expression in cancer tissue was higher than in corresponding normal colorectal epithelial tissue in 124 of 139 cases (89.2%, $p < 0.01$). Tumors with high *STC2* expression showed higher frequencies of lymph node metastasis, lymphatic invasion, tumor depth, tumor size and AJCC Stage classification ($p < 0.01$). Patients with high *STC2* expression also showed significantly worse overall survival rates than those with low *STC2* expression ($p < 0.01$). Furthermore, *STC2* gene appeared to be associated with colorectal cancer progression and may be a useful prognostic indicator for colorectal cancer.

Key words: colorectal cancer; *Stanniocalcin 2 (STC2)*; microarray; laser microdissection (LMD)

Colorectal cancer is one of the most common cancers and is a significant contributor to cancer death.[1] Understanding how colorectal cancer arises, progresses and metastasizes will help the development of novel diagnostic methods and therapies. Therefore, it is important to examine differential gene expression between colorectal cancer and normal colorectal epithelium and elucidate gene function.

Laser microdissection (LMD) is helpful to isolate cells from tissues and microarray is useful for exhaustive analysis.[2] Many reports, including our own, have been published recently using these methods. We reported cancer-related genes relating to esophageal cancer,[3,4] gastric cancer[5,6] and hepatocellular carcinoma[7] using these methods. For colorectal cancer, we reported gene lists comparing 8 samples of colorectal cancer and normal colorectal epithelium using a cDNA microarray containing 12,814 genes.[8,9] In this study, *STC2* gene was identified as upregulated gene in colon cancer tissues.

The *stanniocalcin 2* (*STC2*) gene, located on chromosome 5q35.2, belongs to the *STC* family.[10–13] Stanniocalcins are glycoprotein hormones that are originally found in the corpuscle of Stannius, an endocrine gland of bony fish, involved in calcium and phosphate homeostasis.[14–16] The *STC* family includes 2 genes, *STC1* and *STC2*. It was reported that the *STC1* gene is associated with some cancers.[17–24] *STC2* was reported to be associated with breast cancer,[25–29] ovarian cancer[30] and renal cell carcinoma.[31] However, the relationship between *STC2* expression and clinicopathological factors in colorectal cancer has not yet been investigated.

In our study, we compared differentially expressed genes between colorectal cancer and normal colorectal epithelium and focused on *STC2* to clarify as a potential novel marker or therapeutic target. Additionally, we examined *STC2* gene expression in colorectal cancer tissues and paired normal colorectal tissues, and whether there is an association between *STC2* expression level and clinicopathological significance in colorectal cancer.

Material and methods

Clinical samples

One hundred and thirty-nine patients with colorectal cancer were enrolled into this study. All patients underwent resection of the primary tumor at the Kyushu University Hospital at Beppu and affiliated hospitals between 1993 and 2000. All patients were clearly identified as having colorectal cancer based on the clinicopathologic findings (age 66.1 ± 11.4, male/female = 79:60, tumor size (cm) 4.7 ± 2.0, histological type well/mod/poor = 52:77:8, tumor depth Tis/T1/T2/T3/T4 = 9:11:29:54:36, lymph node metastasis (+)/(−) = 56:83, lymphatic permeation (+)/(−) = 48:91, venous permeation (+)/(−) = 20:119, liver metastasis (+)/(−) = 19:120, Stage 0/I/IIA/IIB/III or IV = 9:31:26:11:62). No patients received chemotherapy or radiotherapy before surgery. Resected tumor and paired nontumor tissue specimens were immediately cut from the resected colorectum and placed in RNA Later (TaKaRa, Japan) or embedded in Tissue Tek OCT medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen and kept at −80°C until RNA extraction. Written informed consent was obtained from all patients. The follow-up period ranged from 3 months to 11.3 years with a median of 2.8 years.

Laser microdissection and RNA extraction

Colon cancer tissues and colon normal epithelial tissues were microdissected using the LMD system (Leica Laser Microdissection System, Leica Microsystems, Wetzlar, Germany) as previously described.[3] Total RNA was extracted using an Rneasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purity and concentration of the RNA samples were determined with a Nano Drop (Nano Drop Technologies, Wilmington) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto) as previously described.[32]

DNA microarray

We used the commercially available Human 1 cDNA Microarray (Agilent Technologies) containing 12,814 genes. All microar-

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***Correspondence to:** Department of Surgery and Molecular Oncology, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu 874-0838, Japan. Fax: +81-977-27-1651.
E-mail: mmori@beppu.kyushu-u.ac.jp

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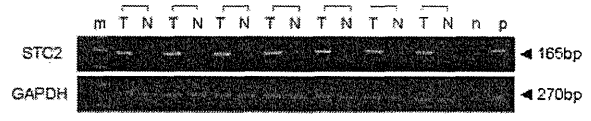

FIGURE 1 – Expression of *STC2* mRNA as assessed by RT-PCR in representative colorectal cancer cases (T, cancer tissue; N, noncancerous tissue; n, negative control; p, positive control; m, marker).

ray data including this study are available from Center for Information Biology Gene Expression database (http://cibex.nig.ac.jp/cibex2/index.jsp).[9]

Oligonucleotide primers for STC2 *gene amplification by RT-PCR*

Total RNA was extracted from each clinical sample and cDNA synthesized from 8.0 μg of total RNA as previously described.[32]

STC2-specific oligonucleotide primers were designed to give a 165 bp PCR product: sense primer 5′-CTTACATGGGATTTG CATGACTT-3′; antisense primer 5′-AATGGATCATCTCCAC TATCACC-3′.[13] Primers were also designed for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (270 bp): sense primer 5′-TTGGTATCGTGGAAGGACTCA-3′; antisense primer 5′-TGT CATCATATTTGGCAGGTT-3′. To avoid amplification of contaminating genomic DNA, the primers spanned more than 2 exons. Amplification was performed for 27 cycles (22 cycles for GAPDH) of 1 min at 95°C, 1 min at 60°C (56°C for GAPDH) and 1 min at 72°C. An 8.0 μl aliquot of each PCR-amplified DNA was electrophoresed on 2% agarose gels containing ethidium bromide.

Real-time quantitative RT-PCR

PCR amplification for quantification of *STC2* and *GAPDH* mRNA in 139 clinical samples was performed using the LightCycler system (Roche Applied Science, IN) and the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Applied Science, IN) as previously described.[33] Amplification conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 64°C (60°C for *GAPDH*) for 10 sec and elongation at 72°C for 10 sec. Melting curve analysis and electrophoresis on 2% agarose gels were performed to ensure that the expected PCR products were generated. To quantitate specific mRNA in the samples, a standard curve was produced for each run based on 3 points from diluted human control cDNA. Relative *STC2* expression levels were then obtained by normalizing the amount of *STC2* mRNA divided by that of *GAPDH* mRNA as an endogenous control in each sample.

Immunohistochemistry

Immunohistochemical studies of STC2 were performed on surgical specimens from representative colorectal cancer patients. Formalin-fixed, paraffin-embedded tissues were deparaffinized, blocked, incubated with specific antibodies for 1 hr at room temperature and detected using ENVISION reagents (ENVISION+ Dual Link/HRP, Dako Cytomation, Denmark). All sections were counterstained with hematoxylin. Primary mouse monoclonal anti-STC2 antibody (Abnova) was used at a dilution of 1:50.

