

Detection of Peritoneal Micrometastases of Gastric Carcinoma with Green Fluorescent Protein and Carcinoembryonic Antigen Promoter

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ABSTRACT

The aim of this study was to specifically visualize micrometastases in the peritoneal cavity, which cannot be detected by conventional methods, by using enhanced Green Fluorescent Protein (EGFP) containing carcinoembryonic antigen (CEA) promoter in an upstream position. In *in vitro* experiments, two cell lines from human gastric cancer, MKN45 and MKN1, and a cell line from human fibrosarcoma, HT1080, were transduced with pCEA-EGFP, which contains the CEA promoter region. MKN45 and MKN1, which expressed CEA mRNA, showed positive fluorescence after transduction of pCEA-EGFP, whereas HT1080 did not. In *in vivo* experiments, 7 days after 10^7 MKN45 had been injected into the peritoneal cavity of BALB/c nude mice, pCEA-EGFP was transduced in the peritoneal cavity using a fusogenic liposome with the envelope protein of Hemagglutinating Virus of Japan on the surface. On the peritoneum of the abdominal wall, fluorescent nodules were detected by fluorescence stereomicroscopy. These nodules had a minimal size of ~ 0.15 mm and could not be detected by conventional stereomicroscopy or macroscopy. They were histologically confirmed to be cancer cells by H&E staining. The results suggest that visualization of peritoneal micrometastasis of gastric cancer using CEA promoter and EGFP can offer a new strategy for diagnosis of micrometastasis.

INTRODUCTION

Gastric cancer is one of the leading causes of cancer death in Japan. Recently, the prognosis of gastric cancer has been improved by early detection and improvement in surgical techniques such as extensive lymphadenectomy (1, 2). However, patients with advanced gastric cancer display poor prognosis even if they undergo curative resection, suggesting that systemic micrometastasis may have already existed at the time of surgery (3). The main cause of recurrence after curative resection of advanced tumors is peritoneal metastasis, which is also the most common cause of noncurative surgery. Once peritoneal metastasis of a visible size is established, a complete cure is not currently possible (4, 5). Neither surgical treatment nor chemotherapy, whether administered systemically or *i.p.*, can be of clinical benefit. However, it is generally believed that the smaller the tumor size is, the more effective the chemotherapy can be. Therefore, if methods that can precisely detect and diagnose peritoneal micrometastasis can be established, it may be possible to improve the prognosis of advanced gastric cancer by appropriate adjuvant therapy.

In trying to find a way to detect micrometastases in the living state, we used a GFP² gene cloned from the jellyfish *Aequorea victoria*, which has been used as a marker of gene expression in many research

fields (6). Because GFP requires no other proteins, substrates, or cofactors to emit fluorescence, it was expected to allow detection of invisible micrometastases in the living state.

To obtain tumor-specific expression of the transduced gene, we used the promoter region of the CEA, which has been used for this purpose in many studies on gene therapy of cancer (7–12). CEA mRNA is specifically expressed in cancer cells derived from epidermal tissues such as cancer of the colon, lung, stomach, breast, and other organs. Schrewe *et al.* (13) cloned the genomic DNA of CEA and demonstrated that a 440-bp segment upstream from the transcriptional start site exhibited an apparently higher promoter activity in CEA-producing cells than in non-CEA-producing HeLa cells.

Gene introduction using a fusogenic liposome with the envelope protein of HVJ on the surface should be more advantageous than other methods. This method should offer high efficiency and low toxicity in *in vivo* transduction (14–16).

The aim of this study was to visualize peritoneal micrometastases in the living state, which cannot be done with conventional methods, such as by using EGFP and CEA promoter.

MATERIALS AND METHODS

Cell Lines. Human gastric cancer cell lines, MKN45 and MKN1, and human fibrosarcoma, HT1080, were obtained from Health Science Research Resources Bank (Tokyo, Japan) and maintained in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% heat-inactivated FCS (Life Technologies, Inc., Grand Island, NY) and streptomycin/penicillin (Life Technologies, Inc.).

Preparation of Vector Plasmids. The mammalian expression vector, pEGFP-N1, which contains EGFP cDNA with cytomegalovirus immediate-early enhancer as a promoter, and pEGFP-1, which contains EGFP cDNA alone with no promoter, were obtained from Clontech Laboratories, Inc. (Palo Alto, CA).

