Colon Neoplastic Cells Do Not Originate from Bone Marrow-derived Cells after Sex-mismatched Bone Marrow Transplantation

GEN SAKAI[1], TOMOHARU YAJIMA[1*], HIROMASA TAKAISHI[1*], TAKEHIKO MORI[2], HAJIME HIGUCHI[1], SHOKO NAKAMURA[1], SHINSUKE FUNAKOSHI[1], MASAYUKI ADACHI[1], MOTOKO IZUMIYA[1], HIDEKO AKAGI[1], YASUO HAMAMOTO[1], TAKANORI KANAI[1], MAKIO MUKAI[3], SHINICHIRO OKAMOTO[2] and TOSHIFUMI HIBI[1]

Divisions of [1]Gastroenterology and Hepatology, [2]Hematology and [3]Diagnostic Pathology, Keio University School of Medicine, Tokyo, Japan

Abstract. *Background: Although previous studies indicate that gastrointestinal (GI) cancer may originate from cells recruited from bone marrow (BM) in mice, whether similar phenomena occur in humans is controversial. In the current study, we evaluated two female patients who developed colonic adenocarcinoma more than 10 years after gender-mismatched BM transplantation, and followingly underwent successful endoscopic mucosal resection. Materials and Methods: Fluorescent in situ hybridization (FISH) analysis was used to determine whether the tumours contained donor-derived BM cells. Results: Approximately 1.2% of the tumour cells contained Y-chromosome-positive signals, and a comparable percentage of normal colonic epithelial cells close to the tumour also contained Y-chromosome-positive signals. Conclusion: These results do not support the concept that GI cancer can originate from BM-derived cells.*

Reportedly, bone marrow (BM)-derived cells can differentiate into cell types other than blood cells, such as intestinal epithelial cells (1). Similarly, donor-derived cells can give rise to colonic mucosa, renal parenchyma, gastric mucosa, and pulmonary epithelium following total-body irradiation (TBI) and allogeneic BM transplantation in humans (1-4). Furthermore, the contribution of BM-derived cells to epithelial carcinogenesis has been recently highlighted. The existence of BM-derived cells within either epithelial cancer or cancer stroma has been reported in patients suffering from secondary cancer after sex-mismatched BM transplantation (5, 6). Several studies using X/Y chromosome fluorescent *in situ* hybridization (FISH) demonstrated a number of donor-derived cells to be identified in a variety of cancer tissues, including gastric cancer, renal cell carcinoma, lung adenocarcinoma, laryngeal squamous cell carcinoma, glioblastoma, and Kaposi sarcoma (7). Recently, one study demonstrated that gastric carcinogenesis originated from donor-derived BM stem cells in a mouse model of *Helicobacter felis* infection (8). In addition, donor-derived BM cells have been identified within colonic adenoma tissues using a murine model of adenomatogenesis and APC min mice (9). Based on these studies, BM-derived cells can develop into or promote the development of cancer (5, 7, 9). Although some reports demonstrate that a similar phenomenon occurs in humans, these findings are still controversial. Here, we evaluated two female patients who developed colonic adenocarcinoma more than 10 years after gender-mismatched BM transplantation and underwent successful endoscopic mucosal resection (EMR) to determine whether the carcinoma contained malignant cells derived from transplanted donor BM stem cells.

*These Authors contributed equally to this work.

Correspondence to: Tomoharu Yajima, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Shinanomachi 35, Shinjuku-ku, Tokyo, 160-8582, Japan. Tel: +81 333531211, Fax: +81 333413631, e-mail: yajimatm@sc.itc.keio.ac.jp

Key Words: Colon cancer, sex-mismatched bone marrow transplantation, cancer stem cell, *in situ* hybridization.

Materials and Methods

Samples. All samples were collected at Keio University Hospital. Written informed consent was obtained from each patient after explanation of the nature and possible consequences of the study. The local Ethics Committee approved the study.

