many of these molecular diagnostic modalities have onerous requirements to obtain specimen, such as needle core biopsies or surgical sampling of tumour tissues that can be invasive.

Circulating tumour cells (CTCs), first described in 1869 by Ashworth,[10] are often present in the peripheral blood of patients with advanced cancers. However, as CTCs are very rare within the bloodstream, detection of CTCs can be difficult. The most commonly used CTC detection method is the CellSearch system,[11 12] which can enrich CTCs using magnetised antibodies that target the major epithelial cell surface marker, epithelial cell adhesion molecule (EpCAM). More recently, genetic analysis of the *EGFR* gene using the EpCAM-dependent CTC-chip detection system has been described for the surveillance of CTCs in patients with lung cancers.[13] CTCs are thought to contain the metastasis-initiating tumour cells that form metastatic colonies at distant organs,[14 15] but recent studies have suggested that there are heterogeneous populations that include CTCs with both epithelial and mesenchymal characteristics,[16] which are associated with the epithelial–mesenchymal transition (EMT).[17] Recently, EpCAM-positive and EpCAM-negative CTCs from patients with breast cancer have been shown to exhibit high potential to metastasise to the lung and brain, respectively, in nude mice.[18 19] In patients with colorectal cancer, not only captured cytokeratin (CK)-positive CTCs, but also co-captured CK-negative cells have been shown to possess complex aneuploidy.[20] Moreover, it has been reported that plastin3 (*PLS3*), which is a novel marker for EMT, was detected in EpCAM-positive and EpCAM-negative CTCs in patients with colorectal cancer with distant metastasis.[21] These findings indicate the presence of CTCs without epithelial markers in patients with colorectal cancer. Therefore, development of a CTC capture system that functions independent of the epithelial cell marker is required to precisely assess the sensitivity of highly metastatic tumour cells to molecularly targeted drugs.

Epithelial and mesenchymal types of malignant tumour cells possess high telomerase activity to maintain the length of telomere during aberrant cell proliferation, suggesting the potential of telomerase activity as a general tumour marker[22] and therapeutic target.[23] We previously developed a green fluorescent protein (GFP)-expressing telomerase-specific replication-competent adenovirus (OBP-401, TelomeScan) that drives the adenoviral *E1A* and *E1B* genes under the *hTERT* gene promoter for telomerase-dependent virus replication. OBP-401 enables the visualisation of viable epithelial and mesenchymal types of human tumour cells with telomerase activity as GFP-positive cells.[24 25] OBP-401-mediated GFP labelling is a useful method to detect viable CTCs in patients with gastrointestinal cancers[26 27] and ovarian cancers.[28] The present study extends our previous work by exploring the potential of an OBP-401-based biological CTC capture system for the surveillance of genetic mutations in viable CTCs as a novel non-invasive companion diagnostic strategy.

MATERIALS AND METHODS

Cell lines

The human colorectal cancer cell lines, SW480, HCT116 and HT29; the human pancreatic cancer cell line, Panc1; the human lung cancer cell line, A549 and H1299; the human gastrointestinal stromal tumour (GIST) cell line, GIST882; and the human normal oesophageal fibroblasts, FEF3, were purchased from the American Type Culture Collection. All cell lines were cultured according to the manufacturer's specifications. There are four types of *KRAS* gene mutations (G12D, G12 V, G12S, G13D) in Panc1, SW480, A549 and HCT116 cells, respectively. HT29 cells have one mutation (V600E) in the *BRAF* gene, whereas GIST882 cells harbour one mutation (K642E) in the *KIT* gene. Normal FEF3 cells have no mutations in the *KRAS*, *BRAF* or *KIT* genes.

To obtain the EMT-induced human cancer cells, A549 cells were treated with transforming growth factor β (TGF-β) (10 ng/mL) for 72 h. EMT induction was defined as a morphological change to spindle type and a change in the EMT-related marker expression, including downregulation of epithelial markers (EpCAM and E-cadherin) and upregulation of the mesenchymal marker (N-cadherin).

Recombinant adenovirus

OBP-401 is a telomerase-specific replication-competent adenovirus variant, in which the *hTERT* gene promoter drives the expression of *E1A* and *E1B* genes that are linked to an internal ribosome entry site and in which the *GFP* gene is inserted into the E3 region under a cytomegalovirus (CMV) promoter (figure 1A).[24–26] OBP-401 was purified by ultracentrifugation using CsCl step gradients. Viral titres were determined by a plaque-forming assay using 293 cells, and the virus was stored at −80°C.

Immunocytochemical staining

The cells seeded on tissue culture chamber slides were fixed in 4% paraformaldehyde for 15 min on ice. The slides were subsequently incubated with the PE-conjugated mouse anti-EpCAM antibody (BioLegend, San Diego, California, USA) for 1 h on ice. Then the slides were analysed using an inverted fluorescence microscope (Olympus; Tokyo, Japan).

Flow cytometry

The cells (1×10^5 cells) were labelled with primary mouse antibodies for EpCAM, E-cadherin, N-cadherin (BioLegend) and coxsackievirus and adenovirus receptor (CAR) for 30 min on ice and were analysed using flow cytometry (FACS Array; Becton Dickinson, Mountain View, California, USA).

CTC model

CTC models were established by incubation with tumour cell lines (SW480, HCT116, HT29, Panc1, EMT-induced A549 and GIST882 cells) in 5 mL of blood (containing approximately 3.5×10^7 white blood cells) from a healthy volunteer.