The *cis*-acting sequences, which convey cell type-specific expression of the CEA gene, are contained in the upstream region between -424 and -2 bp from the transcriptional start as described by Schrewe *et al.* (13). To create a CEA promoter/EGFP chimera gene (pCEA-EGFP), the *Bgl*II/*Hind*III fragment of the CEA promoter subcloned in pCEA-CAT (a gift from Dr. Tadashi Osaki, Department of Medicine III, Osaka University, Osaka, Japan) was inserted into the multiple cloning site of pEGFP-1 upstream of EGFP, resulting in the construct pCEA-EGFP (Fig. 1).

CEA Production by the Cells. The cells were seeded on six-well plates (Iwaki Glass, Chiba, Japan) at a density of 1×10^6 /well and cultured for 48 h under the conditions described previously. The medium was replaced with new medium, and after incubation for 48 h, the supernatant was collected. The CEA content in the supernatant was measured by RIA. In this assay, the detection limit was 2.5 ng/ml.

RNA Extraction. Total cellular RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). The concentration of RNA was determined by measuring the absorbance at 260 nm, and 2 μ g of total RNA was prepared for the next step.

PCR. The RT-PCR was performed as described previously. The 160-bp fragment appeared as the first PCR product, and the 131-bp one as the second PCR product (17). PCR products were analyzed by electrophoresis on 4% NuSieve GTG agarose gel (BioProducts, Rockland, Maine).

In Vitro Transduction. For transduction, 1×10^5 of MKN45, MKN1, and HT1080 cells were seeded in one-well chamber slides. After reaching 50%

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² The abbreviations used are: GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; CEA, carcinoembryonic antigen; RT-PCR, reverse transcription-PCR; HVJ, Hemagglutinating Virus of Japan; RT, reverse transcription; opDC, optimized [N-(N',-N'-dimethylaminoethane)-carbamoyl] cholesterol.

confluence, the cells were incubated with a precipitated mixture of 20 μg of Lipofectin reagent (Invitrogen, Co., Carlsbad, CA) and 2.5 μg each of pEGFP-N1, pCEA-EGFP, and pEGFP1 for 24 h at 37°C, respectively. The medium was then replaced with new medium, and after 48 h, the cells were observed directly under a fluorescence microscope Olympus BX-50 (Olympus, Tokyo, Japan) with FITC filter sets with no additional treatment.

HVJ opDC Liposome. The HVJ liposome-mediated gene transfer method was used for *in vivo* transduction as described elsewhere (14). In brief, 10 mg of lipid mixture and 300 μg of plasmid DNA hydrated in balanced salt solution [137 mM NaCl, 5.4 mM KCl, and 10 mM Tris-HCl (pH 7.5)] were mixed and filtered repeatedly, then incubated with HVJ that had been inactivated with UV irradiation. Next, sucrose balanced salt solution was added, and the mixture was centrifuged for 1.5 h at 25,000 rpm at 4°C. The layer of the HVJ-liposome complex was collected and prepared for *in vivo* transduction.

In Vivo Transduction. Four-week-old female BALB/c-*nu/nu* nude mice were used for *in vivo* experiments (Clea Japan, Inc., Osaka, Japan). MKN45 (1×10^7 cells) was injected into the peritoneal space of nude mice. Four days and 6 days after the injection, 1 ml of HVJ opDC liposome complex from 30- μg DNA plasmids were injected i.p. under general anesthesia.

Seven days after the cells had been introduced, the mice were sacrificed to obtain the peritoneum and organs in the peritoneal cavity for analysis. The organs were immediately observed under a fluorescence stereomicroscope SZX12 (Olympus, Tokyo, Japan) with GFP filter sets.

Nodules with fluorescent activities detected by fluorescence stereomicroscopy were paraffin-embedded and subjected to pathological examination for H&E staining.

RESULTS

CEA Secretion and CEA mRNA Expression by Cell Lines. To examine the CEA expression level in the cell lines used in this study, the culture medium and cell lysate of each cell were subjected to RIA and RT-PCR analysis, respectively. The CEA concentration in the culture medium was 59.5 ± 4.07 ng/ 10^6 cells/48 h in MKN45 cells but was below the detectable level in MKN1 and HT1080 (cutoff level: <5 ng; Table 1). Fig. 2 shows the products of nested PCR. The band of 131 bp indicates the expression of CEA mRNA. The results showed that MKN45 and MKN1 expressed a considerable amount of CEA mRNA, whereas HT1080 did not.