Case 1. When the patient, a 62-year-old woman, was 47 years old (1992), she received a diagnosis of acute promyelocytic leukaemia (APL); therefore, she was treated with all-trans retinoic acid (ATRA) and systemic chemotherapy [two courses of behenoyl cytosine arabinoside, daunorubicin, 6-mercaptopurine plus

prednisolone, six courses of vincristine, endoxan, methotrexate plus prednisone and one course of behenoyl cytosine arabinoside, daunorubicin, 6-mercaptopurine, prednisolone plus daunomycin]. Following recognition of her second complete response (CR) to chemotherapy, she underwent allogeneic BM transplantation [Human Leukocyte Antigen and ABO total match, blood type A/Rh+] on January 17, 1995. The donor was her elder brother, who was 58 years old at the time. On February 13, 1995, a BM puncture examination was used to confirm that the BM cells were donor-derived cells (46,XY). Graft *versus* host disease (GVHD) was tightly controlled by medications, and she was discharged from the hospital on March 12, 1995. Following discharge, she was treated as an outpatient. On June 20, 2007, she underwent a colonic endoscopy following a positive faecal occult blood test. The endoscopy revealed that she had a flat elevation, approximately 10 mm in diameter, in the sigmoidal colon (Figure 1A); therefore, she underwent EMR to remove this lesion. The pathological examination revealed a well-differentiated tubular adenocarcinoma within an adenoma (int, INFα, m, ly0, v0) (Figure 1B).

Case 2. When the patient, a 42-year-old woman, was 28 years old (1994), she received a diagnosis of APL. She underwent ATRA and systemic chemotherapy, comprising of four courses of consolidation therapy and five courses of maintenance therapy, prescribed by the JALSG AML87 protocol (10). Following recognition of her second CR, she underwent allogeneic BM transplantation (HLA and ABO total match, blood type O/Rh+) on March 19, 1996. The donor was her elder brother, who was 44 years old at the time. On April 30, 1996, a BM puncture examination was used to confirm that BM cells were donor-derived cells (46,XY). Graft *versus* host disease (GVHD) was tightly controlled, and she was discharged from the hospital on July 31, 1996. Following discharge she was treated as an outpatient. On December 19, 2007, she underwent a colonic endoscopy following a positive faecal occult blood test. There were two polyps, one in each arm of her ascending colon; the oral-side polyp was about 3 mm, and the anal-side polyp was about 10 mm (Figure 2A and C). Therefore, she underwent EMR; pathological examination revealed that the oral-side polyp was a tubular adenoma with severe dysplasia (Figure 2B) and the anal-side polyp contained a well-differentiated tubular adenocarcinoma within an adenoma (int, INFβ, med, pM, ly0, v0) (Figure 2D).

FISH (11). We analysed the histological and pathological samples using FISH and probes for mammalian X and Y chromosomes. Haematoxylin-eosin-stained colonic tissue sections from both patients described above, who had undergone BM transplantation, were examined. We used a digoxigenin-labelled FISH probe for human Y chromosome and Cy5-labelled FISH probe for rat X chromosome. Following the FISH analysis, the samples were subjected to immunostaining to label for cytokeratin and CD45 to confirm that the cells with Y-positive signals were epithelial cells and not haemocytes.

Analysis of X/Y chromosomes. After removing paraffin from the tissue samples, the samples were soaked in phosphate buffered saline (PBS) for about 5 min, and then treated with protease (0.1% pepsin/0.1 M HCl) for about 3 min. After these reactions, the samples were washed in PBS, and dehydrated in a series of alcohol solutions. Digoxigenin-labelled Y-chromosome and Cy5-labelled X-chromosome FISH probes were applied to the pepsin-treated samples, and the probes were treated simultaneously on a hot plate (80˚C) for 10 min; probes hybridized to targets within the sample, overnight at 37˚C. Tissue samples were stringently washed, the digoxigenin label was then labelled with anti-digoxigenin, and probe signals were analysed.

Immunostaining (CD45, Pan-cytokeratin). Samples that were subjected to FISH analysis were then blocked using PBS with 5% milk and 0.3% Triton X-100. We diluted primary antibodies (FLEX Monoclonal Mouse Anti-Human CD45, Leucocyte Common Antigen and FLEX Monoclonal Mouse Anti-Human Cytokeratin; Dako North America, Inc. Carpinteria, California, USA) in PBS with 5% milk and 0.05% Tween20, which reacted to anti-digoxigenin-Cy5 for 1 h in 37˚C. Samples were then washed in PBS for 15 min, and secondary antibodies (anti-rabbit-Alexa488 and anti-mouse-Alexa594; Life Technologies, Carlsbad, California, USA), diluted at 1/500, were then incubated with the samples for 30 min. Samples were washed in PBS for 15 min, then fixed with 4% paraformaldehyde in PBS for 5 min, and washed in PBS. Finally, samples were stained with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride; DOJINDO Laboratories, Kumamoto, Japan).

