Original Articles

Fluorescent virus-guided capturing system of human colorectal circulating tumor cells for non-invasive companion diagnostics

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ABSTRACT

Background Molecular-based companion diagnostic tests are being used with increasing frequency to predict their clinical response to various drugs, particularly for molecularly targeted drugs. However, invasive procedures are typically required to obtain tissues for this analysis. Circulating tumor cells (CTCs) are novel biomarkers that can be used for the prediction of disease progression and are also important surrogate sources of cancer cells. Because current CTC detection strategies mainly depend on epithelial cell surface markers, the presence of heterogeneous populations of CTCs with epithelial and/or mesenchymal characteristics may pose obstacles to the detection of CTCs.

Methods We developed a new approach to capture live CTCs among millions of peripheral blood leukocytes using a green fluorescent protein (GFP)-expressing attenuated adenovirus, in which the telomerase promoter regulates viral replication (OBP-401, TelomeScan).

Results Our biological capturing system can image both epithelial and mesenchymal tumor cells with telomerase activities as GFP-positive cells. After sorting, direct sequencing or mutation-specific polymerase chain reaction (PCR) can precisely detect different mutations in *KRAS*, *BRAF* and *KIT* genes in epithelial, mesenchymal, or epithelial–mesenchymal transition-induced CTCs as well as in clinical blood samples from colorectal cancer patients.

Conclusion This fluorescent virus-guided viable CTC capturing method provides a non-invasive alternative to tissue biopsy or surgical resection of primary tumors for companion diagnostics.

Significance of this study

What is already known about this subject?

- The molecular characterization of CTCs based on genetic alterations facilitates the administration of molecular targeted drugs for preventing metastatic progression in individual cancer patient.
- Heterogeneous populations of CTCs with epithelial and/or mesenchymal characteristics make difficult to detect the entire CTCs because CTC detection mainly depends on epithelial cell surface markers.

What are the new findings?

- Our fluorescent virus OBP-401 selectively labeled human CTCs with fluorescence among millions of peripheral blood leukocytes.
- Our biological capturing system can image both epithelial and mesenchymal tumor cells with telomerase activities as GFP-positive cells.

How might it impact on clinical practice in the foreseeable future?

- Because current CTC detection strategies mainly depend on epithelial cell surface markers, the presence of heterogeneous populations of CTCs with epithelial and/or mesenchymal characteristics may pose obstacles to the detection of CTCs.
- Fluorescent virus-based biological capture system is a promising tool for monitoring genetic alterations in both epithelial and mesenchymal types of CTCs.

INTRODUCTION

The rapid evolution of genetic and genomic technologies in regards to predictive pharmacogenetic biomarkers for molecularly targeted therapies (e.g., monoclonal antibodies and small-molecule tyrosine kinase inhibitors) have resulted in tremendous advances in personalized oncologic treatment[1]. The current commonly used biomarkers include human epidermal growth factor receptor 2 (HER2**)** for the use of trastuzumab in breast and gastric cancer[2,3], KRAS for the use of cetuximab and panitumumab in colorectal cancer[4], echinoderm microtubule associated protein like 4-anaplastic lymphoma kinase (EML4-ALK) for the use of crizotinib, and epidermal growth factor receptor (EGFR) for the use of erlotinib and gefitinib, in non-small cell lung cancer[5,6], and BCR–ABL for the use of tyrosine kinase inhibitors in chronic myeloid leukemia[7]. Companion diagnostic assays are designed to accompany specific therapies and help guide selection of patients according to expected drug responses. While the use of these assays has led to a shift in paradigms from disease-based therapeutic regimens to molecular target-based protocols[8,9], many of these molecular diagnostic modalities have onerous specimen requirements, such as needle core biopsies or surgical sampling of tumor tissues that can be invasive.