Statistical analysis

For continuous variables, data were expressed as the means ± SD. We classified the 139 colorectal cancer cases into 2 groups according to a cutoff value at median + SD of *STC2* mRNA expression level in nontumor tissues as determined by quantitative RT-PCR, to give high- (n = 84) and low-(n = 55) expression groups. Differences between groups were estimated using Student's t-test and χ^2 test. Overall survival curves and disease-free survival curves were plotted according to the Kaplan-Meier method, and measured from the day of surgery, with the log-rank test applied for comparisons. Variables with a value of $p < 0.05$ by univariate analysis were used in subsequent multivariate analyses based on Cox's proportional hazards model. All differences were deemed significant at the level of $p < 0.05$. Statistical analyses were performed using the JMP 5 for Windows software package (SAS Institute, Cary, NC).

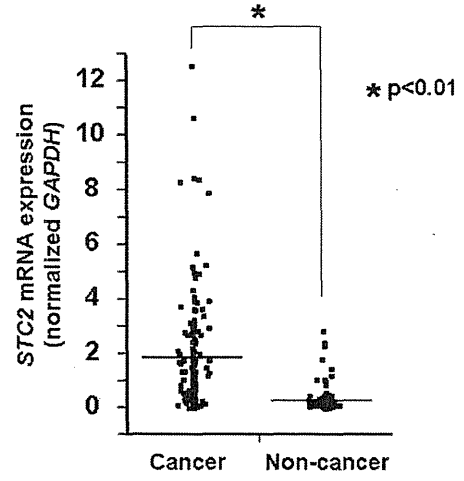

FIGURE 2 – *STC2* mRNA expression in cancer and noncancerous tissues from colorectal cancer patients as assessed by real-time quantitative PCR (n = 139). Horizontal lines indicate mean value of each group (T, cancer tissue; N, noncancerous tissue).

Results

Expression of STC2 *mRNA in clinical tissue specimens*

STC2 mRNA expression in cancerous and noncancerous tissues of colorectal cancer patients was examined by RT-PCR and real-time quantitative PCR and quantified using *STC2/GAPDH* expression ratios. Results indicated that *STC2* mRNA expression levels were higher in cancerous tissues (1.89 ± 2.13; mean ± SD) than in noncancerous tissues (0.29 ± 0.43) in 124 of the 139 cases (89.2%). This resulted in a significant difference in mRNA expression level between cancer and normal tissues ($p < 0.01$) (Figs. 1 and 2). To investigate protein expression of STC2, immunohistochemical staining was performed 5 each cases of high *STC2* mRNA expression group and low *STC2* mRNA expression group. STC2 staining was stronger in colorectal cancer tissues than in corresponding normal colorectal epithelial tissues. STC2 staining of cancer tissues in high mRNA expression group was stronger than in low mRNA expression group (Fig. 3).

The clinicopathological significance of STC2 *mRNA expression*

Clinicopathological features were analyzed in relation to *STC2* expression status (Table I). The incidence of deeply invading tumors was also significantly higher ($p < 0.01$) in the high-expression group (77.4%, 65/84) than in the low-expression group (45.4%, 25/55). Likewise, the incidence of lymph node metastasis and lymphatic permeation was higher ($p < 0.01$) in the high-expression group (51.2%, 43/84, 42.8%, 36/84) than in the low-expression group (23.6%, 13/55, 21.8%, 12/55). Furthermore, stage according to AJCC Stage classification correlated with higher *STC2* expression levels ($p < 0.01$). Incidence of larger tumors (>3 cm) was significantly higher ($p < 0.01$) in the high-expression group (89.3%, 75/84) than in the low-expression group (63.6%, 35/55). No other significant differences were observed with respect to age, gender, histology, venous permeation and liver metastasis.

The 5-year actuarial overall survival rates in patients with high *STC2* mRNA expression levels and those with low levels were 50.3 and 91.4%, respectively. Analysis of overall survival curves

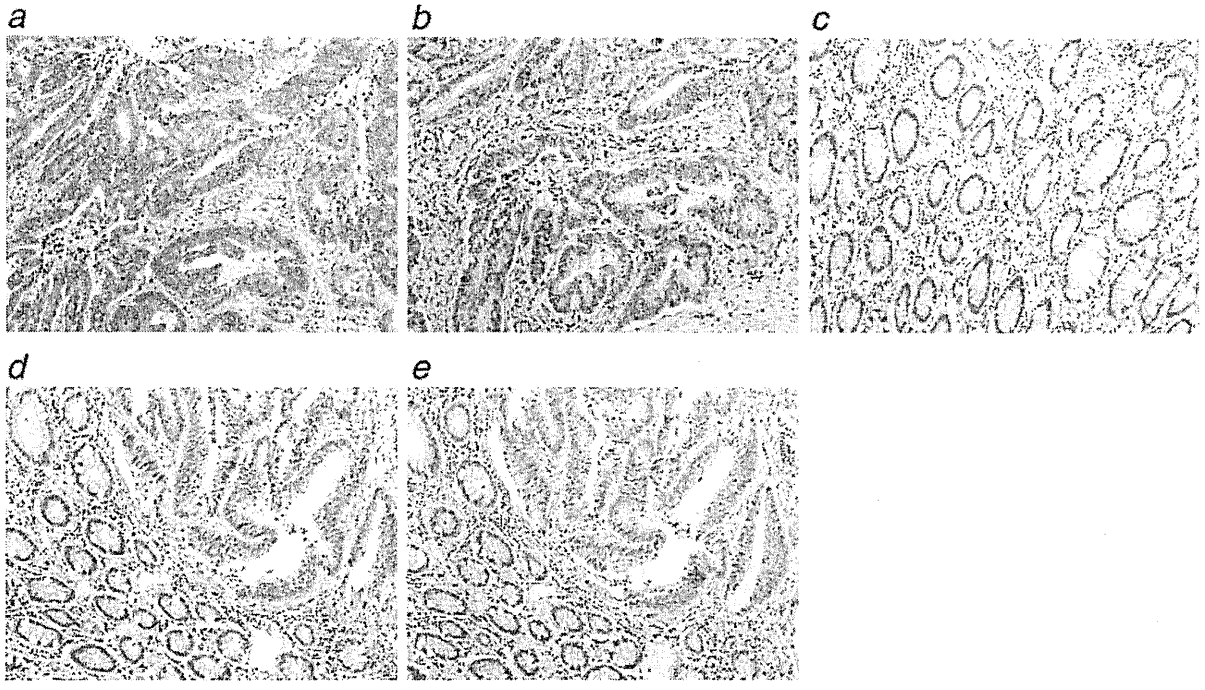

FIGURE 3 – Immunohistochemistry with STC2 antibody on colorectal cancer patient samples. The majority of staining occurred in cancer cells. (*a*) cancer tissue (mRNA high-expression group), STC2 stain; (*b*) cancer tissue (mRNA high-expression group), control stain (secondary antibody only); (*c*) noncancerous tissue (mRNA high-expression group), STC2 stain; (*d*) cancer and noncancerous tissue (mRNA low-expression group), STC2 stain; (*e*) cancer and noncancerous tissue (mRNA low-expression group), control stain (secondary antibody only), original magnification ×100. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I – *STC2* GENE EXPRESSION AND CLINICOPATHOLOGICAL FEATURES FOR 139 COLORECTAL CANCER PATIENTS