Microscopic observation. Samples were mounted on slides using mounting medium that included an anti-bleaching agent. Signals from FISH probes and data were analysed using the Leica CW-4000 system (Leica Microsystems GmbH, Wetzlar, Germany). We collected and merged 10 images of probe signals by moving the microscopical stage by 0.5 μm in depth on the sections, combining the signal by depth from the upper side to the lower side of sections into one image.

Results

Most cancer cells were derived from the recipients. In this study in FISH, X-chromosome signals were purple dots and Y-chromosome signals were yellow dots; DAPI was used as a counterstain; CD45 signals were red dots and cytokeratin signals were in green dots. Furthermore, not every cell necessarily had chromosomal signals because some optical sections may not have included target loci.

In case 1, only a small percentage (1.2%; 4/340) of cancer epithelial cells in the colonic tumour (Figure 1A), identified as well-differentiated adenocarcinoma (Figure 1B), had donor-derived Y chromosome signals (Figure 3A) (Table I); this finding indicated that most cancer cells were derived from the female patient, the BM transplantation recipient. Some normal (non-neoplastic) cells in normal tissue surrounding the tumour also had X- and Y-chromosome signals; this observation indicated that these were donor-derived cells (Figure 3A). Furthermore, approximately 1.4% of the non-cancerous epithelial cells in the adenoma were donor-derived cells, with Y-chromosome signals (Figure 3B) (Table II); this finding is consistent with previous reports, specifically the finding that about 1% of epithelial cells in adenomas, from a patient that had undergone BM transplantation, were donor-derived (Figure 3B) (Table I) (6, 9). In addition, the interstitial cells within cancerous and non-cancerous areas of the lesion were

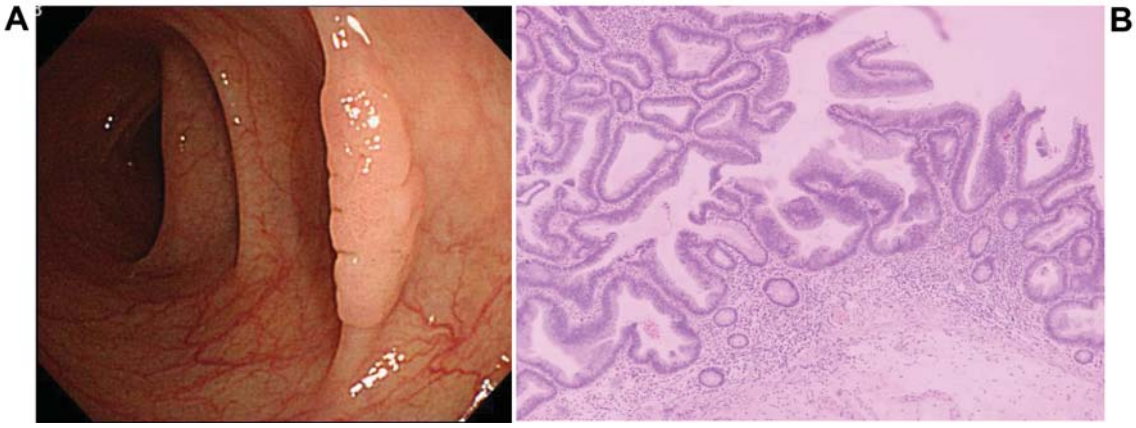

Figure 1. *Case 1 of colonic adenocarcinoma and adenoma. The patient had undergone endoscopic mucosal resection. Colonic endoscopy was performed 12 years after sex-mismatched bone marrow transplantation. A: Endoscopic image. A flat elevation of approximately 10 mm was found in the sigmoidal colon. B: Haematoxylin and eosin-stained sample from colonic tumour of this patient; histological examination of the samples revealed a well-differentiated tubular adenocarcinoma in an adenoma (int, INFα, m, ly0, v0, and surgical margin negative). Original magnification: ×100.*