In Vitro Transduction. Three plasmid DNAs, pEGFP-N1, pCEA-EGFP, and pEGFP-1, were transduced to three tumor cell lines,

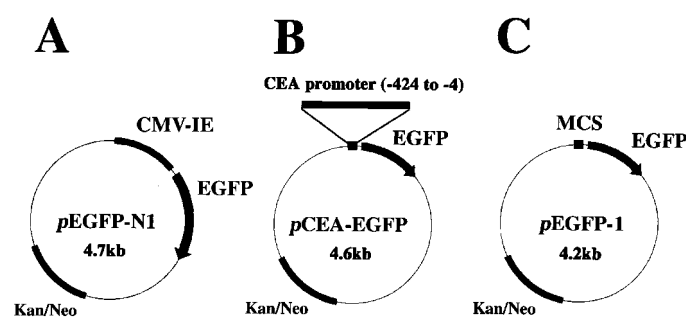


Fig. 1. Plasmid constructs. A, pEGFP-N1. The cytomegalovirus immediate-early enhancer is inserted in the MCS of plasmid pEGFP-1 containing EGFP. B, pCEA-EGFP. The CEA promoter region between -424 and -2 bp from the transcriptional start site is inserted in the MCS of plasmid pEGFP-1. C, pEGFP-1. The MCS is upstream of EGFP, and no promoter lesion is present in plasmid pEGFP-N1. MCS, multicloning site.

Table 1 CEA secretion of human gastric cancer cell lines, MKN45 and MKN1 and human fibrosarcoma cell line HT1080 measured by RIA

Cell line	CEA secretion (ng/ 10^6 cells/48 hr)
MKN45	59.9 ± 4.1
MKN1	ND ^a
HT1080	ND

^a ND, not detected.

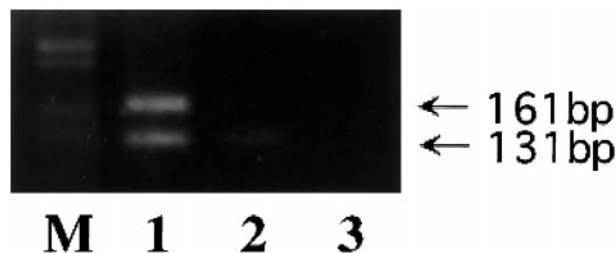


Fig. 2. Detection of mRNA CEA by nested PCR after reverse transcription. The 161-bp band shows the product of the first PCR, and the 131-bp band shows the product of second PCR that presents the expression of mRNA CEA. Lane 1, human gastric cell line MKN45 expresses a large amount of mRNA CEA. Lane 2, human gastric cell line MKN1 expresses mRNA CEA. Lane 3, human fibrosarcoma cell line HT1080 does not express mRNA CEA.

MKN45, MKN1, and HT1080, using Lipofectin reagents as described previously (Fig. 3). All three cell lines became fluorescence-positive when transduced with pEGFP-N1 (positive control) but were fluorescence-negative when transduced with pEGFP-1 (negative control). When the cells were transduced with pCEA-EGFP, which contains the CEA promoter region, only MKN45 and MKN1 expressed fluorescence, but HT1080 expressed no fluorescence as found with the negative control. These results suggest that transduction with pCEA-EGFP causes cells that express CEA mRNA to become fluorescence-positive regardless of the CEA expression.

In Vivo Transduction. Seven days after i.p. injection of 1×10^7 MKN45 cells, the mice were transduced twice with EGFP containing plasmids using HVJ opDC liposome. The mice were sacrificed 24 h later to harvest the peritoneum and organs in the peritoneal cavity for fluorescence and macroscopic analysis.

Under normal stereomicroscopic observation, no metastatic nodule was detected on the peritoneum of the abdominal wall regardless of the plasmid used for transduction (pEGFP-N1, pEGFP-1, or pCEA-EGFP). On the other hand, under fluorescence stereomicroscopic observation, several fluorescent nodules were detected on the peritoneum of the abdominal wall in the mice transduced with pEGFP-N1 and pCEA-EGFP (Fig. 4). No fluorescent nodule was detected in the mice transduced with pEGFP-1. The minimal detectable size of the fluorescence-positive nodule on the peritoneum in mice transduced with pCEA-EGFP was ~ 150 μm .