Clinicopathologic variable	High-expression group (n = 84)	Low-expression group (n = 55)	p-value
Age	65.7 ± 12.1	66.7 ± 10.2	0.63
Gender			
Male	44	35	0.19
Female	40	20	
Tumor size			
<3 cm	7	19	<0.01
>3 cm	75	35	
Histology			
Well	27	25	0.23
Moderate	50	27	
Poor	6	2	
Depth			
Tis, T1, T2	19	30	<0.01
T3, T4	65	25	
Lymph node metastasis			
Absent	41	42	<0.01
Present	43	13	
Lymphatic permeation			
Absent	48	43	<0.01
Present	36	12	
Venous permeation			
Absent	70	49	0.34
Present	14	6	
Liver Metastasis			
Absent	70	50	0.23
Present	14	5	
AJCC Stage classification			
0, I, II	37	40	<0.01
III, IV	47	15	

High-expression group (*STC2/GAPDH* ≥ cutoff value), low-expression group (*STC2/GAPDH* < cutoff value), well, well differentiated; poor, poorly differentiated; moderate, moderately differentiated.

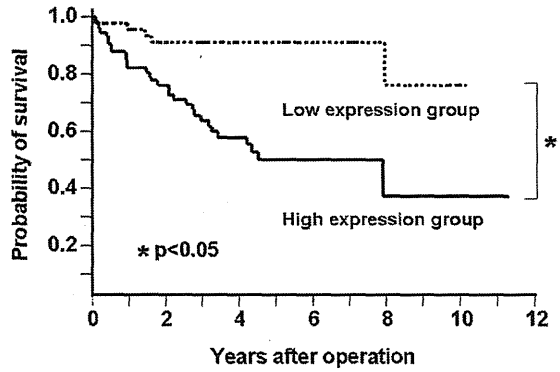

FIGURE 4 – Kaplan-Meier overall survival curves for colorectal cancer patients according to the level of *STC2* mRNA expression. The overall survival rate for patients in the high-expression group was significantly higher than that for patients in the low-expression group ($p < 0.01$). High-expression group (n = 84): *STC2/GAPDH* ≥ cutoff value, low-expression group: *STC2/GAPDH* < cutoff value (n = 55).

showed that patients in the high-expression group had a significantly poorer prognosis than those in the low-expression group ($p < 0.01$) (Fig. 4).

Univariate analysis identified *STC2* expression (low- or high-expression), tumor size, depth, lymph node metastasis, lymphatic permeation, venous permeation and liver metastasis as adverse prognostic factors for overall survival after colorectal resection. Variables with a p-value of less than 0.05 by univariate analysis were selected for multivariate analysis using Cox's proportional

TABLE II – RESULTS OF UNIVARIATE AND MULTIVARIATE ANALYSES OF CLINICOPATHOLOGICAL FACTORS AFFECTING OVERALL SURVIVAL RATE AFTER SURGERY

Clinicopathologic variable	*n*	5-year survival rate (%)	Univariate analysis *p*-value	Multivariate analysis	
				Relative risk (CI)	*p*-value
Gender					
Male	79	63.1	0.41		
Female	60	69.9			
Tumor size					
<3 cm	26	96.0	0.004	1.54	0.36
>3 cm	110	58.8		(0.65–6.66)	
Histology					
Well	52	69.5	0.61		
Mod + poor	85	32.6			
Depth					
Tis, T1, T2	49	90.7	<0.0001	1.78	0.04
T3, T4	90	51.9		(1.02–3.75)	
Lymph node metastasis					
Absent	83	82.9	<0.0001	1.97	0.0003
Present	56	43.1		(1.34–3.04)	
Lymphatic permeation					
Absent	91	78.7	<0.0001		
Present	48	43.9			
Venous permeation					
Absent	119	71.4	0.0053	1.29	0.21
Present	20	39.3		(0.86–1.87)	
Liver metastasis					
Absent	120	75.4	<0.0001		
Present	19	15.5			
STC2					
Low	55	91.3	0.0002	1.76	0.023
High	84	50.3		(1.07–3.31)	

n, number of patient; CI, confidence interval, *STC2*, *STC2* expression; high, high-expression group (*STC2/GAPDH* ≥ cutoff value); low, low-expression group (*STC2/GAPDH* < cutoff value).

hazards model. *STC2* expression (relative risk: 1.76, confidence interval: 1.07–3.31, $p = 0.023$) was found to be a factor affecting overall survival rate after lymph node metastasis (Table II).

Discussion

This study identified differentially expressed genes between colorectal cancer and normal colorectal epithelial tissues. We had previously reported that the expression of *FABP6* was higher in primary colorectal cancers and adenomas than in normal epithelium, and *FABP6* might play an important role in early carcinogenesis.[34] Here, we focused on *STC2* as it was one of the upregulated genes.

*STC*s represent a small family of secreted glycoprotein hormones, consisting of *STC1* and *STC2*, which are conserved from fish to mammals. The STC2 protein may play a role in the regulation of renal and intestinal calcium and phosphate transport, cell metabolism or cellular calcium/phosphate homeostasis, but the precise physiological function of *STC2* has not been clearly elucidated.[16]

Some studies on *STC2* gene expression relating to cancer have been reported. In breast cancer, *STC2* expression was found to be associated with tumor estrogen receptor (ER) status. High *STC2* expressions were associated with a good prognosis (disease-free survival) in ER-positive breast cancer patients.[22–24] It was reported that a significantly higher expression level of *STC2* in metastases after 5 and 10 years after surgery was shown when compared to the primary breast cancers displaying early metastatic lesions.[28] In ovarian cancer, *STC2* was one of the overexpressed genes as investigated by immunohistochemistry-guided laser capture microdissection and microarray, and overexpression of *STC2* was associated with a decreased disease-free interval.[30] Increased cytoplasmic STC2 expression correlated with aggressiveness of renal cell carcinoma and shorter overall patient survival times.[31] In colorectal cancer, it was reported that expression of *STC1* increased 10-fold in tumors compared to normal mucosa by microarray,[20] and *STC2* was one of the overexpression genes in colorectal cancer cells by microarray.[35] However, *STC2* expression status in clinical samples and clinicopathologic factors in colorectal cancer has not been investigated before this study.

Our study indicated that in colorectal cancer patients, *STC2* was more frequently overexpressed in cancerous tissues than in noncancerous tissues, and high mRNA expression of *STC2* was significantly associated with tumor size, depth, lymph node metastasis, lymphatic permeation, AJCC Stage classification and overall survival. Additionally, *STC2* expression was found to be a factor affecting overall survival rate by multivariate analysis. It was shown by means of immunohistochemical staining that STC2 protein expression was correlated with mRNA expression. Therefore, these results suggest that high *STC2* expression has an association with progression and malignancy of colorectal caner cells.

In human breast cancer cell lines, *STC2* represents a downstream target of estrogen and retinoic acid signaling pathways and constitutive *STC2* expression resulted in significant impairment of cell growth, migration and cell viability after serum withdrawal.[29] Additionally, it was reported that *STC2* expression was sporadically abrogated in human cancer cells by transcriptional silencing associated with CpG island promoter hypermethylation.[36] Constitutive expression of human *STC2* in transgenic mice acts as a potent growth inhibitor *in vivo* and results in a significant reduction of intramembranous and endochondral bone development, as well as high neonatal morbidity.[37] It seems that these findings correspond to clinical data of breast cancer. Meanwhile, in ovarian cancer, renal cell carcinoma and colorectal cancer, high *STC2* expression correlated with a poor prognosis. The reason why colorectal cancer with high *STC2* expression shows aggressive behavior remains unclear.