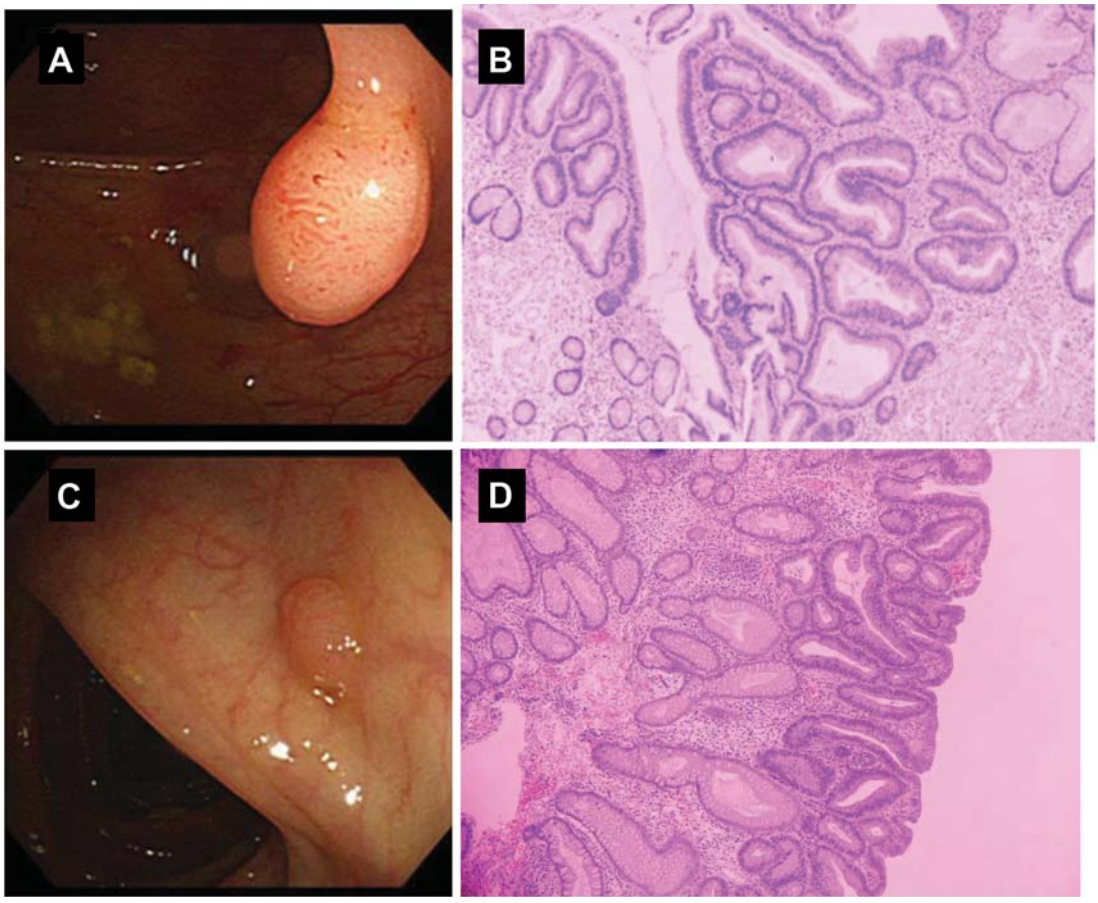

Figure 2. *Case 2 of colonic adenocarcinoma and adenoma. The colonic endoscopy was performed 11 years after sex-mismatched bone marrow transplantation. A: Endoscopic image. An Isp polyp of approximately 10 mm was found in the oral-side ascending colon. B: Haematoxylin and eosin (HE)-stained sample from the colonic tumour, shown in Figure 2A; the patient had undergone endoscopic mucosal resection (EMR), and the histological examination revealed a well-differentiated tubular adenocarcinoma in adenoma (int, INFβ, med, pM, ly0, v0, surgical margin-negative). Original magnification: ×100. C: Endoscopic image. A polyp of approximately 3 mm was found in the anal-side ascending colon. D: HE-stained sample from the colonic tumour shown in Figure 2C; the patient underwent EMR, and the histological examination revealed only adenoma (surgical margin-negative). Original magnification: ×100.*