On the liver surface, nodules were detected in all of the mice under normal stereomicroscopy, and the nodules on the liver of pEGFP-N1- and pCEA-EGFP-transduced mice also showed fluorescence.

Compared with metastatic nodules on the peritoneal wall, those on the mesenterium were difficult to detect because of autofluorescence produced by the intestines and colon. On the mesenterium of mice transduced with pEGFP-N1 and pCEA-EGFP, several nodules with substantial fluorescence activity were detected. The fluorescence intensity did not differ much from that of the mesenterium of mice transduced with pEGFP-1. These results suggest that the mesenterium has a high background level of fluorescence, making it difficult to distinguish tumor fluorescence and autofluorescence.

The minimal-size fluorescence-positive nodule detected on the peritoneal wall was excised and histologically examined. Microscopic observation revealed a nest of adenocarcinoma cells (Fig. 5).

The peritoneal metastases that was undetectable by normal stereomicroscopic observation could be detected specifically by using pCEA-EGFP and fluorescent stereomicroscopy.

DISCUSSION

In this study, we succeeded in making peritoneal micrometastatic lesions visible by gene transfer techniques, which had not been

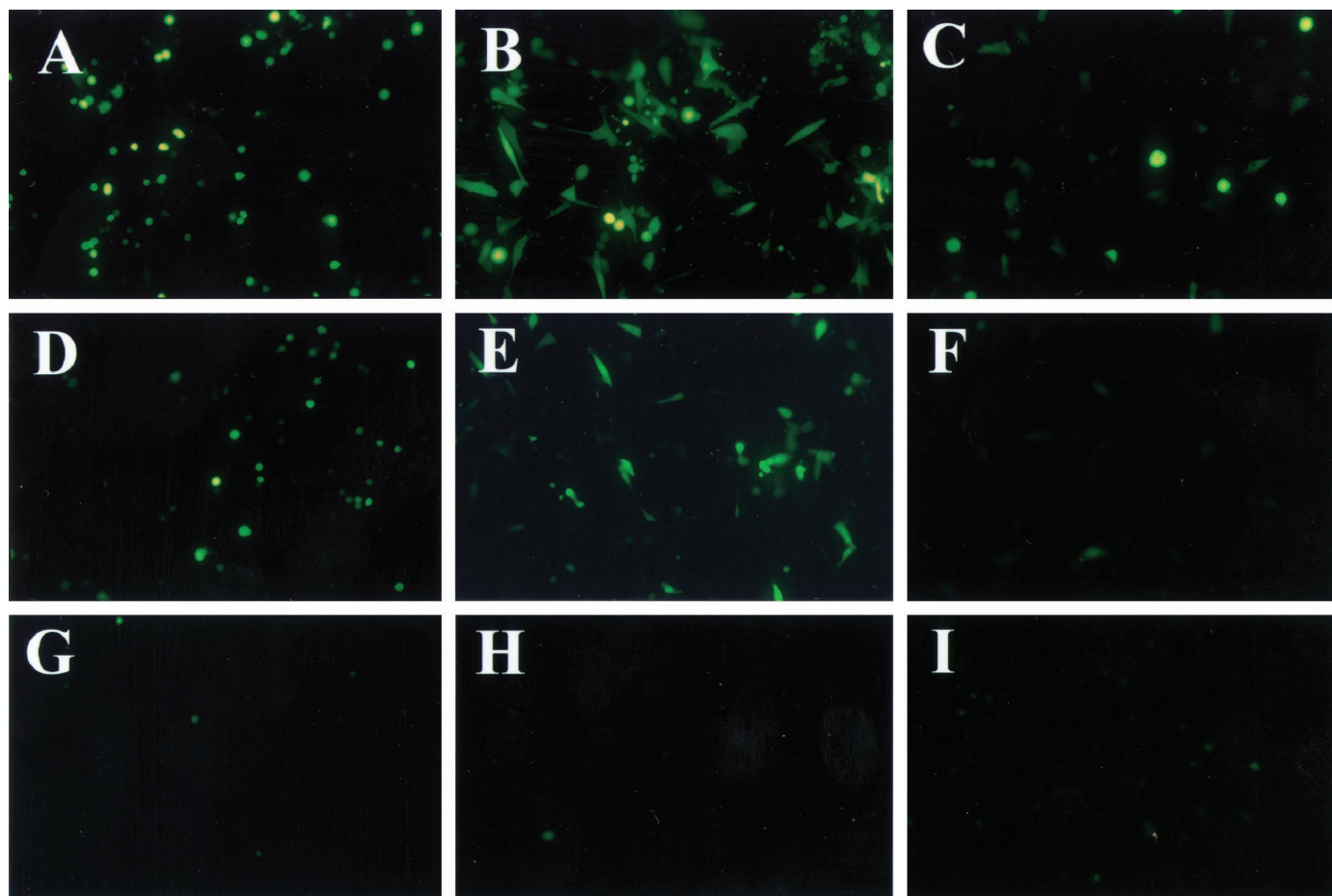