DNA extraction from CTC models and clinical samples

The protocol for DNA extraction from the CTC models or clinical samples is shown in figure 2A and online supplementary figure S1. Approximately 5 mL of blood was incubated with lysis buffer containing ammonium chloride to remove the red blood cells (RBCs). These cancer cells were then infected with OBP-401 at 1×10^6 plaque-forming units (PFU) and incubated for 24 h. Thereafter, the cell pellets were labelled with anti-CD45 antibody conjugated with PE and sequentially sorted by FACS Aria (Becton Dickinson, San Jose, California, USA). We set the P1 gate to obtain viable cells, the P2 gate to detect GFP-positive cells without intrinsic fluorescence and the P3 gate to detect only GFP-positive tumour cells without the haematopoietic CD45 marker. Cells in the P2 or P3 gates were collected and stored temporarily at −30°C. DNA was extracted from captured cells using a QiaAMP DNA Mini kit (Qiagen, Valencia, California, USA). The DNA solution was mixed with DNA polymerase, and each primer was subjected to PCR analysis. Five-millilitre blood samples were collected with consent from patients with colorectal cancer, according to a protocol

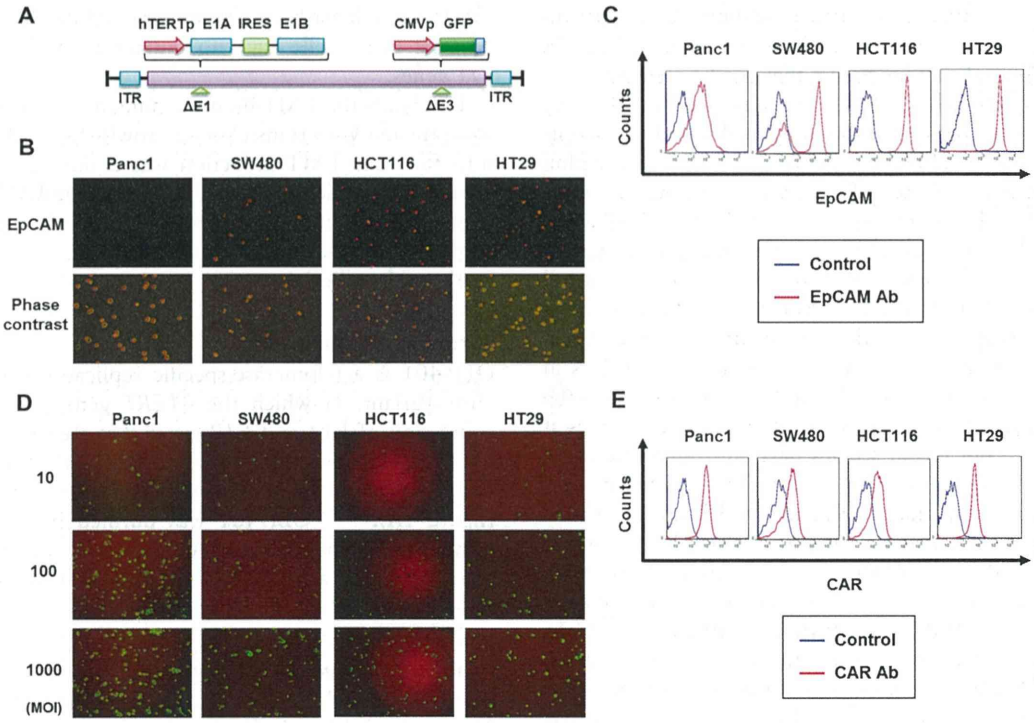


Figure 1 OBP-401-mediated green fluorescent protein (GFP) expression in human cancer cells with different levels of EpCAM expression. (A) Schematic DNA structure of OBP-401 (TelomeScan). OBP-401 is a telomerase-specific replication-competent adenovirus variant in which the hTERT promoter element drives expression of the *E1A* and *E1B* genes linked with internal ribosome entry sites (IRES). The *GFP* gene is inserted under the cytomegalovirus (CMV) promoter into the E3 region. (B) Immunofluorescence staining of epithelial cell adhesion molecule (EpCAM) in four human cancer cell lines (Panc1, SW480, HCT116, and HT29 cells). EpCAM expression under fluorescence microscopy (top panels) and phase-contrast microscopy (bottom panels). Original magnification: ×100. (C) Flow cytometric analysis of EpCAM expression in four human cancer cell lines. Cells are incubated with anti-EpCAM antibody. An isotype-matched normal mouse IgG1 is used as a control. (D) Cells re-infected with OBP-401 at multiplicity of infection (MOI) of 10, 100 or 1000 plaque-forming units per cell and assessed for GFP expression under fluorescence microscopy 24 h after infection. (E) Expression of CAR is analysed using flow cytometry in four human cancer cell lines.

approved by the institutional review board at Okayama University Graduate School (Receipt No. 1537).

Gene mutation analysis by direct sequencing

Taq polymerase, forward primer and reverse primer were mixed with eluted DNA solution, and DNA was amplified using the PCR Thermal Cycler. Primer sequences and PCR settings are shown in online supplementary table S1. Using the PCR products, the sequence of each gene was analysed with an ABI PRISM 3100 Genetic Analyzer (Life Technologies, Carlsbad, California, USA).

Gene mutation analysis by ASB-PCR

ASB-PCR for the KRAS and BRAF genes was performed with a primer set of a TaqMan Mutation Detection Assays (Applied Biosystems, Foster City, California, USA), as described in a previous report.[29] This assay amplifies only mutant alleles with mutant-specific primers and prevents the amplification of wild-type alleles using blocking oligonucleotides. Genetic mutations of target genes were analysed with the StepOnePlus real-time PCR system (Applied Biosystems). Genotyping Master mix and Mutation Detection Assay were mixed with two sets of eluted DNA solution, and this mixture was applied to real-time PCR analysis. The mutation detection method was customised as follows. The PCR cycle number was set to 70 for the efficient amplification of small copy numbers of target genes. A total cell count was restricted to less than 50 cells/well to prevent non-specific amplification of wild-type alleles. The sensitivity and specificity were analysed using a mixture of KRAS/BRAF wild-type and mutant cells. Genetic mutation was recognised as positive when the amplification for mutant alleles using a specific primer was detected.

RESULTS

Fluorescence imaging of human cancer cells with differential EpCAM expression

OBP-401 (TelomeScan) was previously constructed by inserting the *GFP* gene under the control of the CMV promoter at the deleted E3 region of the telomerase-specific replication-selective type 5 adenovirus OBP-301 (Telomelysin) (figure 1A). To assess the potential of OBP-401-mediated biological imaging, we used four epithelial types of human cancer cell lines (Panc1, SW480, HCT116 and HT29) that differentially express EpCAM in immunocytochemistry (figure 1B) and fluorescence-activated cell sorting (FACS) analysis (figure 1C). All cell lines could be visualised by OBP-401-induced GFP expression in a dose-dependent manner, independently of EpCAM expression (figure 1D). The expression level of CAR, which is associated with adenovirus infectivity, was similar among all cell lines (figure 1E). These results suggest that OBP-401-mediated biological imaging is a useful method to detect human cancer cells regardless of high or low EpCAM expression.