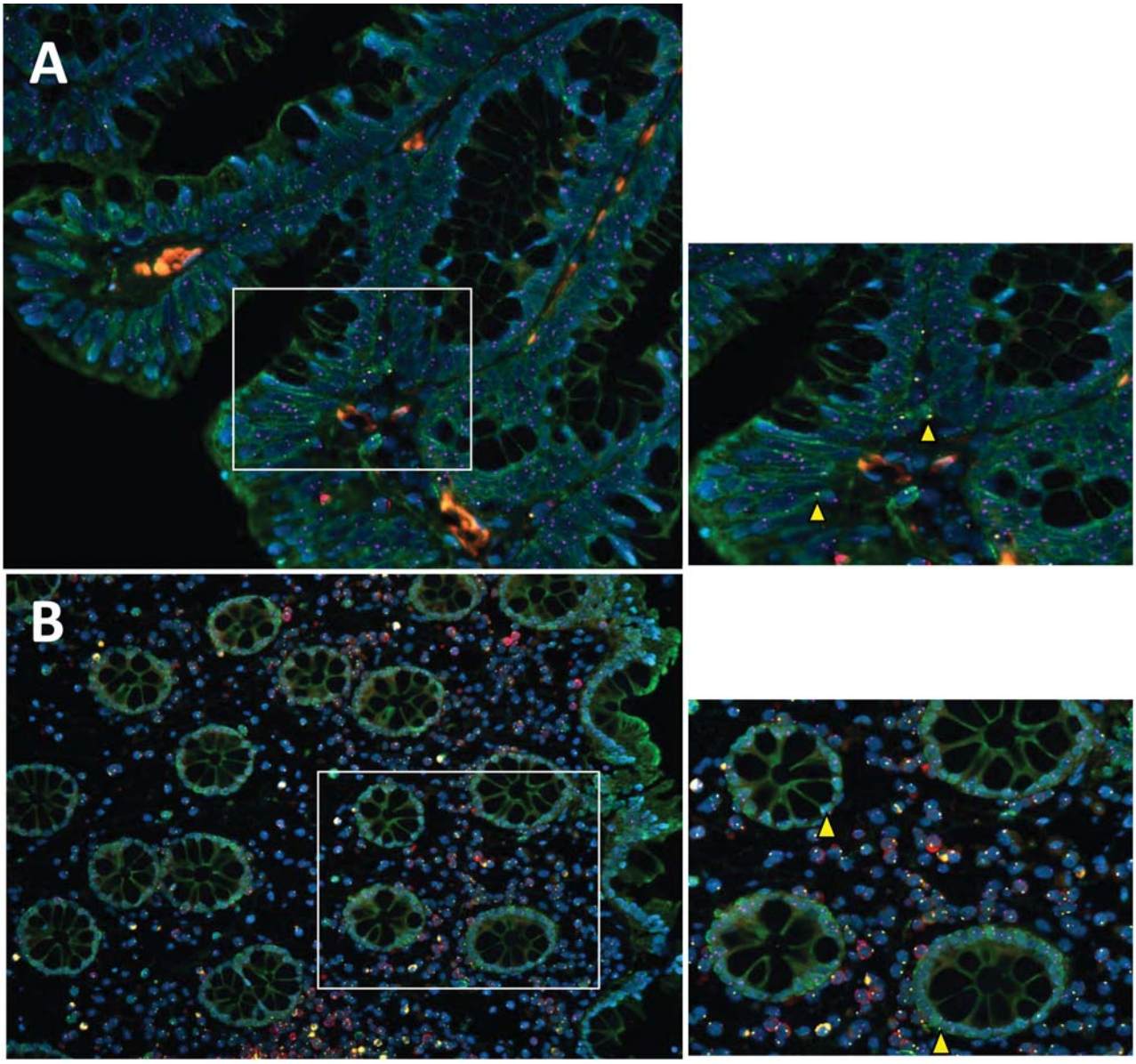

Figure 3. *FISH analysis of samples from case 1. FISH signals, DAPI, CD45 signal (red), and cytokeratin (green) signals are shown in cancerous (A) and non-cancerous (B) areas. Yellow arrowheads indicate Y-chromosome-positive cells. Only a small percentage of cancerous epithelial cells (1.2%) and non-cancerous epithelial cells (1.4%) have Y-chromosome signals derived from the donor. The interstitial cells in the cancerous (A) and non-cancerous (B) epithelial cell lesions were comprised of almost all donor-derived CD45$^+$ cells (approximately 76%). Original magnification: main image, ×100 ; inset, ×200.*

predominantly donor-derived cells [76% (215/280) and 75% (210/280), respectively] (Figure 3B) (Table I); this finding indicated that almost all donor-derived cells in the cancer lesion were haematopoietic cells. Consistently, the analysis of CD45 staining revealed that donor-derived Y-positive cells were almost all CD45-positive; this observation also indicated that the donor-derived cells in the lesion were haemocyte-derived cells.