Circulating tumor 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 magnetized 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 tumor 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 epithelial-mesenchymal transition (EMT)[17]. Recently, EpCAM-positive and EpCAM-negative CTCs from breast cancer patients have been shown to exhibit high potential to metastasize to the lung and brain, respectively, in nude mice[18,19]. In colorectal cancer patients, 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 colorectal cancer patients with distant metastasis[21]. These findings indicate the presence of CTCs without epithelial markers in colorectal cancer patients. 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 tumor cells to molecularly targeted drugs.

Epithelial and mesenchymal types of malignant tumor cells possess high telomerase activity to maintain the length of telomere during aberrant cell proliferation, suggesting the potential of the telomerase activity as a general tumor 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 visualization of viable epithelial and mesenchymal types of human tumor cells with telomerase activity as GFP-positive cells[24,25]. OBP-401-mediated GFP labeling is a useful method to detect viable CTCs in patients with gastrointestinal cancers[26,27] and ovarian cancers[28]. The present study extends on 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 GIST cell line, GIST882; and the human normal esophageal 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, G12V, G12S, G13D) in Panc1, SW480, A549 and HCT116 cells, respectively. HT29 cells have one mutation (V600E) in the *BRAF* gene, whereas GIST882 cells harbor 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 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 titers 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 minutes on ice. The slides were subsequently incubated with the PE-conjugated mouse anti-EpCAM antibody (BioLegend, San Diego, CA, USA) for 1 h on ice. Then the slides were analyzed using an inverted fluorescent microscope (Olympus; Tokyo, Japan).

Flow cytometry

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

CTC model

CTC models were established by incubation with tumor 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 model and clinical samples

The protocol for DNA extraction from the CTC model or clinical samples is shown in figure 2A and Supplementary figure 1. Approximately 5 ml of blood was incubated with lysis buffer containing ammonium chloride to remove the red blood cells. These cells were then infected

with OBP-401 at 1×10^6 PFU and incubated for 24 h. Thereafter, the cell pellets were labeled with anti-CD45 antibody conjugated with PE and sequentially sorted by FACS Aria (Becton Dickinson, San Jose, CA, 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 tumor cells without the hematopoietic 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, CA, USA). The DNA solution mixed with DNA polymerase, and each primer was subjected to PCR analysis. Five ml of blood samples were collected with consent from patients with colorectal cancer, according to a protocol approved by the institutional review board at Okayama University Graduate School.

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 **Supplementary Table S1**. Using the PCR products, the sequence of each gene was analyzed with ABI PRISM 3100 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

Gene mutation analysis by ASB-PCR

ASB-PCR for the KRAS and BRAF genes was performed with a primer set of TaqMan Mutation Detection Assays (Applied Biosystems, Foster City, CA, 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 analyzed with 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 customized as follows. The PCR cycle number was set to 70 cycles 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 analyzed using a mixture of KRAS/BRAF wild-type and mutant cells. Genetic mutation was recognized as positive when the amplification for mutant alleles using specific primer was detected.

RESULTS

Fluorescent 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 visualized by OBP-401-induced GFP expression in a dose-dependent manner independently with EpCAM expression (figure 1D). The expression level of coxsackievirus and adenovirus receptor (CAR), which is associated with adenovirus infectivity, was almost similar among 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 CTC in the peripheral blood for genetic 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 volunteer, we made CTC models with different types of genetic mutations. As illustrated in figure 2A and Supplementary figure 1, following the lysis of red blood cells (RBC) 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 plaque-forming units (PFU) for 24 h, labeled 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 (APC)-positive cells at the P2 gate and hematopoietic CD45-positive cells at the P3 gate (figure 2B). The GFP-positive cells could be detected in the CTC model under a fluorescent 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 analyzed genetically by direct sequencing (Supplementary Table 1). The expected genetic mutations in the *KRAS* or *BRAF* gene were precisely detected in all CTC models containing four human cancer cell lines by direct sequencing (figure 3A and Supplementary figure 2), indicating that the OBP-401-based biological capture system is effective for the collection of CTCs expressing various levels of EpCAM marker. Recent studies have demonstrated that a heterogeneous population of CTCs are present within individual cancer patients and that these CTCs have both 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 in the genetic analysis using direct sequencing, SW480 cells (KRAS G12V mutant) were mixed with H1299 cells (KRAS wild-type) at a 50%, 40%, 30%, 20% or 10% purity ratio. *KRAS* gene mutation could be detected by direct sequencing only in the 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-labeled GFP-positive cells using ASB-PCR