Endoplasmic reticulum (ER) stress results from physiological, pathological and experimental conditions that perturb ER function because of accumulation of misfolded proteins within the ER. Accumulation of misfolded protein in the ER induces a highly conserved homeostatic response in all eukaryotic cells, termed the unfolded protein response (UPR).[38,39] It was reported that *STC2* is a novel target of the UPR. *STC2* expression is upregulated after exposure to tunicamycin and thapsigargin and induced in cultured

cells downstream of *PERK-ATF4* activation by ER stress agents, oxidative stress and hypoxia.[40] Recent report revealed that endogenous *HIF-1α* plays an essential role in hypoxia-induced *STC2* expression and the direct binding of *HIF-1α* to *STC2* promoter with the chromatin immunoprecipitation assay.[36] These findings support the notion that *HIF-1α* is a potent stimulator of *STC2* expression. *STC2* may be a critical survival component of the UPR, and in colorectal cancer, cells may overexpress *STC2* for tolerance to the extreme microenvironment of tumor and ER stress including hypoxia. The contradictory findings in different types of cancer may be due to differences in variations of organs.

In conclusion, here, we identified several upregulated genes in colorectal cancer cells compared to normal colorectal cancer epithelial cells and focused on one of the most upregulated genes, *STC2*. *STC2* may play an important role in the progression of colorectal cancer and may prove useful as a novel prognostic marker and target for molecular treatment of patients with colorectal cancer. Furthermore, understanding the biological function of *STC2* in colorectal tissue may help to delineate its role in the pathophysiology of colorectal cancer.

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Bone marrow and peripheral blood expression of *ID1* in human gastric carcinoma patients is a *bona fide* indicator of lymph node and peritoneal metastasis

M Iwatsuki[1,2], T Fukagawa[3], K Mimori[1], H Nakanishi[4], S Ito[5], H Ishii[1], T Yokobori[1], M Sasako[3], H Baba[2] and M Mori*[,1]

[1]Department of Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu 874-0838, Japan; [2]Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan; [3]Gastric Surgery Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; [4]Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan; [5]Department of Gastroenterological Surgery, Aichi Cancer Center Central Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan

Recent studies have showed that the bone marrow-derived endothelial progenitor cells play critical roles in metastasis and that *ID1* is required in metastasis as regulator of angiogenesis. Therefore, we investigated the clinical significance of *ID1* mRNA expression in bone marrow and peripheral samples in patients with gastric cancer. Two hundred and eighty-nine bone marrow and 196 peripheral blood samples from gastric cancer patients were collected and analysed by quantitative RT–PCR for *ID1*. The ID1 protein expression in one bone marrow, three metastatic lymph nodes and three peritoneal disseminated tumours was examined by immunohistochemical methods. In both bone marrow and peripheral blood samples, *ID1* mRNA expression in the metastatic group was significantly higher than in any other group ($P = 0.003$, $P = 0.0001$, respectively) and significantly associated with lymph node metastasis and peritoneal dissemination. The cells in bone marrow with metastatic cancer stained strongly with ID1 compared with those of healthy volunteers. The expression of *ID1* mRNA in bone marrow and peripheral blood was significantly associated with lymph node metastasis and peritoneal dissemination, and therefore constitutes a predictable marker for lymph node metastasis and peritoneal dissemination.

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The presence of isolated tumour cells (ITCs) is an important factor in the metastasis of solid cancers. Recently, we investigated the presence of ITCs in peripheral blood and in bone marrow, using quantitative RT–PCR in more than 800 cases of gastric cancer (Mimori *et al*, 2008). We found that ITCs circulate in a range of clinical stages of gastric cancer. These data suggested that host cells might play a supportive role for metastasis.

Recently, Kaplan *et al* reported that bone marrow-derived haematopoietic progenitor cells that express vascular endothelial growth factor receptor 1 (VEGFR-1) home to tumour-specific pre-metastatic sites and form cellular clusters before the arrival of tumour cells (Kaplan *et al*, 2005). In a large-scale study of gastric cancer cases, we recently reported that the simultaneous expression of ITC-associated genes and high levels of expression of *VEGFR-1* in bone marrow were significantly associated with haematogeneous metastases (Mimori *et al*, 2008). Gao *et al* determined that the bone marrow-derived endothelial progenitor cells (EPCs) were critical regulators of angiogenic switching (Gao *et al*, 2008). Furthermore, they showed that tumours induce expression of *ID1* in EPCs and that suppression of *ID1* after metastatic colonisation blocked EPC mobilisation, inhibited angiogenesis and impaired pulmonary macrometastases. ID proteins are inhibitors of DNA binding of basic helix-loop-helix (bHLH) transcription factors by heterodimerisation with the bHLH proteins (Benezra *et al*, 1990). *ID1* has been reported to be associated with the undifferentiation of cancer cells, severe malignant grade of tumour, invasion of tumours and worse prognosis in several tumours (Fong *et al*, 2003).

In this study, we investigated the clinical significance of the *ID1* mRNA expression in bone marrow and peripheral blood samples obtained from gastric cancer patients. The results showed that the *ID1* mRNA expression in bone marrow and peripheral blood was significantly associated with lymph node metastasis and peritoneal dissemination. Thus, *ID1* is a *bona fide* predictive marker for both pathologic parameters, each of which is an established definitive prognostic indicator in gastric cancer.

MATERIALS AND METHODS

Patients

Physicians (TF and MS) collected bone marrow and peripheral blood samples from 289 Japanese gastric cancer patients who underwent surgery from 2001 to 2004 at the Central Hospital, the

*Correspondence: Dr M Mori; E-mail: mmori@gesurg.med.osaka-u.ac.jp
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Table 1 Clinicopathlogial significance of *ID1* mRNA expression in gastric cancer patients

Features	Bone marrow			Peripheral blood		
	Number	*ID1* mRNA expression (mean ± s.d.)	*P*-value	Number	*ID1* mRNA expression (mean ± s.d.)	*P*-value
Age	289	62.3 ± 11.9	—	196	62.9 ± 12.2	—
Sex (M:F)	289	190:99	—	196	131:65	—
Tumour size			0.01			0.002
⩽5 cm	126	311 ± 146		85	24.8 ± 12.2	
⩾5 cm	160	795 ± 129		110	64.6 ± 10.7	
Depth of tumour invasion[a]			0.01			0.004
m, sm	90	210 ± 171		62	12.9 ± 14.2	
mp, ss, se, si	199	744 ± 115		134	62.8 ± 9.6	
Venous invasion			0.97			0.007
Positive	72	580 ± 199		50	77.8 ± 15.2	
Negative	188	571 ± 123		128	28.9 ± 9.5	
Lymphatic invasion			0.001			0.02
Positive	156	850 ± 132		110	57.8 ± 10.3	
Negative	104	158 ± 162		68	18.2 ± 13.1	
Lymph node metastasis			0.001			0.02
Positive	188	800 ± 121		132	56.7 ± 9.6	
Negative	79	58.7 ± 186		50	12.4 ± 15.6	
Peritoneal dissemination[b]			0.002			<0.0001
Positive	70	1102 ± 194		40	119 ± 17.1	
Negative	218	412 ± 110		156	28.6 ± 8.6	

[a]Tumour invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se) and invasion of adjacent strucures (si). [b]Peritoneal dissemination: peritoneal cytology or metastasis positive.