The findings from the analysis of case 2 were similar to those from case 1; only a small percentage (1.2%; 4/330) of cancerous epithelial cells in case 2 had Y chromosome signals, but cells in the other normal areas were derived from the donor, as they had X- and Y-chromosome signals. Interstitial cells within both lesion cases comprised of about 80% donor-derived CD45$^+$ haematopoietic cells (Figure 4).

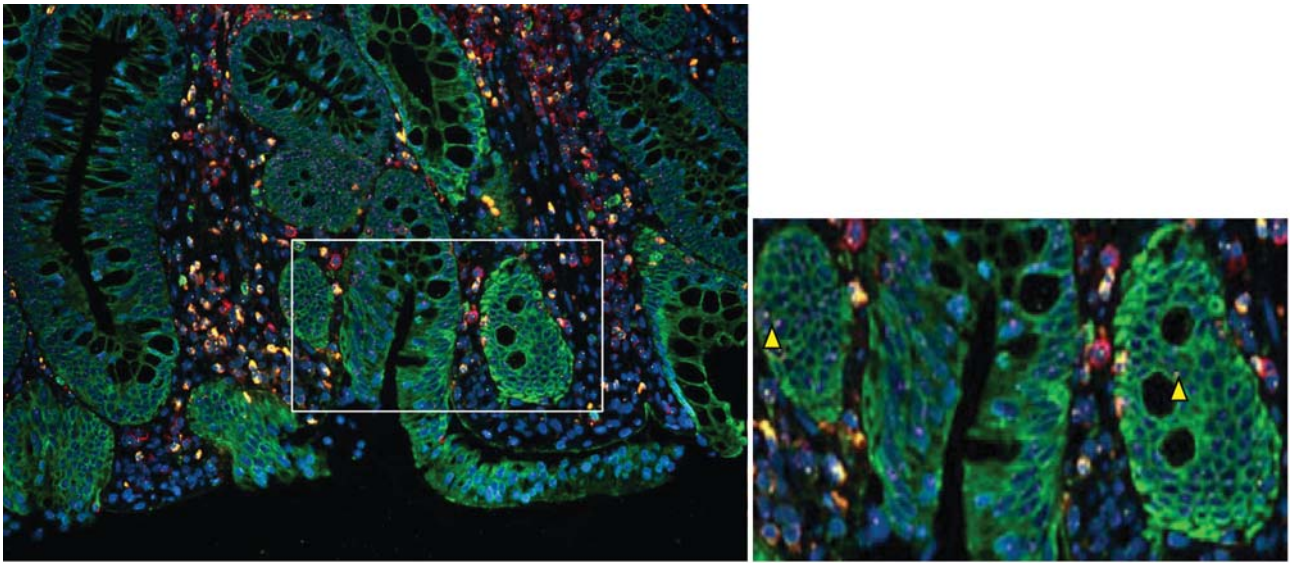

Figure 4. *FISH analysis of samples from case 2. FISH signals, DAPI, CD45 signals, and cytokeratin signals in a cancerous area. Yellow arrowheads indicate Y-chromosome-positive cells. Only a small percentage of cancerous epithelial cells (1.2%) and non-cancerous epithelial cells (1.5%) have Y-chromosome signals derived from the donor. The interstitial cells in the cancerous lesion comprised of almost all donor-derived CD45$^+$ cells (approximately 80%). Original magnification: main image, ×100; inset, ×200.*

Table I. *Y-Chromosome-positive rate of each lesion.*

Y-Chromosome-positive rate (%)	Cancer lesion	Non-cancerous lesion	CD45(+) cells
Case 1	1.2% (4/340)	1.4% (5/350)	95% (95/100)
Case 2	1.2% (4/330)	1.5% (5/350)	92% (92/100)