To further increase the sensitivity to detect genetic alterations in the 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 analyzing the human cancer cells, we confirmed that there was no amplification of PCR products in the human normal fibroblast with wild-type *KRAS* and *BRAF* genes or in blood obtained from normal healthy volunteers by ASB-PCR with mutation-specific primers (Supplementary figure 3).

When we analyzed 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 (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 tumor cells were required for direct sequencing in the 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 the heterogeneous populations of CTCs.

Fluorescence-guided capture of EMT-induced and mesenchymal CTCs

nduction 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 must be detected to predict metastatic progression in cancer patients. We used A549 human lung cancer cells with *KRAS* gene mutation (G12S) and EpCAM-negative GIST882 mesenchymal human tumor cells with *KIT* gene mutation (K642E), which is frequently mutated in more than 70% of gastrointestinal stromal tumors (GIST)[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, transforming growth factor-β (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) and, 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 the 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 colorectal cancers patients

Finally, the blood samples obtained from eight patients with KRAS- or BRAF-mutated colorectal cancers were analyzed by the OBP-401-based CTC capture system and by ASB-PCR 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 tumors were detected in the CTCs of two advanced colorectal cancer patients (figure 4H and Table 2). The other six patients showed no detectable genetic abnormalities in blood samples, although the *KRAS* gene mutations were observed in their primary tumors. Three patients without metastatic lesions did not have large CTC count, and chemotherapeutic treatment in the other three patients with metastatic disease may have resulted in 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 the blood samples from cancer patients.

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 personalized medicine. The selection of targeted therapies for individual patients is currently made by analyzing the primary tumors, although there are very few cells within the primary tumors 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 tumor tissues for real-time molecular characterization. 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 both epithelial and mesenchymal types of tumor cells[24,25]. Recent studies have demonstrated that highly metastatic tumor cells are involved in both EpCAM-positive and EpCAM-negative subpopulations of CTCs in the blood of breast cancer patients[18,19]. During anticancer treatment, the characteristics of CTCs dynamically change between epithelial and mesenchymal types of CTCs within individual cancer patients[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 stabilization of the telomere in epithelial and mesenchymal malignant tumor cells during aberrant proliferation. In fact, high *hTERT* mRNA levels have been detected in the blood samples of cancer patients[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 (i.e., apoptosis or senescence). Thus, the telomerase activity may be superior to the unstable epithelial cell marker as a general biomarker for the detection of viable CTCs in the blood. Moreover, GFP-labeled CTCs by OBP-401 infection are considered to be useful for direct determination of drug sensitivity and metastatic potential, as well as determination of tumor heterogeneity[36-39].

A number of approaches based on the physical and biological properties of CTCs have been studied to distinguish CTCs from the surrounding normal hematopoietic cells and to capture them for further analysis. The CellSearch system, which is the only test approved by the US Food and Drug Administration (FDA) to detect CTCs, uses magnetized 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 analyze CTCs using EpCAM-coated microposts. Our OBP-401-based CTC detection has been previously compared with the CellSearch assay in metastatic breast cancer patients[40]. Although both assays exhibited comparable detection rates, the numbers of CTC-positive cells between both assays were 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 tumor cells while the CellSearch method detects epithelial non-tumor 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 tumor-specific probe, because the fluorescent signal can be amplified only in viable human tumor cells by viral

replication. We excluded the autofluorescence-positive cells at the P2 gate and the hematopoietic 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 tumor 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 cancer patients. Mostert *et al.* 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[41]. 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, the ASB-PCR is a suitable assay for non-invasive companion diagnostics in cancer patients. 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 colorectal cancer patients[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 colorectal cancer patients, the frequency of the *KRAS* and *BRAF* gene mutations is significantly higher in liver metastasis than in primary tumors[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 the metastatic potential in patients with KRAS wild-type colorectal cancers.