Fig. 3. *In vitro* transduction of plasmid pEGFP-N1, pCEA-EGFP, and pEGFP-1 to MKN45, MKN1, and HT1080 by lipofection, respectively. Cell lines were incubated on one-well chamber slides and observed by fluorescence microscopy with $\times 200$. A–C, MKN45, MKN1, and HT1080 were transduced with plasmid pEGFP-N1 as a positive control. The three cell lines clearly expressed fluorescence. D–F, MKN45, MKN1, and HT1080 were transduced with plasmid pCEA-EGFP. Human gastric cancer cell lines, MKN45 and MKN1, which express mRNA CEA, showed fluorescence; however, human fibrosarcoma cell line HT1080 did not. The fluorescent levels of MKN45 and MKN1 transduced with plasmid pCEA-EGFP were slightly lower than that of MKN45 and MKN1 transduced with plasmid pEGFP-N1. G–I, MKN45, MKN1, and HT1080 were transduced with plasmid pEGFP-1 as a negative control. None of the cell lines expressed fluorescence.

possible by conventional methods. Our strategy was to use a CEA promoter and EGFP reporter gene.

Many kinds of promoters have been used thus far to enable tumor-specific transcription in experiments of gene therapy including the α -fetoprotein promoter for hepatocellular carcinoma and the CEA

promoter for lung carcinoma, gastric carcinoma, pancreatic carcinoma, and colon carcinoma (7–12). It is reported that 50% of patients with gastric cancers display elevation of serum CEA, and most of the gastric cancer cells express CEA. However, CEA mRNA is produced by most epithelial tissues, even normal ones, whereas nonepithelial