Fluorescence-guided isolation of CTCs with multi-laser FACS

We used OBP-401 to establish a simple ex vivo method to capture viable human CTCs in the peripheral blood for genetic

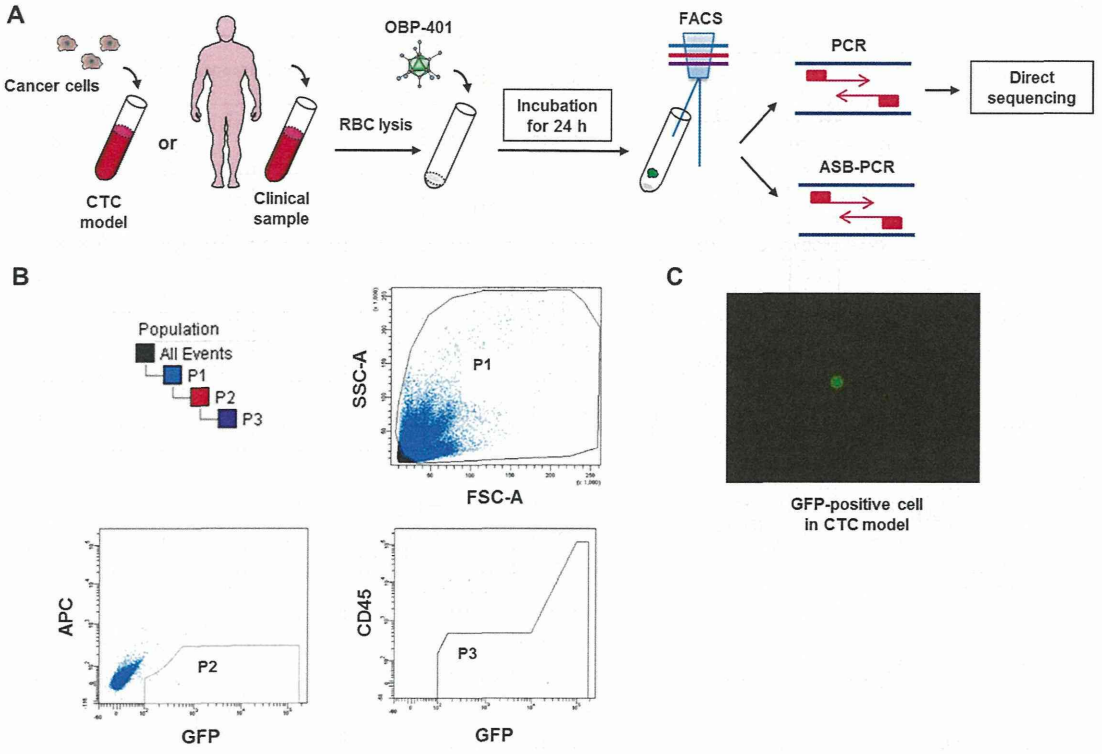


Figure 2 A simple fluorescence virus-guided capturing system of circulating tumour cells (CTCs). (A) Cell isolation steps in the OBP-401-based CTC capturing system. CTC models containing spiked human cancer cells in 5 mL of blood sample or clinical blood samples obtained from patients with cancer are incubated with red blood cell (RBC) lysis buffer for 6 min. After centrifugation, cell pellets are then infected with OBP-401 at 1×10^6 plaque-forming units and incubated for 24 h. Thereafter, cells are incubated with anti-CD45 antibody, and the cell pellet is sorted by fluorescence-activated cell sorting (FACS). DNA extracted from FACS-sorted green fluorescent protein (GFP)-positive cells is subjected to direct sequencing or allele-specific blocker PCR (ASB-PCR) analysis. (B) Each gate is set to capture the GFP-positive CTCs by FACS analysis. After isolating only viable cells at the P1 gate, the P2 and P3 gates are set to exclude the intrinsic fluorescence-positive cells and CD45-positive normal blood cells, respectively. (C) Representative image of GFP-positive CTCs in blood sample containing SW480 cells after infection with OBP-401. Original magnification: ×200.