It is also worth noting that 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 harbor 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 tumor, 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 osteosarcoma patients remains to be elucidated. Thus, the characterization 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 in those with epithelial malignant tumors.

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 colorectal cancer patients, and these mutations were identical to those seen in the primary tumors. This novel "liquid biopsy" via a simple blood test could be carried out in real time and enables optimized 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 the small number 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 the entire CTCs using array-comprehensive genomic hybridization (aCGH)[49]. Moreover, genetic analysis in single CTC has been recently used to clarify the global gene alterations using aCGH and next-generation sequencing[50,51]. Thus, the comprehensive analysis of genetic alterations in individual CTCs from cancer patients would provide novel insight into the identification of the genetic signature in association 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 cancer patients, and large-scale clinical studies of this strategy are warranted.

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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.

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Figure Legends

Figure 1 OBP-401-mediated 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), and the *GFP* gene is inserted under the CMV promoter into the E3 region. (B) Immunofluorescence staining of 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 MOIs of 10, 100 or 1000 PFU per cell and assessed for GFP expression under fluorescence microscopy 24 h after infection. (E) Expression of CAR is analyzed using flow cytometry in four human cancer cell lines.

Figure 2 A simple fluorescent virus-guided capturing system of CTC. (A) Cell isolation steps in the OBP-401-based CTC capturing system. CTC models containing the spiked human cancer cells in 5 ml of blood sample or clinical blood samples obtained from cancer patients are incubated with RBC lysis buffer for 6 minutes. After centrifugation, cell pellets are then infected with OBP-401 at 1×10^6 PFU and incubated for 24 h. Thereafter, cells are incubated with anti-CD45 antibody, and the cell pellet was sorted by FACS. DNA extracted from FACS-sorted 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 CTC in blood sample containing SW480 cells after infection with OBP-401. Original magnification: ×200.

Figure 3 Genetic mutation analysis of human 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 GFP-positive cells at the P3 gate. The number of cells in the P3 gate and the mutation pattern in each model is indicated. (B) The minimal purity of tumor cells for direct sequencing to detect the expected gene mutations is evaluated. SW480 (*KRAS* G12V) cells re 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 analyzed by direct sequencing. (C) 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.

Figure 4 Fluorescent virus-guided capture and genetic mutation analysis of human mesenchymal or EMT-induced tumor cells. (A) A549 human lung cancer cells and GIST882 human gastrointestinal stromal tumor cells are infected with OBP-401 at MOIs of 10, 100 or 1000 PFU per cell. GFP expression is assessed under the fluorescent microscope 24 h after virus infection. (B) Morphological change of A549 cells treated with 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 (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 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 marker and 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 ASB-PCR. Mutation-specific curves for *KRAS* gene cross their threshold of detection. (H) Representative computed tomography images of colon cancer patient with lung, spleen, and ovary metastases. The primary tumors and CTCs show the *BRAF* V600E mutation.

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

CTC model				FACS analysis			Genetic analysis			
Cancer cells	Cell type	Gene status	Number of cancer cells	Gate	Number of GFP-positive cells	Purity of cancer cells (%)	Primer	Amplification	Ct values	
									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

NA: not amplified.

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

Patients				FACS analysis		Genetic analysis			
Tumor site	Stage	Gene status of primary tumor	Metastasis	Gate	Number of GFP-positive cells	Primer	Amplification	Ct values	
								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

NA: not amplified.