National Cancer Center, Tokyo, Japan. The documented informed consent was obtained from all patients and the protocol of the study was approved by the local ethics committee. There were 190 male and 99 female patients with an average age of 62.3 and a range of 24–86 years (Table 1). Seventy of the patients showed peritoneal dissemination at the time of surgery or at postoperative follow-up. Among the 289 cases, 76, 60, 62 and 91 were classified as stages I, II, III or IV, respectively, according to the Treaty for Japanese Gastric Cancer Association (Maruyama *et al*, 2006).

Bone marrow and peripheral blood samples from gastric cancer patients

Aspiration of both bone marrow and peripheral blood was conducted under general anaesthesia immediately before surgery as described earlier (Mimori *et al*, 2008). The bone marrow aspirate was obtained from the sternum using a bone marrow aspiration needle and peripheral blood was obtained through a venous catheter. The first 1.0 ml of bone marrow and peripheral blood were discarded to avoid contamination by the skin. The second collected 1.0 ml of bone marrow and peripheral blood were put into 4.0 ml of Isogen-LS (Nippon Gene, Toyama, Japan) and stored at −80 °C until RNA extraction.

Total RNA extraction and first-strand cDNA synthesis

Samples transferred from Tokyo to Beppu remained frozen while in transit. Total RNA was extracted from bone marrow and peripheral blood according to the manufacturer's protocol as described elsewhere (Iinuma *et al*, 2006). The reverse transcriptase reaction (RT) was performed as described earlier (Mori *et al*, 1995). The first-strand cDNA was synthesised from 2.7 μg of total RNA in 30 μl reaction mixtures containing 5 μl 5 × RT buffer (BRL, Gaithersburg, MD, USA), 200 μM dNTP, a 100 μM solution of a random hexadeoxynucleotide mixture, 50 units of Rnasin (Promega, Madison, WI, USA), 2 μl of 0.1 M dithiothreitol and 100 units of Maloney leukemia virus RT (BRL). The mixture was incubated at 37°C for 60 min, heated to 95°C for 10 min and then chilled on ice.

Quantitative real-time RT–PCR

The sequences of *ID1* mRNA were as follows: sense, 5′-CC AGTGGCAGCACCGCCACC-3′, and anti-sense, 5′-CGGATTCCG AGTTCAGCTCC-3′. We used glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) as an internal control. The primers were as follows: sense, 5′-TTGGTATCGTGGAAGGACTCTA-3′, and anti-sense, 5′-TGTCATATTTGGCAGGTT-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 Diagnostics, Tokyo, Japan) to detect *ID1* in bone marrow and peripheral blood. Monitoring was performed according to the manufacturer's instructions, as described earlier (Ogawa *et al*, 2005). 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 s, annealing at 62°C for 10 s and extension at 72°C for 10 s. After amplification, products were subjected to a temperature gradient from 67°C to 95°C at 0.2°C s^{-1}, under continuous fluorescence monitoring, to produce a melting curve of products.

Data analysis for RT-PCR

We used the LightCycler Software version 3.5 program (Roche Molecular Biochemicals, Basel, Switzerland) to calculate the cycle numbers. After proportional baseline adjustment, a fit point method was used 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 the concentration. Concentrations of unknown samples were calculated by plotting their crossing points against the standard curve and dividing by *GAPDH* content. The results of RT-PCR were sent from Beppu to Tokyo for analyses.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded specimens obtained from patients with metastatic gastric cancer and two healthy volunteers. Tissue sections were deparaffinised, 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 antibody against ID1 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which detects ID1 in paraffin-embedded human tissue sections and does not crossreact with ID2, ID3 or ID4 (Maruyama *et al*, 1999), was used at a dilution of 1 : 100. The blocking peptide to ID1 (sc-488P, Santa Cruz Biotechnology) was used as a negative control (Supplementary Figure 4). Tissue sections were immunohistochemically stained using the avidin-biotin-peroxidase method (LSAB+ system-HRP; DAKO, Kyoto, Japan). All sections were counterstained with haematoxylin.

Statistical analysis

The expression of *ID1* was adjusted in each case for *GAPDH* expression. For continuous variables, data were expressed as the means ± s.d. The relationship between *ID1* mRNA expression and clinicopathlogical factors was analysed using a χ^2 test and Student's *t*-test. All tests were analysed using JMP software (SAS Institute Inc., Cary, NC, USA) and the findings were considered significant when the *P*-value was <0.05.

RESULTS

Expression of *ID1* mRNA in bone marrow of gastric cancer

Figure 1A shows expression of *ID1* mRNA in bone marrow according to staging classification. In bone marrow, the mean expression level of *ID1* mRNA in stage IV (957 ± 169) was significantly higher than other stages ($P=0.003$). Specifically, the levels of stages I, II and III were 54 ± 185, 472 ± 208, and 767 ± 205, respectively. To confirm the specificity of *ID1*, we performed RT-PCR analysis of six representative cases in each stage, which was very close to the average value (Figure 1C). In addition, sequencing of these transcripts confirmed that it was the product of *ID1* (Supplementary Figure 1).

Expression of *ID1* mRNA in peripheral blood of gastric cancer

In the peripheral blood samples, there was a significant relationship between the expression level of *ID1* mRNA and the progression of gastric cancer cases (Figure 1B). The mean expression level of *ID1* mRNA in stage IV (105 ± 15) was significantly higher ($P=0.0001$) than stages I, II and III (12.4 ± 15.4, 29.6 ± 15.5, and 38.3 ± 16.0, respectively). In addition, there was a significant correlation between the expression of *ID1* mRNA in bone marrow and peripheral blood ($r=0.23$, $P=0.002$, data not shown).

ID1 expression and clinicopathological features of gastric cancer patients

We examined the clinicopathlogical significance of *ID1* mRNA in samples from bone marrow and peripheral blood (Table 1). In both bone marrow and peripheral blood, there are significant associations with many cliniopathlogical features such as tumour size and depth of tumour invasion. Especially, in patients with evidence of lymphatic invasion, lymph node metastasis or peritoneal dissemination, we found significantly higher expression of *ID1* mRNA in bone marrow samples compared to patients without metastasis. ($P=0.001$, $P=0.001$ and $P=0.002$, respectively, Figures 2A-C). Similarly, in peripheral blood samples, the cases with lymphatic invasion, lymph node metastasis or peritoneal dissemination had significantly higher expression of *ID1* mRNA compared to patients without metastasis. ($P=0.02$, $P=0.02$ and $P<0.0001$ respectively, Figures 3A-C).