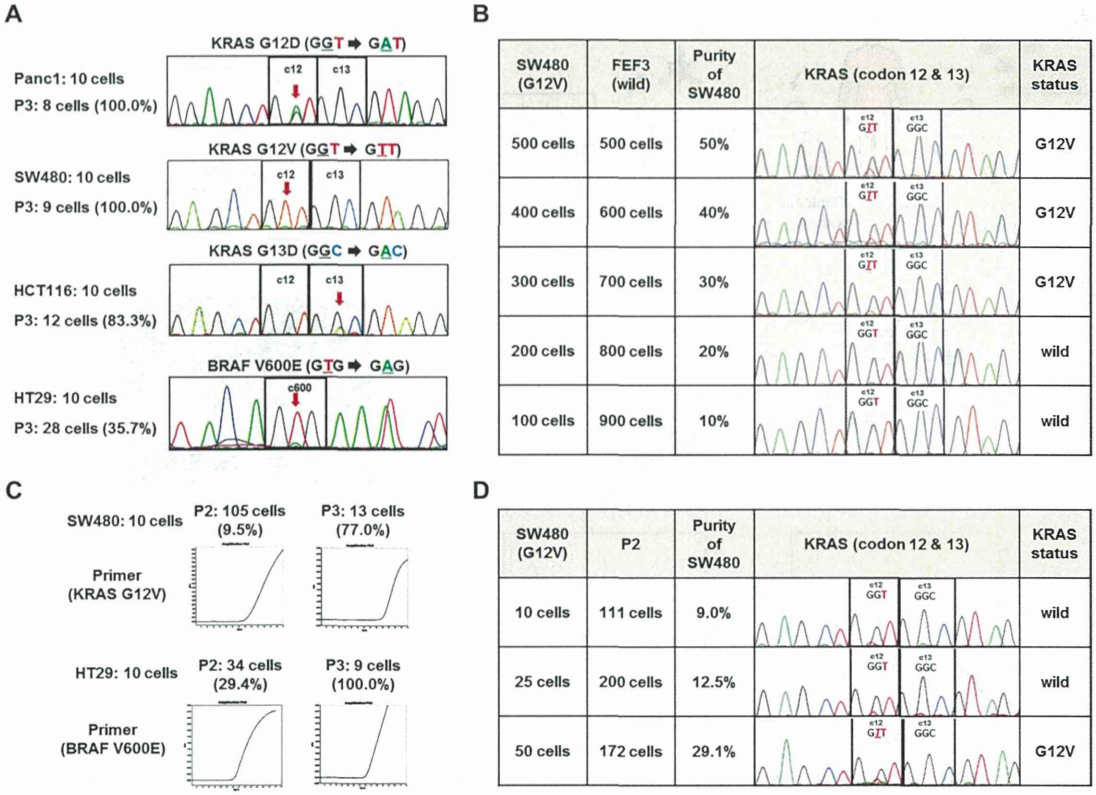
analysis. By spiking a certain number of human cancer cells that have different types of genetic mutations in the *KRAS* or *BRAF* gene in 5 mL of blood from healthy volunteers, we made CTC models with different types of genetic mutations. As illustrated in figure 2A and online supplementary figure S1, following the lysis of RBCs in 5 mL aliquots of CTC models or whole blood samples obtained from patients, the cell pellets were subsequently incubated with OBP-401 at 1×10^6 PFU for 24 h, labelled with anti-CD45 antibody conjugated with phycoerythrin (PE), and sequentially sorted by FACS. In preliminary experiments using CTC models, we found suitable conditions for sorting only GFP-positive CTCs by excluding auto-fluorescent allophycocyanin-positive cells at the P2 gate and haematopoietic CD45-positive cells at the P3 gate (figure 2B). The GFP-positive cells could be detected in the CTC model under a fluorescence microscope (figure 2C).

Genetic analysis of OBP-401-labelled GFP-positive cells using direct sequencing

FACS-isolated GFP-positive CTCs at the P3 gate were analysed genetically by direct sequencing (see online supplementary table 1). The expected genetic mutations in the *KRAS* or *BRAF* gene were precisely detected in all CTC models comprising four human cancer cell lines by direct sequencing (figure 3A and see online supplementary figure S2), indicating that the OBP-401-based biological capture system is effective for the collection of CTCs expressing various levels of the EpCAM marker. Recent studies have demonstrated that a heterogeneous population of CTCs is present within individual patients with cancer and that these CTCs have epithelial and mesenchymal markers, suggesting the diverse genetic variations with wild-type and mutant-type genes in the populations of CTCs. To evaluate the minimum purity limitation of mutant-type CTCs for genetic analysis using direct sequencing, SW480 cells (KRAS G12 V mutant) were mixed with H1299 cells (KRAS wild-type) at a 50%, 40%, 30%, 20% or 10% purity ratio. *KRAS* gene mutations could be detected by direct sequencing only in samples containing more than a 30% purity ratio of SW480 cells (figure 3B). Thus, high purity of mutant-type CTCs in heterogeneous populations is necessary for detection of genetic alterations by direct sequencing.

Genetic analysis of OBP-401-labelled GFP-positive cells using ASB-PCR

To further increase the sensitivity to detect genetic alterations in heterogeneous populations of CTCs, we next evaluated the potential of the Allele-Specific Blocker (ASB)-PCR method using four types of mutation-specific primers for the *KRAS* or *BRAF* genes. Before analysing the human cancer cells, we confirmed that there was no amplification of PCR products in human normal fibroblasts with wild-type *KRAS* and *BRAF* genes or in blood obtained from normal healthy volunteers by ASB-PCR



SW480 (G12V)	FEF3 (wild)	Purity of SW480	KRAS (codon 12 & 13)	KRAS status
500 cells	500 cells	50%		G12V
400 cells	600 cells	40%		G12V
300 cells	700 cells	30%		G12V
200 cells	800 cells	20%		wild
100 cells	900 cells	10%		wild

SW480 (G12V)	P2	Purity of SW480	KRAS (codon 12 & 13)	KRAS status
10 cells	111 cells	9.0%		wild
25 cells	200 cells	12.5%		wild
50 cells	172 cells	29.1%		G12V

Figure 3 Genetic mutation analysis of human circulating tumour cells (CTCs) by direct sequencing and mutation-specific PCR. (A) Detection of *KRAS* or *BRAF* gene mutation in the CTC models containing 10 human cancer cells by direct sequencing of green fluorescent protein (GFP)-positive cells at the P3 gate. The number of cells in the P3 gate and the mutation pattern in each model are indicated. (B) The minimal purity of tumour cells for direct sequencing to detect the expected gene mutations is evaluated. SW480 (*KRAS* G12 V) cells were mixed with H1299 (*KRAS* wild-type) cells at 50%, 40%, 30%, 20% and 10% of purity ratios. DNA is extracted from cell mixtures, and the *KRAS* gene mutation is analysed by direct sequencing. (C) Allele-specific blocker (ASB)-PCR-mediated detection of *KRAS* and *BRAF* gene mutations in GFP-positive cells at the P2 or P3 gate in the CTC models containing as few as 10 SW480 cells and HT29 cells. When *KRAS* and *BRAF* genes contain targeted mutations, mutation-specific curves cross their threshold of detection. (D) Detection of *KRAS* gene mutation by direct sequencing of GFP-positive cells at the P2 gate without CD45 depletion requires at least 50 SW480 cells in the CTC model.

with mutation-specific primers (see online supplementary figure S3).