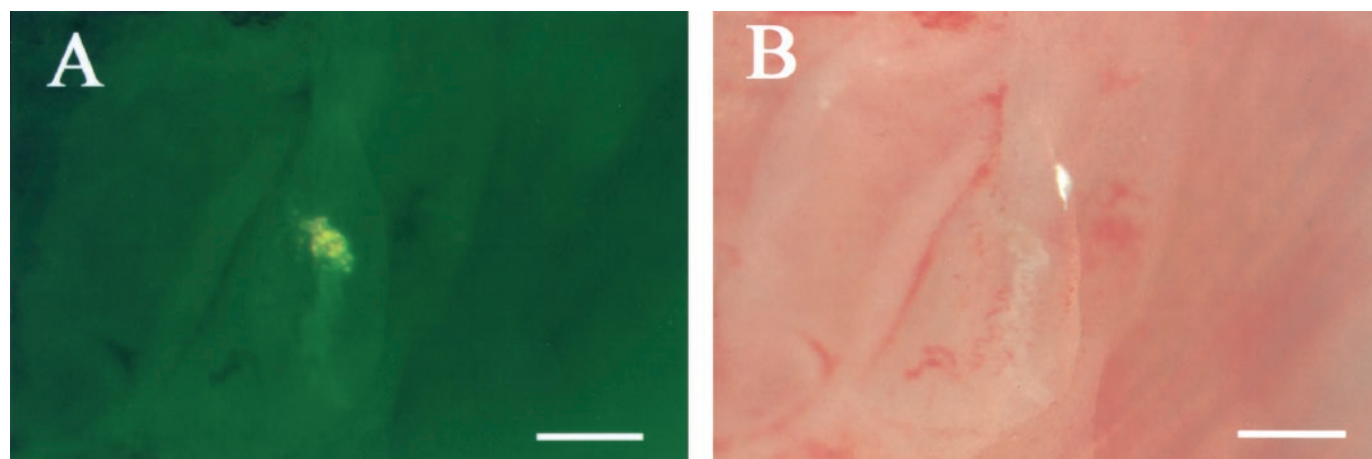
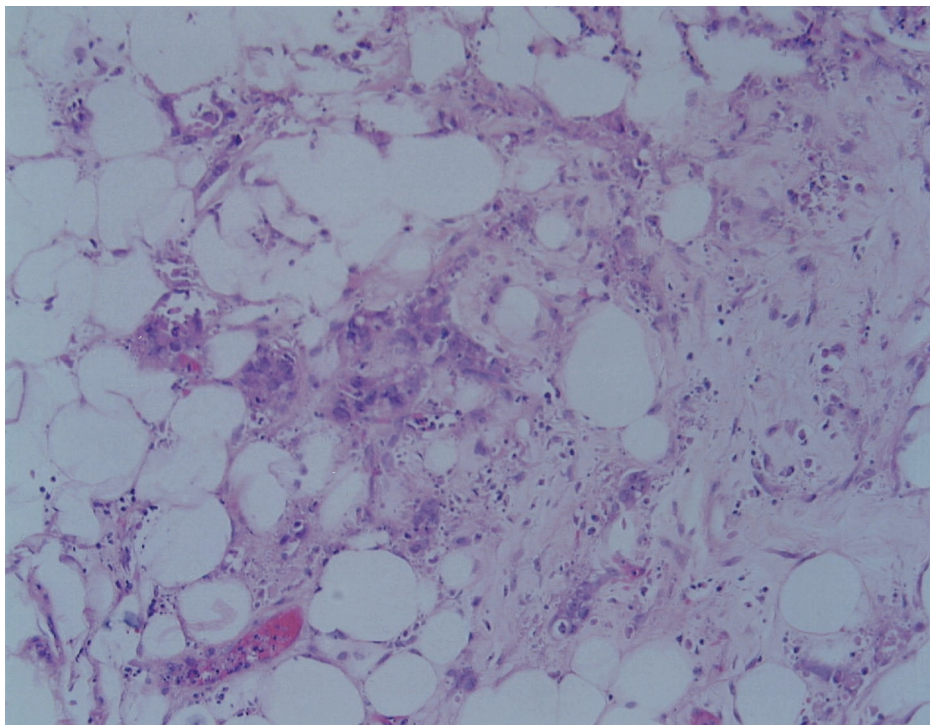


Fig. 4. *In vivo* transduction of plasmid pCEA-EGFP. A, a fluorescent nodule was detected on the peritoneum of the abdominal wall by fluorescence stereomicroscopy after transduction of plasmid pCEA-EGFP by the HVJ-liposome method. B, normal stereomicroscopic observation was conducted over the region containing the fluorescent nodule shown in A. Peritoneal metastasis could not be detected by normal stereomicroscopic observation at 90-fold magnification; bar = 200 μ m.

Fig. 5. H&E staining of fluorescence-positive nodules. After dissection of fluorescent nodules under fluorescence stereomicroscopic observation, the tissues were paraffin-embedded and subjected to pathological examination for H&E staining. A tumor nest from MKN45 was observed in the abdominal wall.



tissues do not produce CEA mRNA (18). This characteristic is thus considered to be advantageous when its promoter is used for tissue-specific expression of a certain reporter gene in the abdominal cavity. Because all of the cells except for cancer cells are of nonepithelial tissue origin, a reporter gene driven by CEA promoter should be expressed only in cancer cells.

The MKN45 and MKN1 cell lines used in this *in vitro* experiment, both of which are derived from human gastric cancer, expressed CEA mRNA and became fluorescent by transduction with pCEA-EGFP. On the other hand, HT1080 from a nonepithelial cell line did not express CEA mRNA and did not become fluorescent by transduction of pCEA-EGFP. The results of *in vitro* experiments suggested that pCEA-EGFP could make the peritoneal metastasis nodules become fluorescent in a tumor-specific manner.

We next conducted an *in vivo* experiment to make invisible peritoneal metastases become detectable by *in vivo* transduction of pCEA-EGFP plasmid DNA using the HVJ opDC liposome method.