Table II. *The ratio of BMDC in secondary cancer after BMT in previous reports.*

Author (Ref)	Age (years) /gender	Primary diagnosis	Year of BMT	Donor	Secondary cancer	Year of diagnosis	BMDC (%)
Itzhak *et al*. (7)	62/M	ALL	2002	Un-related	Kaposi sarcoma	2003	2.5
	48/M	Mantle cell lymphoma	1998	Sister	Lung adenocarcinoma	2001	4.0
	36/M	CML	1999	Sister	Laryngeal carcinoma	1999	6.0
	10/M	ALL	1993	Sister	Glioblastoma	1996	5.0
Pawelek *et al*. (17)	32/M	CLL	1987	Son	RCC	1989	10.0
Krause et al. (16)	43/F	ALL	-	Brother	Skin cancer (squamous)	7 years (after BMT)	0.0

ALL: Acute lymphocytic leukemia; BMDC: bone marrow derived cels; BMT: bone marrow transplantation; CML: chronic myelogenous leukemia; RCC: renal cell carcinoma.

Discussion

Previous reports illustrate that in patients who had undergone BM transplantation, some BM-derived cells were induced to migrate to sites of inflammation, following injury to repair damaged epithelial cells (1, 7-9). Furthermore, accumulating evidence supports the existence of cancer stem cells (CSCs) (12-15). However, the nature, origin, and function of CSCs are largely unknown. Some recent case reports have demonstrated that BM-derived stem cells are the origin of some types of human epithelial cancer, exhibiting the malignant phenotype of skin cancer (9), renal cell carcinoma (6), lung adenocarcinoma (7), laryngeal squamous cell carcinoma (7), glioblastoma (7) and Kaposi sarcoma (7). Additionally,

patients who underwent BM transplantation developed these carcinomas within 1 to 7 years of sex-mismatched BM transplantation; however, BM-derived cells represented fewer than 10% of the cells in the cancer lesions (Table II).

To further assess whether BM-derived stem cells contribute to human carcinogenesis, two human colon adenocarcinomas, discovered in two patients who had both undergone sex-mismatched allogeneic BM transplantation, were examined to determine whether the carcinoma contained malignant cells derived from transplanted donor stem cells.

Although Houghton *et al*. reported that nearly 100% of the gastric cancer cells examined in a murine model of induced gastric cancer cells were derived from donor stem cells (8), only a small percentage (approximately 1% to 2%) of the neoplastic cells in gland tissue samples from the patients in both cases examined here were donor-derived cells with Y-chromosome signals. Therefore, we conclude that almost all cancer cells in these samples were derived from the patients, *i.e*. the BM transplantation recipient, not the donor. Moreover, approximately 1.7% of the non-neoplastic epithelial cells in these samples were donor-derived; this observation indicates that, as shown in previous studies, BM-derived cells may be induced to repair inflammation and injury within patients who have undergone BM transplantation (1, 7-9, 16).

The reasons for the marked differences in the findings from Houghton *et al*.'s study of a mouse model and those from our human study are unknown. The differences may be due to differences in the species, differences in the doses of total-body irradiation, differences in the efficacy of stem cell ablation both in the BM and the periphery, or to combinations of any or all of these possibilities (7). Additionally, the duration of the interval between BM transplantation and the development of secondary cancer may be important; there may be as many as 10 to 20 years between the emergence of cancer and its clinical detection. Therefore, both tumours examined in this study may have been initiated before BM transplantation, and consequently be derived from the recipients' own BM cells rather than from either donor. Nevertheless, our report dealt with colonic cancer from two patients who were diagnosed with adenocarcinoma 11 or 12 years after BM transplantation, and we demonstrated that the adenocarcinoma and adenoma cells, which were pre-cancerous, were tissues derived from only the recipients' cells and contained only the recipient's sex karyotype, and that these lesions did not contain donor-derived Y chromosomes. Further studies are needed in order to address this issue.

Interestingly, interstitial cells in these lesions were derived from donor-derived BM. Therefore, we concluded that donor-derived cells in the cancer lesions were haematopoietic cells based on the percentage of donor-derived cells among all interstitial cells in both lesions and because the donor-derived interstitial cells in both lesions were almost all CD45-positive haemocytes.

Taken together, our findings demonstrate theoretically that BM-derived cells do not appear to contribute to carcinogenesis, despite previous reports which were insufficient to firmly establish the origin of carcinogenesis.

Conflicts of Interest

This study was supported in part by grants-in-aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research and Creative Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology; the Japanese Ministry of Health, Labour and Welfare; the Japan Medical Association; the Foundation for Advancement of International Science; and the Foundation for Research by Taiho Pharmaceutical Co. Ltd. None of the Authors have any financial relationships relevant to this publication to disclose.