When we analysed five human cancer cells mixed with 100 human normal fibroblasts at a purity ratio of approximately 5%, ASB-PCR, using all types of primers, detected the expected mutations in the GFP-positive cells (see online supplementary table 2). In the CTC models containing 10 human cancer cells with different types of *KRAS* and *BRAF* gene mutations, ASB-PCR analysis detected the expected genetic mutations in the GFP-positive cells at the P3 gate (table 1). Moreover, ASB-PCR analysis could detect the genetic alterations in the GFP-positive cells at the P2 gate without exclusion of CD45-positive normal blood cells (figure 3C), whereas at least 50 tumour cells were required for direct sequencing in the

Table 1 Data for mutation-specific PCR for the genetic analysis of CTC models

CTC model				FACS analysis			Genetic analysis			
									Ct values	
Cancer cells	Cell type	Gene status	Number of cancer cells	Gate	Number of GFP-positive cells	Purity of cancer cells (%)	Primer	Amplification	1st PCR	2nd PCR
Panc1	Epithelial	KRAS G12D	10	P2	29	34.5	KRAS G12D	+	37.1	36.1
				P3	6	100.0	KRAS G12D	+	35.3	38.2
SW480	Epithelial	KRAS G12V	10	P2	105	9.5	KRAS G12V	+	45.0	56.5
				P3	13	76.9	KRAS G12V	+	41.6	52.0
HCT116	Epithelial	KRAS G13D	10	P2	23	43.5	KRAS G13D	+	47.5	37.2
				P3	18	55.6	KRAS G13D	+	37.0	44.0
HT29	Epithelial	BRAF V600E	10	P2	34	29.4	BRAF V600E	+	34.0	NA
				P3	9	100.0	BRAF V600E	+	40.0	NA
EMT-induced A549	Mesenchymal	KRAS G12S	10	P2	77	13.0	KRAS G12S	+	41.9	43.5
				P3	17	58.8	KRAS G12S	+	51.1	64.7

CTC, circulating tumour cell; EMT, epithelial–mesenchymal transition; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; NA, not amplified.

presence of CD45-positive cells at the P2 gate (figure 3D). These results suggest that the ASB-PCR method is more simple and sensitive than direct sequencing for detection of genetic alterations in heterogeneous populations of CTCs.

Fluorescence-guided capture of EMT-induced and mesenchymal CTCs

Induction of EMT in CTCs has recently been demonstrated in patients with advanced breast cancers.[17] EMT-induced CTCs frequently formed metastatic colonies in the brain and lung of nude mice,[19] suggesting that highly malignant EMT-induced CTCs can be detected to predict metastatic progression in patients with cancer. We used A549 human lung cancer cells with *KRAS* gene mutation (G12S) and EpCAM-negative GIST882 mesenchymal human tumour cells with the *KIT* gene mutation (K642E), which is frequently mutated in more than 70% of GISTs.[30] OBP-401 infection efficiently induced GFP expression in both cell lines in a dose-dependent manner (figure 4A).

When treated with the EMT inducer, TGF-β, A549 cells showed spindle-shape morphological changes (figure 4B) and altered EMT-related biomarker expression, such as EpCAM and E-cadherin downregulation and N-cadherin upregulation (figure 4C). In contrast, CAR expression was not affected after TGF-β treatment (figure 4C). Therefore, OBP-401 efficiently induced GFP expression in the TGF-β-treated A549 cells (figure 4D). In addition, GIST882 cells were confirmed to be EpCAM negative (figure 4E). When 10 EMT-induced A549 cells were spiked in blood samples, the expected genetic mutation (G12S) in the *KRAS* gene was detected by direct sequencing and by ASB-PCR analysis (figure 4F–G and table 1). In contrast, the expected *KIT* gene mutation could be detected at the P3 gate by direct sequencing in the CTC model containing 100 GIST882 cells (figure 4F) but not in that with 10 cells, presumably due to low expression of CAR. These results suggest that the targeted genetic mutations in EMT-induced and mesenchymal CTCs are also detectable by the OBP-401-based CTC capture system, although the sensitivity is dependent on the CAR expression.

Detection of genetic mutations in CTCs in patients with colorectal cancer

Finally, the blood samples obtained from eight patients with *KRAS*- or *BRAF*-mutated colorectal cancers were analysed by the OBP-401-based CTC capture system and by ASB-PCR