The nodules on the peritoneal surface of the abdominal wall are easily detectable by fluorescent microscopy. The minimal detectable size of the tumor was 150 μm . On the other hand, nodules on the mesentery were difficult to detect because autofluorescence in fat tissue of the mesentery and the contents of the intestinal organs can interfere with detection of fluorescence produced by EGFP. Autofluorescence was not detected in peritoneal mesothelium at all. Autofluorescence produced by fat tissue is not a serious problem, because its yellowish color is quite distinct from that produced by EGFP. Appropriate observation filter or a transgene that can emit other colors of fluorescence will result in easier discrimination. On the other hand, the contents of the intestinal organs had a similar color of autofluorescence as EGFP, which may cause lower detection of nodules on the mesentery. In the future clinical application, fasting or a laxative for removal of intestinal contents will be required. To improve the detection level for the mesentery samples, the fluorescence intensity must be increased. More specific and stronger promoters than a CEA promoter are needed.

We considered the EGFP to be the most suitable for our *in vivo*

experiment for the following reasons. EGFP does not need fixation, proteins, substrate, or other cofactors; its optimal temperature is near body temperature; and it expresses ~ 35 -fold stronger fluorescence than wild-type GFP (19).

Several gene delivery systems have been reported in gene therapy studies (20). A retroviral vector system would seem to be advantageous because of low immunogenicity and integration of the plasmid DNA to the host genome. However, there are problems such as mutagenesis and efficiency of transduction (21). The adenoviral vector system also offers advantages such as high efficiency of transduction but poses the problems of immunogenic response and toxicity (22, 23). Other viral vector systems also have problems that remain unsettled such as the difficulty of transducing plasmids of large size and the issue of safety (20). The HVJ-liposome method is characterized by safe and high efficiency of transduction with a short incubation time, which avoids the risks of pathogenesis or cytotoxicity and, thus, would allow repeated transduction (14, 16). In this study, *in vitro* transduction efficiency for cultured cancer cells was 1–3%, when HVJ liposome was used for transfection. *In vivo* transduction efficiency to disseminated metastases was unable to be determined. *In vivo* transduction would be clinically useful enough to detect micrometastatic clusters if it had a similar efficiency to *in vitro* transduction.

A fluorescence stereomicroscope equipped with GFP filter sets enabled direct observation of fluorescence without any treatment. We reported the data that showed MKN45 cells that were stably transduced with EGFP genes could be detected at a single-cell level by fluorescence stereomicroscopy at 90-fold magnification (23). In this study, the minimal size detected by fluorescence stereomicroscopic observation in the peritoneal cavity was 150 μm , which corresponds to 10^2 – 10^4 cells. To increase detection sensitivity, a higher transduction efficiency and more fluorescent GFP are needed. To improve specificity, some way must be devised to distinguish between fluorescence and autofluorescence, for example, by adding coloring. If these problems can be resolved, it could be possible to observe the peritoneal cavity by fluorescence laparoscopy (24).

Various studies have been reported on the assessment of microme-

tastases in the peritoneal cavity of gastric cancer patients. These include examination of i.p. lavage cytology by pathological examination, immunohistochemistry, or RT-PCR using tumor-specific primers, and detection of metastasis using antitumor antibody labeled with radioisotope. However, all of the methods have their drawbacks. For example, peritoneal lavage cytology detects free cancer cells in the peritoneal cavity, and thus positive cytology does not always coincide with the presence of peritoneal metastasis. Our method using a CEA promoter and EGFP is preferable to the methods described previously because it allows direct detection of metastatic nodules in the peritoneal cavity. In addition, because we used a cationic HVJ liposome in this study, it is possible to easily deliver a gene of interest not only into tumor cells on the peritoneum but also into those infiltrating the peritoneum.

In this study, we visualized only i.p. micrometastatic lesions. However, other investigators have reported that micrometastases can be detected in lymph nodes or the bone marrow (25–28). Our method can thus be used to directly observe metastasis in such tissues.

In conclusion, we successfully visualized peritoneal micrometastatic lesions of gastric cancer using a CEA promoter and EGFP. This may provide a new tool for research on peritoneal metastases, diagnosis of micrometastases at the time of surgery, and assessment of anticancer therapy of metastases.

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