Expression of ID1 protein in bone marrow from patients with metastatic gastric cancer and healthy volunteers

The ID1 protein expression in bone marrow was evaluated immunohistochemically in studies of metastatic gastric cancer patients and healthy volunteers. In bone marrow of healthy volunteer (Figure 4A), the ID1 expression was localised mainly in the nuclei of bone marrow cells. The population of ID1-positive cells in healthy volunteer is lower than that in metastatic patient (Figure 4B). The ID1 expression of bone marrow cells with metastatic patient was also localised mainly in the nuclei. We also examined the ID1 expression of bone marrow carcinomatosis resulting from gastric cancer. The metastasized cancer cells were confirmed to be epithelial cells by HE (Supplementary Figure 2A) stain and AE1/AE3 (Supplementary Figure 2B). These cells were stained slightly with ID1 antibody in the cytoplasm (Figure 4C).

ID1 expression in primary lesions and metastatic lesions of gastric cancer

We examined the ID1 protein expression immunohistochemically in 30 primary lesions, 3 metastatic lymph nodes and 3 peritoneal disseminated lesions of gastric cancer cases. We found that 20 cases have high ID1 expression in primary lesions (Figure 5A). Some of the cases showed weak (Supplementary Figure 3A) or moderate (Supplementary Figure 3B) ID1 staining. In addition, two of three metastatic lymph nodes and peritoneal disseminated lesions were stained slightly with the ID1 antibody and the ID1 expression was localised in the cytoplasm of cancer cells in primary lesion, metastatic lymph node metastasis and peritoneal dissemination (Figures 5B and C).

DISCUSSION

Peritoneal dissemination is recognised as the most critical factor in assessing the prognosis of gastric cancer cases (Bando *et al*, 1999). There is no conclusive evidence, however, whether peritoneal dissemination might be established by the lymph node metastasis as well as direct dissemination from the serosal layer of stomach (Yonemura *et al*, 2007). In this study, the *ID1* mRNA expression in bone marrow and peripheral blood was significantly associated with lymph node metastasis and peritoneal dissemination. Therefore, we suggest that peritoneal dissemination of gastric cancer is mediated through lymph node metastasis combined with the *ID1*-expressing endothelial cells from bone marrow. From a clinical point of view, there are no convincing markers for

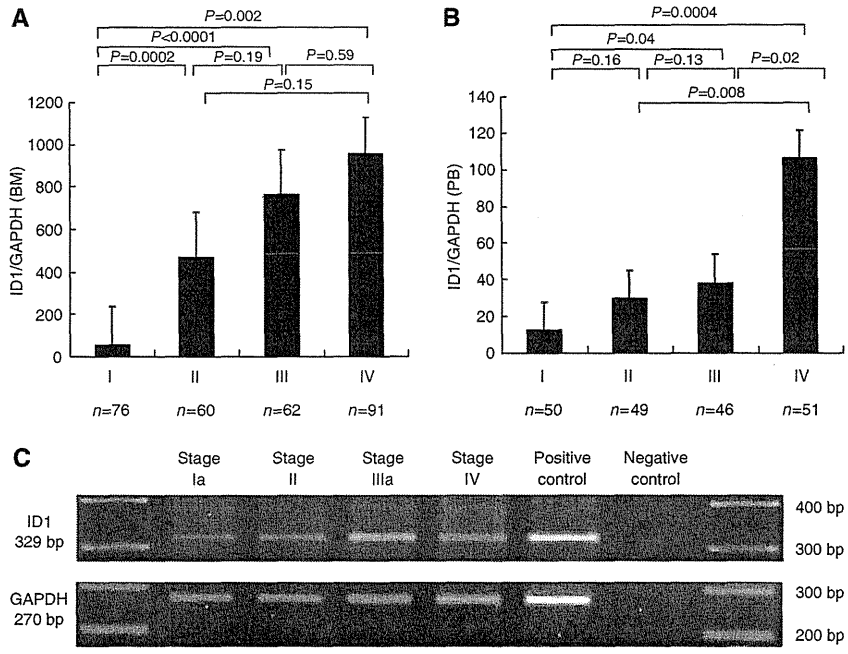

Figure 1 The mean value of *ID1* mRNA expression normalised *GAPDH* in bone marrow (**A**) and peripheral blood (**B**) according to staging classification. Group stage I consisted of patients with tumours that invaded less than the sub-mucosal layer and no lymph node metastasis (BM: $n=76$; PB: $n=50$). Group stage II consisted of patients with tumours that penetrated serosa or lymph node metastasis (Group 1) (BM: $n=60$; PB: $n=49$). Group stage III consisted of patients with tumours invasion of adjacent structures or lymph node metastasis (Group 2 or 3) (BM: $n=62$; PB: $n=46$). Group stage IV consisted of patients with distant metastasis (BM: $n=91$; PB: $n=51$). The mean value of *ID1* mRNA expression in bone marrow and peripheral blood increased along with the progression of stage. The RT–PCR analysis of four representative bone marrow samples was performed in each stage (**C**: upper; *ID1* product size 329 bp, lower; *GAPDH* product size 270 bp).

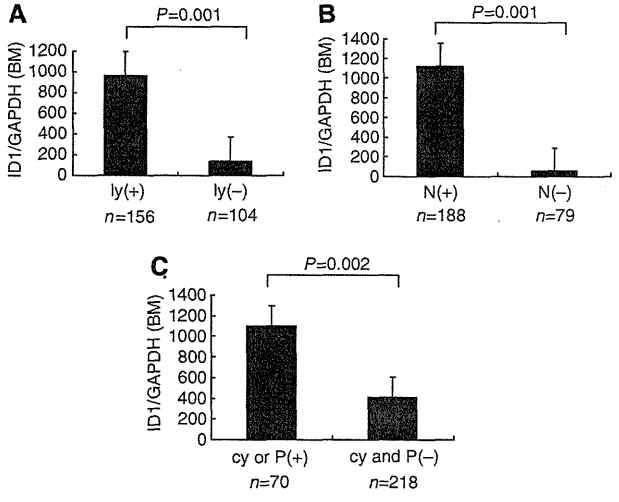

Figure 2 Comparison of the *ID1* mRNA expression in bone marrow of patients with or without lymphatic invasion (ly) (+: $n=156$, –: $n=104$; **A**), lymph node metastasis (*N*) (+: $n=188$, –: $n=79$; **B**) and peritoneal cytology (cy) or peritoneal metastasis (P) (+: $n=70$; –: $n=218$; **C**). In patients with evidence of lymphatic invasion, lymph node metastasis or peritoneal dissemination, the expression of *ID1* mRNA in bone marrow was significantly higher compared to patients without metastasis.

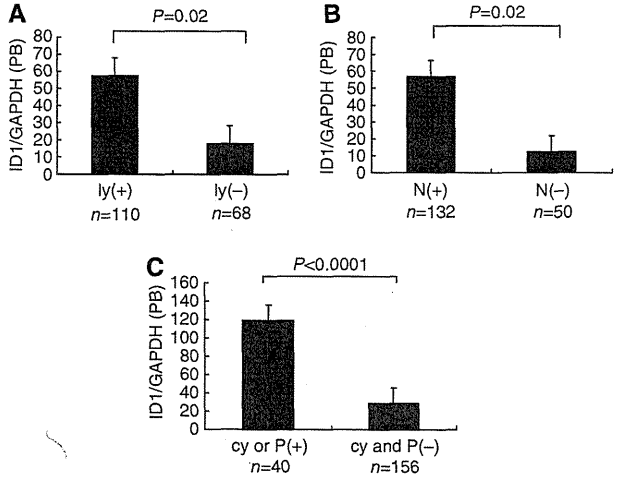

Figure 3 Comparison of the *ID1* mRNA expression in peripheral blood of patients with or without lymphatic invasion (ly) (+: $n=110$, –: $n=68$; **A**), lymph node metastasis (N) (+: $n=132$, -: $n=50$; **B**) and peritoneal cytology (cy) or peritoneal metastasis (P) (+: $n=40$, –: $n=156$; **C**). In patients with evidence of lymphatic invasion, lymph node metastasis or peritoneal dissemination, the expression of *ID1* mRNA in peripheral blood was significantly higher compared to patients without metastasis.

peritoneal dissemination before surgery. Therefore, it is significant that the *ID1* expression in bone marrow and peripheral blood can be used as a reliable marker before surgery to determine which gastric cancer patients are likely to have peritoneal dissemination mediated through lymph node metastasis.