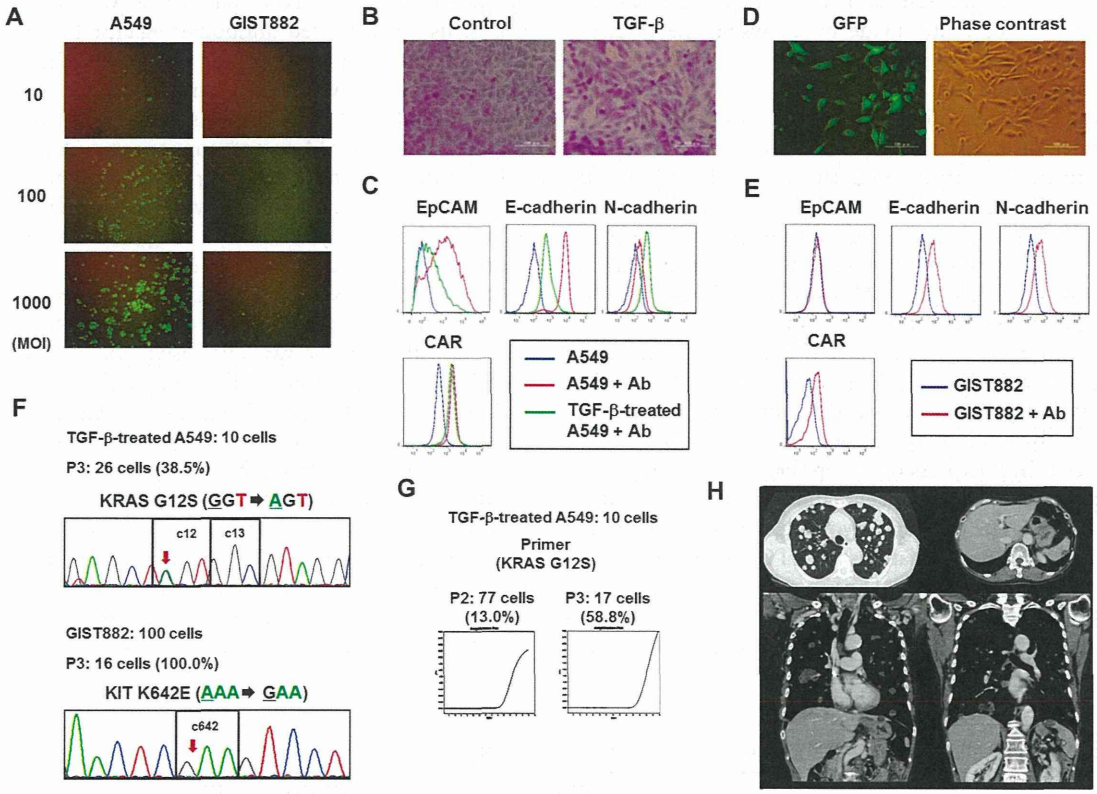


Figure 4 Fluorescence virus-guided capture and genetic mutation analysis of human mesenchymal or epithelial–mesenchymal transition (EMT)-induced tumour cells. (A) A549 human lung cancer cells and GIST882 human gastrointestinal stromal tumour cells are infected with OBP-401 at a multiplicity of infection (MOI) of 10, 100 or 1000 plaque-forming units (PFU) per cell. Green fluorescent protein (GFP) expression is assessed under fluorescence microscope 24 h after virus infection. (B) Morphological change of A549 cells treated with transforming growth factor β (TGF-β). A549 cells are treated with TGF-β (10 ng/mL) for 72 h and stained with crystal violet. Original magnification: ×200. (C) Flow cytometric analysis of epithelial (epithelial cell adhesion molecule (EpCAM) and E-cadherin) and mesenchymal (N-cadherin) cell surface marker and CAR expression in A549 cells treated with or without TGF-β. (D) GFP expression in TGF-β-treated A549 cells after infection with OBP-401 at an MOI of 100 PFU per cell for 24 h. Original magnification: ×200. (E) Flow cytometric analysis of epithelial (EpCAM and E-cadherin) and mesenchymal (N-cadherin) cell-surface markers and coxsackievirus and adenovirus receptor (CAR) expression in GIST882 cells. (F) Detection of *KRAS* and *KIT* gene mutations by direct sequencing of GFP-positive cells at the P3 gate requires 10 TGF-β-treated EMT-induced A549 cells and 100 GIST882 cells in the CTC models, respectively. (G) Detection of *KRAS* gene mutations in GFP-positive cells at the P2 or P3 gate in the CTC models containing as few as 10 of TGF-β-treated A549 cells by allele-specific blocker (ASB)-PCR. Mutation-specific curves for the *KRAS* gene cross their threshold of detection. (H) Representative CT images of patients with colon cancer and lung, spleen and ovary metastases. The primary tumours and CTCs show the *BRAF* V600E mutation.

technology. In preliminary experiments, the number of GFP-positive cells at the P3 gate was less than 10 cells in some clinical blood samples and, therefore, we performed ASB-PCR analysis using GFP-positive cells at the P2 gate. Among the eight blood samples from patients with various stages of colorectal cancer, the same *KRAS* and *BRAF* gene mutations as in the primary tumours were detected in the CTCs of two patients with advanced colorectal cancer (figure 4H and table 2). The other six patients showed no detectable genetic abnormalities in blood samples, although *KRAS* gene mutations were observed in their primary tumours. Three patients without metastatic lesions did not have a large CTC count, and chemotherapeutic treatment in the other three patients with metastatic disease may have resulted in a reduced number of CTCs. Although further large-scale clinical trials are required, our results suggest that the OBP-401-based telomerase-dependent biological CTC capture system is useful for genetic analysis of CTCs in blood samples from patients with cancer.

DISCUSSION

The co-development of a targeted therapy together with its companion diagnostic test, which guides selection of patients and provides surrogate markers to monitor responses, is a key part of personalised medicine. The selection of targeted therapies for individual patients is currently made by analysing the primary tumours, although there are very few cells within the primary tumours that are responsible for metastasis or recurrence, and these cells may have additional genetic abnormalities. The present study demonstrated that CTCs obtained non-invasively are a promising alternative to surgically resected or biopsied tumour tissues for real-time molecular characterisation. A telomerase-dependent biological CTC capture system was clinically useful for the detection of mutations in different target genes, such as *KRAS*, *BRAF* and *KIT*, even in EpCAM-negative cells among highly heterogeneous CTC populations.

We applied telomerase-specific OBP-401 to selectively label human neoplastic cells with GFP signals and confirmed its broad infectivity independent of EpCAM expression, which was consistent with observations from our previous reports that OBP-401 induced GFP expression in epithelial and mesenchymal types of tumour cells.[24 25] Recent studies demonstrated that highly metastatic tumour cells are involved in EpCAM-positive and EpCAM-negative subpopulations of CTCs in the blood of patients with breast cancer.[18 19] During anticancer treatment, the characteristics of CTCs dynamically alternate between epithelial and mesenchymal types of CTCs within individual patients with cancer.[17] Further, platelet-derived TGF-β secretion induces EMT with metastatic potential in CTCs.[31] These findings indicate that single CTCs frequently turn the EMT switch on or off in the microenvironment of the bloodstream. In contrast, high telomerase activity is a general functional biomarker for stabilisation of the telomere in epithelial and mesenchymal malignant tumour cells during aberrant proliferation. In fact, high *hTERT* mRNA levels have been detected in the blood samples of patients with cancer.[32–34] Moreover, hTERT overexpression has been shown to be positively associated with EMT induction in human cancer cells.[35] When the telomerase activity in the CTCs is suppressed in circulating cells, these CTCs undergo programmed cell death (ie, apoptosis or senescence). Thus, the telomerase activity may be superior to unstable epithelial cell marker as a general biomarker for the detection of viable CTCs in the blood. Moreover, GFP-labelled CTCs by OBP-401 infection are considered to be useful for direct determination of drug sensitivity and metastatic potential, and determination of tumour heterogeneity.[36–39]

A number of approaches based on the physical and biological properties of CTCs have been studied to distinguish CTCs from surrounding normal haematopoietic cells and to capture them for further analysis. The CellSearch system, which is the only test approved by the US Food and Drug Administration to detect CTCs, uses magnetised antibodies against EpCAM for positive selection and uses CD45 for leukocyte depletion. Another popular technology for CTC enrichment is a microfluidic-based device called the CTC-chip; this device can isolate and analyse CTCs using EpCAM-coated microposts. Our OBP-401-based CTC detection has been previously compared with the CellSearch assay in patients with metastatic breast cancer.[40] Although both assays exhibited comparable detection rates, the number of CTC-positive cells between both assays was not significantly correlated. Nine out of 50 (18%) cases were positive by both methods, while 12 (24%) and 18 (36%) patients showed positive cells with the OBP-401 assay and the CellSearch assays individually, respectively. We speculate that CTCs detected by OBP-401 primarily detect EpCAM-negative tumour cells while the CellSearch method detects epithelial non-tumour cells as well, including circulating fibroblasts.