Acknowledgements

This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education and Culture of Japan.

References

1 Okamoto R, Yajima T and Watanabe M: Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. Nat Med *8*: 1011-1017, 2002.

2 Spyridonidis A, Schmitt-Graff A and Tomann T: Epithelial tissue chimerism after human hematopoietic cell transplantation is a renal phenomenon. Am J Pathol *164*: 1147-1155, 2004.

3 Poulsom R, Forbes SJ and Hodivala-Dilke K: Bone marrow contributes to renal parenchymal turnover and regeneration. J Pathol *195*: 229-235, 2001.

4 Suratt BT, Cool CD and Serls AE: Human pulmonary chimerism after hematopoietic stem cell transplantation. Am J Respir Crit Care Med *168*: 318-322, 2003.

5 Chakraborty A, Lazova R and Davies S: Donor DNA in a renal cell carcinoma metastasis from a bone marrow transplant recipient. Bone Marrow Transplant *34*: 183-186, 2004.

6 Yilmaz Y, Lazova R and Qumsiyeh M: Donor Y chromosome in renal carcinoma cells of a female BMT recipient: Visualization of putative BMT-tumor hybrids by FISH. Bone Marrow Transplant *35*: 1021-1024, 2005.

7 Avital I, Moreira AL and Downey RJ: Donor-derived human bone marrow cells contribute to solid organ cancers developing after bone marrow transplantation. Stem Cells *25*: 2903-2909, 2007.

8 Houghton J, Stoicov C and Nomura S: Gastric cancer originating from bone marrow-derived cells. Science *306*: 1568-1571, 2004.

9 Cogle CR, Theise ND and Fu D: Bone marrow contributes to epithelial cancers in mice and humans as developmental mimicry. Stem Cells *25*: 1881-1887, 2007.

10 Ohno R, Kobayashi T and Kodera K: Randomized study of individualized induction therapy with or without VCR, and of maintenance of 4 or 12 courses in adult AML:JALSG-AML87. Japan Adult Leukemia Study Group (JALSG). Leukemia *6 (Suppl 2)*: 92-95, 1992.

11 Caroline J, Fabien M, Michel RN and Claire V: Optimization of nuclear transcript detection by FISH and combination with fluorescence immunocytochemical detection of transcription factors. J Histochem Cytochem *45*: 1585-1592, 1997.
12 Al-Hajj M, Wicha MS and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA *100*: 3983-3988, 2003.
13 O'Brien CA, Pollett A and Dick JE: A human colon cancer cell capable of initiating tumor growth in immunodeficient mice. Nature *445*: 106-110, 2007.
14 Ricci-Vitiani L, Lombardi DG and De Maria R: Identification and expansion of human colon cancer-initiating cells. Nature *445*: 111-115, 2007.
15 Chenwei L, Heidt DG and Simeone DM: Identification of pancreatic cancer stem cells. Cancer Res *67*: 1030-1037, 2007.
16 Krause DS, Theise ND and Collector MI: Multiorgan, multilineage engraftment by a single bone marrow-derived stem cell. Cell *105*: 369-377, 2001.
17 Pawelek JM and Chakraborty AK: The cancer cell-leukocyte fusion theory of metastasis. Adv Cancer Res *101*: 397-444,2008.
18 Au WY, Chan EC and Pang A: Nonhematologic malignancies after allogeneic hematopoietic stem cell transplantation: Incidence and molecular monitoring. Bone Marrow Transplant *34*: 981-985, 2004.
19 Golfinopouloss V, Pentheroudakis G and Pavlidis N: Donor-derived breast cancer in a bone marrow transplantation recipient. Breast Cancer Res Treat *113*: 211-213, 2009.
20 Jiang Y, Jahagirdar BN and Reinhardt RL: Pluripotency of mesenchymal stem cells derived from adult marrow. Nature *418*: 41-49, 2002.
21 Li H-C, Stoicov C, Rogers AB and Houghton J: Stem cells and cancer: Evidence for bone marrow stem cells in epithelial cancers. World J Gastroenterol *12*: 363-371, 2002.
22 Peters BA, Diaz LA and Polyak K: Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med *11*: 261-262, 2005.

Received May 25, 2012
Revised August 13, 2012
Accepted August 14, 2012