There are two possible sources of the *ID1*-positive cells: ITC and host cells (such as EPCs, as stated by Gao *et al*). With regard to tumour cells, Tsuchiya *et al* showed that the number and size of

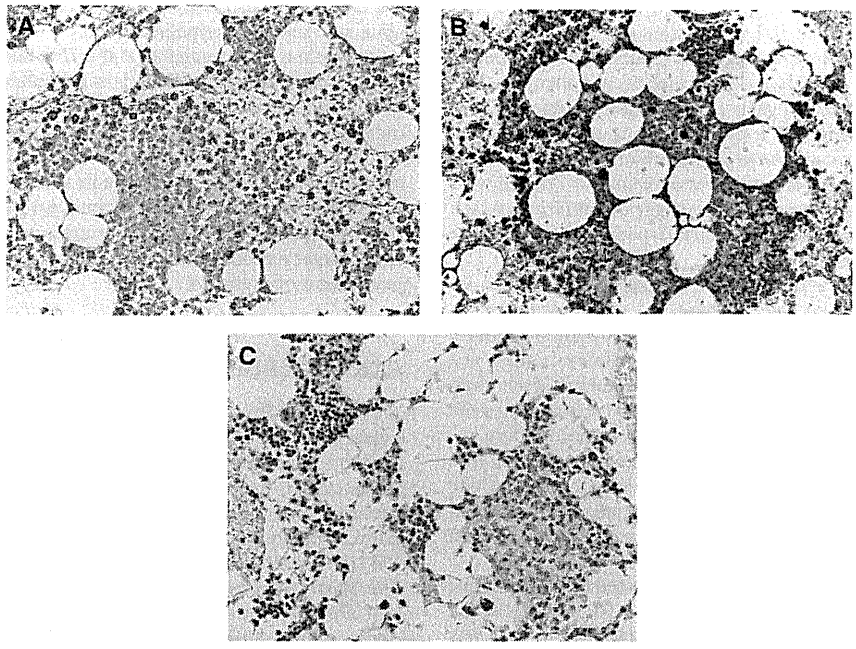

Figure 4 Immunohistochemistry with ID1 antibody, assessing bone marrow from a representative healthy volunteer and metastatic gastric cancer patient. In the bone marrow of healthy volunteer (**A**), the ID1 expression was localised mainly in the nuclei of bone marrow cells. The population of ID1-positive cells in healthy volunteer is lower than that in metastatic patients (**B**). The metastasized cells confirmed to be epithelial cells by HE stain and AE1/AE3 (Supplementary Figures 2A and B) that originated from gastric cancer were stained slightly with ID1 in cytoplasm (**C**). (**A**–**C**: original magnification: × 100).

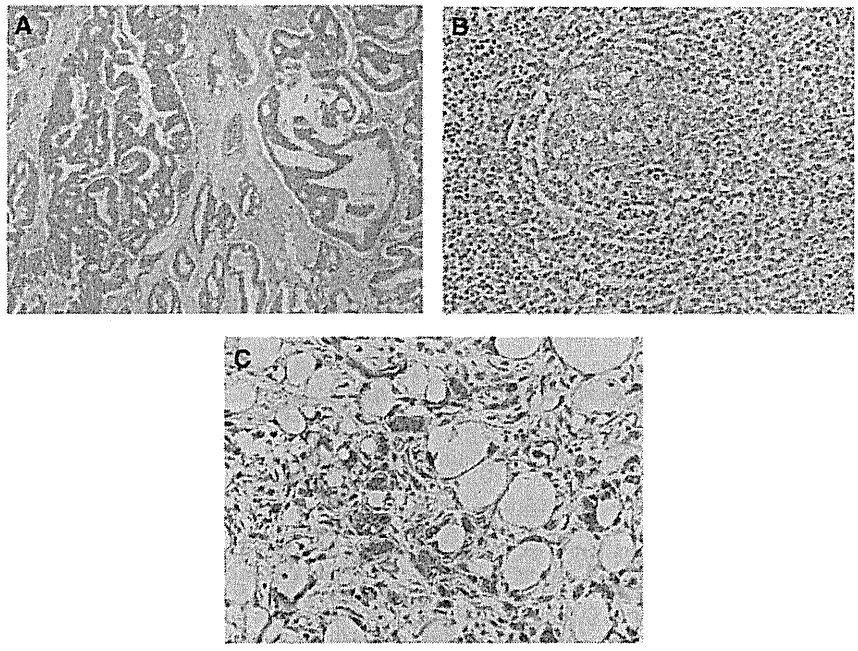

Figure 5 Immunohistochemistry with ID1 antibody assessing primary and metastatic lesions. Most of the primary lesions of gastric cancer were stained strongly with the ID1 antibody. A representative case was shown in (**A**). The ID1 expression was localised in the cytoplasm of cancer cells in primary lesion, metastatic lymph node metastasis (**B**) and peritoneal dissemination (**C**). (Original magnification: **A**: × 40, **B** and **C**: × 100).

peritoneal metastatic nodules formed by ID1 and ID3 double-knockdown gastric cancer cells were reduced in comparison to mock-transfected control cells *in vivo* (Tsuchiya *et al*, 2005). Furthermore, Kim *et al* reported that transgenic mice expressing a thymocyte-specific Id1 gene developed T-cell lymphoma *in vivo* (Kim *et al*, 1999). In addition, overexpression of ID1 in the primary cancer cells relative to normal mucosa has been observed in primary human oesophageal (Hu *et al*, 2001) and colorectal cancers (Wilson *et al*, 2001). Those reports found that the ID1 expression was significantly associated with the differentiation of

cells and a poor prognosis. In gastric cancer, Han *et al* found that strong immunohistochemical ID1 expression was associated with poorer differentiation and more aggressive behaviour of tumour cells (Han *et al*, 2004). In this study, we also examined the ID1 expression in primary lesion of gastric cancer cases. We found that two-thirds cases have high ID1 expression in primary lesions (Figure 5A). Furthermore, we showed that the metastasized cancer cells from gastric cancer in bone marrow were slightly stained with ID1 antibody (Figure 4C). These findings suggest that *ID1* may be a potential oncogene. As for the origin of ID1-positive cells in bone marrow, these seem to represent ITCs.