Our strategy involves conventional FACS to capture OBP-401-labelled GFP-positive CTCs. OBP-401 infection increases the signal-to-background ratio as a tumour-specific probe, because the fluorescent signal can be amplified only in viable human tumour cells by viral replication. We excluded the autofluorescence-positive cells at the P2 gate and the

Table 2 Data for mutation-specific PCR for the genetic analysis of patient samples

Patients				FACS analysis		Genetic analysis			
								Ct values	
Tumour site	Stage	Gene status of primary tumour	Metastasis	Gate	Number of GFP-positive cells	Primer	Amplification	1st PCR	2nd PCR
Colon	I	KRAS G13D	None	P2	6	KRAS G13D	–	NA	NA
Colon	II	KRAS G13D	None	P2	20	KRAS G13D	–	NA	NA
Colon	II	KRAS G12D	Liver	P2	95	KRAS G12D	+	55.1	61.0
Colon	III	KRAS G13D	None	P2	913	KRAS G13D	–	NA	NA
Colon	III	BRAF V600E	Lung, spleen, ovary	P2	138	BRAF V600E	+	63.0	NA
Colon	IV	KRAS G12D	Liver	P2	14	KRAS G12D	–	NA	NA
Colon	IV	KRAS G12V	Liver	P2	74	KRAS G12V	–	NA	NA
Colon	IV	KRAS G12V	Lung	P2	53	KRAS G12V	–	NA	NA

CTC, circulating tumour cell; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; NA, not amplified.

haematopoietic CD45-positive cells at the P3 gate. When at least 10 human cancer cells were spiked in 5 mL of blood from a healthy volunteer, the number of GFP-positive cells detected at the P3 gate was almost the same as the number of spiked tumour cells, suggesting that the P3 gate contains pure CTCs. However, the P2 gate may be contaminated with non-CTC cells. Indeed, ASB-PCR analysis detected the expected gene mutations in the *KRAS* and *BRAF* genes at the P2 gate, whereas the P3 gate was necessary when direct sequencing was applied. Recently, a combination of the CellSearch system and genetic analysis was also performed to detect genetic mutations in rare CTCs from patients with cancer. Mostert *et al*[41] compared the three types of PCR-based genetic analysis of CTCs, and ASB-PCR, used in our study, was the most sensitive method for detecting *KRAS* and *BRAF* gene mutations in the CTCs from patients with metastatic colorectal cancers. In addition, as our data demonstrated that direct sequencing was limited if CTC-derived DNA had more than 30% purity, we conclude that, together with FACS-isolated OBP-401-infected GFP-expressing CTCs, ASB-PCR is a suitable assay for non-invasive companion diagnostics in patients with cancer. The specificity of the ASB-PCR assay allowed us to use the P2 gate for clinical samples even in the presence of non-CTC cells.

Mutation in *KRAS* and *BRAF* genes is highly associated with resistance to the anti-EGFR antibody, cetuximab, in patients with colorectal cancer.[42 43] In fact, the appearance of *KRAS* gene mutant DNA is associated with resistance to cetuximab in patients with KRAS wild-type colorectal cancers.[44] In patients with colorectal cancer, the frequency of the *KRAS* and *BRAF* gene mutations is significantly higher in liver metastasis than in primary tumours,[45] and *KRAS* and *BRAF* gene mutant status is significantly associated with poor outcomes.[46] These findings suggest that genetic analysis for the *KRAS* and *BRAF* gene mutation in CTCs can be used as a 'liquid biopsy' to monitor resistance to cetuximab and to predict metastatic potential in patients with KRAS wild-type colorectal cancers.

It is also worth noting that the OBP-401-based biological CTC capture system is applicable to the genetic analysis of CTCs with mesenchymal characteristics, including GISTs and osteosarcomas,[25] although the CellSearch system is also useful for detection of epithelial CTCs. Approximately 80% of GIST cells harbour a mutation in the *KIT* gene,[30] which is significantly associated with disease recurrence and poor outcomes.[47] Recently, the small-molecule tyrosine-kinase inhibitor imatinib has been shown to be effective against KIT-mutated GIST that is refractory to conventional chemotherapy.[48] In contrast, bone and soft tissue sarcoma cells, which make up one of the most notorious types of malignant mesenchymal tumours, are also detectable as GFP-positive cells by OBP-401 infection.[25] Frequent lung metastasis has been shown to be a poor prognostic factor in patients with osteosarcoma, but the potential of CTC enumeration in patients with osteosarcoma remains to be elucidated. Thus, the characterisation of CTCs, using the OBP-401-based biological CTC capture system, may be a useful strategy for monitoring metastatic progression in patients with GIST or osteosarcomas, as well as those with epithelial malignant tumours.

The combination of the OBP-401-based CTC capture system and genetic analysis using ASB-PCR detected *KRAS* and *BRAF* mutations in blood samples obtained from patients with colorectal cancer, and these mutations were identical to those seen in the primary tumours. This novel 'liquid biopsy' via a simple blood test could be carried out in real time and enables optimised and timely decisions for therapeutic intervention. However, the technology has to be further validated in large clinical studies with defined endpoints. In addition, one limitation of our study was that it was difficult for ASB-PCR to detect uncommon genetic abnormalities. Regardless, when frequently occurring genetic mutations are targeted for the surveillance of CTCs, the ASB-PCR method would be a useful and highly sensitive method for detecting small numbers of CTCs with genetic mutations. In contrast, if the identification of genetic traits in highly metastatic CTCs is the main goal, a genome-wide approach should be considered for the genetic analysis of CTCs. For example, genome-wide transcriptome analysis has been performed to identify a wide range of copy number alterations in entire CTC populations, using array-comprehensive genomic hybridisation (aCGH).[49] Moreover, genetic analysis in a single CTC has been recently used to clarify global gene alterations using aCGH and next-generation sequencing.[50 51] Thus, the comprehensive analysis of genetic alterations in individual CTCs from patients with cancer would provide novel insight into the identification of genetic signatures associated with metastatic progression.

In summary, we established a telomerase-dependent biological CTC capture system for genotyping of epithelial, mesenchymal, and EMT-induced types of CTCs using OBP-401 and FACS analysis. This technology facilitates the surveillance of genetic alterations in viable CTCs in patients with cancer. Large-scale clinical studies of this strategy are warranted.

Acknowledgements We thank Yukinari Isomoto and Tomoko Sueishi for their technical support.

Contributors Conception and design: HT, SK, TF; development of methodology: KS, HT, YH, TN; acquisition of data: KS, YH, YM; analysis and interpretation of data: KS, HT, HK, SK, TF; writing, review and/or revision of the manuscript: KS, HT, AG, TF; administrative, technical, or material support: MN, SK, YU; study supervision: TF.

Funding This study was supported by grants-in-aid from the Ministry of Education Culture, Sports, Science and Technology, Japan (T. Fujiwara, H. Tazawa, S. Kagawa) and grants from the Ministry of Health, Labour and Welfare, Japan (T. Fujiwara).

Competing interests Yasuo Urata is the president and CEO of Oncolys BioPharma, Inc., the manufacturer of OBP-401 (TelomeScan). Hiroshi Tazawa and Toshiyoshi Fujiwara are consultants for Oncolys BioPharma, Inc. The other authors have no real or potential conflicts of interest to declare.

Ethics approval Okayama University.

Provenance and peer review Not commissioned; externally peer reviewed.

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GUT

Fluorescence virus-guided capturing system of human colorectal circulating tumour cells for non-invasive companion diagnostics

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Gut 2015 64: 627-635 originally published online May 28, 2014
doi: 10.1136/gutjnl-2014-306957

Biological Ablation of Sentinel Lymph Node Metastasis in Submucosally Invaded Early Gastrointestinal Cancer

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Currently, early gastrointestinal cancers are treated endoscopically, as long as there are no lymph node metastases. However, once a gastrointestinal cancer invades the submucosal layer, the lymph node metastatic rate rises to higher than 10%. Therefore, surgery is still the gold standard to remove regional lymph nodes containing possible metastases. Here, to avoid prophylactic surgery, we propose a less-invasive biological ablation of lymph node metastasis in submucosally invaded gastrointestinal cancer patients. We have established an orthotopic early rectal cancer xenograft model with spontaneous lymph node metastasis by implantation of green fluorescent protein (GFP)-labeled human colon cancer cells into the submucosal layer of the murine rectum. A solution containing telomerase-specific oncolytic adenovirus was injected into the peritumoral submucosal space, followed by excision of the primary rectal tumors mimicking the endoscopic submucosal dissection (ESD) technique. Seven days after treatment, GFP signals had completely disappeared indicating that sentinel lymph node metastasis was selectively eradicated. Moreover, biologically treated mice were confirmed to be relapse-free even 4 weeks after treatment. These results indicate that virus-mediated biological ablation selectively targets lymph node metastasis and provides a potential alternative to surgery for submucosal invasive gastrointestinal cancer patients.

Received 21 October 2014; accepted 14 December 2014; advance online publication 20 January 2015. doi:10.1038/mt.2014.244

INTRODUCTION

Due to recent advances in endoscopic technology, early gastrointestinal cancers, which are defined as those that invade no more deeply than the submucosa, are treated endoscopically.[1–3] Endoscopic submucosal dissection (ESD) or local tumor excisions that allow en bloc resection, which lead to more precise histological evaluation and more potential for cure, are considered clinically relevant for early gastrointestinal cancer. A complete local resection of *in situ* or intramucosal tumor is acceptable as a curative treatment due to little risk of lymph node metastasis.[4–7] However, lymph node metastasis is typically found in submucosal invasive gastrointestinal cancer such as esophageal, gastric and colorectal cancer, at an approximate frequency of greater than 10%.[8–11] Since it is difficult to determine submucosally invaded lesions with the risk of lymph node metastasis without pathological evaluation, these patients are treated surgically to remove possibly metastasized lymph nodes, even though primary early gastrointestinal cancer itself is technically resectable with ESD. This means that most submucosal invasive gastrointestinal cancer patients, who are node-negative, routinely undergo unnecessary surgery. Thus, a less invasive way to selectively treat lymph node metastasis would benefit these patients by allowing them to avoid a prophylactic surgery.

Sentinel lymph node metastasis represents the initial spread of malignant tumors from the primary site. Metastatic lymph nodes as well as migrating tumor cells in the draining lymph vessels have to be treated to prevent recurrence and, therefore, anticancer agents that spread over the regional lymphatic area are required. For sentinel lymph node mapping, submucosal injection of a visible dye such as methylene blue or indocyanine green (ICG) allows an adequate regional diffusion in the lymphatic area.[12] It has also been reported that human adenovirus can be effectively transported into the lymphatic circulation in murine models.[13,14] Oncolytic viruses that selectively replicate in tumor cells and lyse infected cells have been developed as anticancer agents.[15–18] These viruses are designed to induce virus-mediated lysis of infected cells after selective viral replication within the tumor cells.

In this study, we evaluated whether a telomerase-dependent, tumor-killing replicating adenoviral agent (OBP-301) that was administered submucosally prior to the primary tumor resection could purge lymph node metastasis in an orthotopic early rectal cancer xenograft model with spontaneous lymph node metastasis. The steps of this procedure mimic the procedures of ESD

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