On the other hand, in this study, we present two lines of evidence indicating that the origin of the ID1 expression is from host cells, perhaps originating from bone marrow or peripheral blood. First, immunohistochemical studies showed that the population of ID1-positive cells in healthy volunteer is lower than that in metastatic patients (Figure 4A). Thus, ID1-expressing cells are particularly numerous in the bone marrow in which there are relatively few cancer cells. The current findings may suggest that ID1 is not a component of the aggregated cancer cells in the metastatic lymph nodes and peritoneal disseminated tumours, but instead plays a supportive role for gastric cancer cells to form lymph node metastasis and peritoneal dissemination.

Secondary, Gao *et al* found that Id1 was expressed by EPC positive for VE-cadherin and CD31 in peripheral blood (Gao *et al*, 2008). As we expected, the *ID1* expression in peripheral blood was significantly related to the incidence of peritoneal dissemination. In addition, there were significant association between *ID1* expression in peripheral blood and those in bone marrow from gastric cancer cases. This finding may indicate that the expression of *ID1* in peripheral blood originates from the circulating progenitor cells (CPCs), including EPC and from the bone marrow (Lyden *et al*, 2001). Those results may suggest that the origin of *ID1* expression is not only from cancer cells but also from host cells, such as CPCs in bone marrow and peripheral blood.

In summary, we found that the *ID1* mRNA expression in bone marrow and peripheral blood is a reliable predictive marker for lymph node metastasis and peritoneal dissemination, which indicates a poor prognostic outlook in gastric cancer. In addition, our findings suggest that the *ID1* expression originates from not only the cancer cells but also the host side progenitor cells with the cancer-bearing condition. Therefore, we propose that targeting the *ID1*-expressing cells in the bone marrow and/or peripheral blood after surgery represents a new concept for the treatment and/or prevention of metastasis.

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Molecular Bases of Myelodysplastic Syndromes: Lessons from Animal Models

YUKIKO KOMENO, JIRO KITAURA, AND TOSHIO KITAMURA*

Division of Cellular Therapy, Institute of Medical Science, the University of Tokyo, Tokyo, Japan

Myelodysplastic syndrome (MDS) is a clonal disorder of hematopietic stem cells characterized by ineffective hematopoiesis, peripheral blood cytopenia, morphologic dysplasia, and susceptibility to acute myeloid leukemia. Several mechanisms have been suggested as causes of MDS: unbalanced chromosomal abnormalities reflecting a gain or loss of chromosomal material, point mutations of transcription factors, and inactivation of p53. However, appropriate animal models that mimic MDS have long been lacking. We recently reported a novel murine model of MDS that recapitulates trilineage dysplasia and transformation to AML. In this review, we summarize the animal models of MDS and discuss the molecular bases of MDS as well as those of leukemia and myeloproliferative disorders (MPD).

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Acute myeloid leukemias (AML) are characterized by accumulation of immature "blasts" in the bone marrow and peripheral blood. A variety of gene alterations have been found in AML cells, including chromosomal translocations and deletions, point mutations, and frameshift mutations. Although fusion genes generated by chromosomal translocations—such as *AML1/ETO*, *PML/RARα*, and *MLL* rearrangements—impair myeloid differentiation in vitro, they fail to cause AML by themselves in vivo. On the other hand, *Bcr/Abl*, oncogenic *Ras*, *FLT3-ITD*, *KIT* mutations, and *JAK2/3* mutations confer a proliferative advantage on hematopoietic cells. These gene alterations are divided into two groups: one that confers proliferative or survival advantage (class I mutations) and another that impairs hematopoietic differentiation (class II mutations) (Gilliland, 2001; Gilliland and Griffin, 2002). Transgenic mice with the mutant genes, bone marrow transplantation (BMT) models of mice, and clinical data from patients with hematological disorders have revealed that both class I and class II mutations are required for development of AML. Indeed, two mutations are found in leukemic cells of patients (Kiyoi et al., 1997; Gilliland, 2001; Kottaridis et al., 2001; Gilliland and Griffin, 2002; Thiede et al., 2002; Bacher et al., 2006; Muller et al., 2008) (Table 1). This "two-hit model of leukemogenesis" was first demonstrated by Gilliland's group using an in vivo BMT model (Fig. 1) (Kelly et al., 2002). *PML/RARα* transgenic mice under the control of the cathepsin G promoter develop nonfatal myeloproliferative disorders (MPD), progressing in about 15–20% of cases to APL-like disease after 6–13 months (Grisolano et al., 1997; Pollock et al., 1999). Kelly et al. demonstrated that retroviral transduction of *FLT3-ITD* into BM cells obtained from *PML/RARα* transgenic mice, followed by BMT, resulted in APL-like disease with a higher penetrance (100%) and a shorter latency (40–210 days). The "two-hit model" was also confirmed by several other BMT models using combinations of *FLT3-ITD* and *AML1/ETO* or *MLL/SEPT6* (Ono et al., 2005; Schessl et al., 2005). Some frequent combinations of class I and class II mutations occur clinically, such as *FLT3-ITD/PML/RARα* and *Ras* mutations/*MLL* fusions (Table 1). It is extremely rare to find multiple mutations in the same class (class I or class II) in a patient. These lines of evidence all support the "two-hit model of leukemogenesis."

There are some exceptions to the two-hit model. *C/EBPα* mutations are frequently found in both alleles in AML with a normal karyotype and are regarded as class II mutations. In fact, AML with class II mutations such as t(8;21), inv(16) or t(15;17) have never been found to be associated with *C/EBPα* mutations (Nerlov, 2004). Interestingly, even class I mutations are infrequently associated with *C/EBPα* mutations. In patients with mutations in both alleles of *C/EBPα*, each mutation is located in a different domain and therefore has a distinct functional effect. Since C/EBPα is involved in both differentiation and growth arrest, and no patients have null alleles, two kinds of *C/EBPα* mutations in the same cell may functionally mimic the class I and class II mutations (Kirstetter et al., 2008). Another example of gene mutation is thought to induce leukemia by itself: a novel truncated isoform of AML1/ETO, AML1/ETO9a, was detected in samples from t(8;21) positive AML (Yan et al., 2006). AML1/ETO9a lacks the C-terminal NHR3 and NHR4 domains of AML1/ETO, and expression of AML1/ETO9a induced leukemia without additional genetic events in a mouse BMT model. The molecular mechanism of AML1/ETO9a-induced leukemia awaits further study (Yan et al., 2008).

While the combination of class I and class II mutations induces leukemogenesis, a class I mutation alone, such as *Bcr/Abl* or *JAK2/3* mutations, will induce MPD, where autonomous cell growth or inhibition of apoptosis is a major problem (Fig. 1). On the other hand, in patients with myelodysplastic syndrome (MDS), cell differentiation is mainly affected. MDS is a clonal disorder of hematopoietic stem cells characterized by ineffective hematopoiesis, peripheral blood cytopenia, morphologic dysplasia, and susceptibility to acute myeloid leukemia (Malcovati and Nimer, 2008). MDS can occur de novo or secondary to prior cytotoxic chemotherapy and radiation. The two-hit model would predict that while class I mutations induce MPD, class II mutations are expected to induce MDS. However, the hypothesis depicted in Figure 1 is oversimplified: class II mutations such as *AML1/ETO* and *PML/RARα* are not observed in patients with MDS. In addition, mice expressing *AML1/ETO* or *PML/RARα* develop MPD, not MDS (Grisolano

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*Correspondence to: Toshio Kitamura, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.
E-mail: kitamura@ims.u-tokyo.ac.jp

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