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# Circulating tumour cells detected by a novel adenovirus-mediated system may be a potent therapeutic marker in gynaecological cancers

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BACKGROUND: Recently developed detection system for circulating tumour cells (CTCs) using a telomerase-specific replicative adenovirus generated nonspecific green fluorescent protein (GFP) signals because of the co-presence of white blood cells (WBCs) nonspecifically infected by viruses. Here, we established a unique detection system for CTCs that completely excludes nonspecific signals.

METHODS: Blood obtained from the patients was subjected to haemolytic processes to eliminate red blood cells. The cell pellets were then infected with OBP-401, fixed, incubated with fluorescence-labelled anti-CD45 antibody to mark white blood WBCs, and examined on slides under a microscope.

RESULTS: Preparatory experiments with cancer cells artificially added to healthy donor samples confirmed that CD45 labelling could distinguish GFP-positive cancer cells from WBCs. In 53 patients with gynaecological cancers, CTCs were detected in 21 patients (39.6%) when CD45-positive cells were excluded as WBCs among GFP-positive cells. No CTCs were detected in samples from healthy volunteers. There was no significant correlation between CTC counts and known clinicopathological factors. The CTCs rapidly vanished after surgery or chemotherapy in most patients whose treatments were effective. In contrast, the persistence of CTCs even after treatments was tightly associated with poor response to the treatments ($P < 0.005$).

CONCLUSION: The presence of CTCs in our system may potentially be a novel therapeutic marker in gynaecological cancers.

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Blood-borne metastasis responsible for most cancer-related deaths is initiated by intravasation of cancer cells from the primary lesion into the systemic circulation. Efforts have been made to identify the circulating tumour cells (CTCs) in peripheral blood (Racila *et al*, 1998; Yu *et al*, 2011). The clinical relevance of detecting CTCs as a prognostic and/or surrogate marker of treatment response has been established in several cancer types such as breast cancer, colorectal cancer, and prostate cancer (Cristofanilli *et al*, 2004, 2005; Cohen *et al*, 2008; de Bono *et al*, 2008). Core techniques have been applied to immunologically recognise CTCs with epithelial cell-specific markers, combined with reverse transcription PCR, cell size filtration, or other cytometric procedures (Attard and de Bono, 2010; Sun *et al*, 2011). CellSearch (Veridex, Raritan, NJ, USA) is the only method approved by the US Food and Drug Administration as an automated detection system for CTCs (Allard *et al*, 2004; Riethdorf *et al*, 2007); it consists of immunomagnetic enrichment with ferrofluids conjugated with anti-epithelial cell adhesion molecule (EpCAM) antibody and detection with a triple-stain procedure (positive staining for cytokeratin (CK8/18/19) and DAPI, and negative staining for CD45, a leucocyte common antigen).

Telomerase-specific replication-selective adenovirus is an onco-lytic adenovirus vector that expresses a green fluorescent protein (*GFP*) gene and was developed by us (named Telomescan or OBP-401). It contains the *human telomerase reverse transcriptase* (*hTERT*) gene promoter upstream of the *E1* gene in the adenovirus type 5 genome (Figure 1A). As hTERT expression is highly specific to cancer cells (Takakura *et al*, 1998; Kyo *et al*, 1999a, 1999b; Takakura *et al*, 2005) and the *hTERT* promoter has stringent cancer specificity (Takakura *et al*, 1999, 2005), this virus can express *E1* genes preferentially in cancer cells and thereby replicate there with much higher efficiency than in healthy cells (Kawashima *et al*, 2004; Takakura *et al*, 2010). Cancer cells infected with OBP-401 have been confirmed to express GFP in not only *in vivo* mouse xenografts but also cytological samples in clinical practice (Kishimoto *et al*, 2006; Maida *et al*, 2009). Recently, OBP-401 was successfully applied to detect CTCs in the clinical blood samples of patients with gastric, breast, and other cancers (colon cancer, hepatocellular carcinoma, and non-small cell lung cancer) (Kojima *et al*, 2009; Kim *et al*, 2011). These hTERT-based adenoviral-mediated systems have a great advantage for detecting
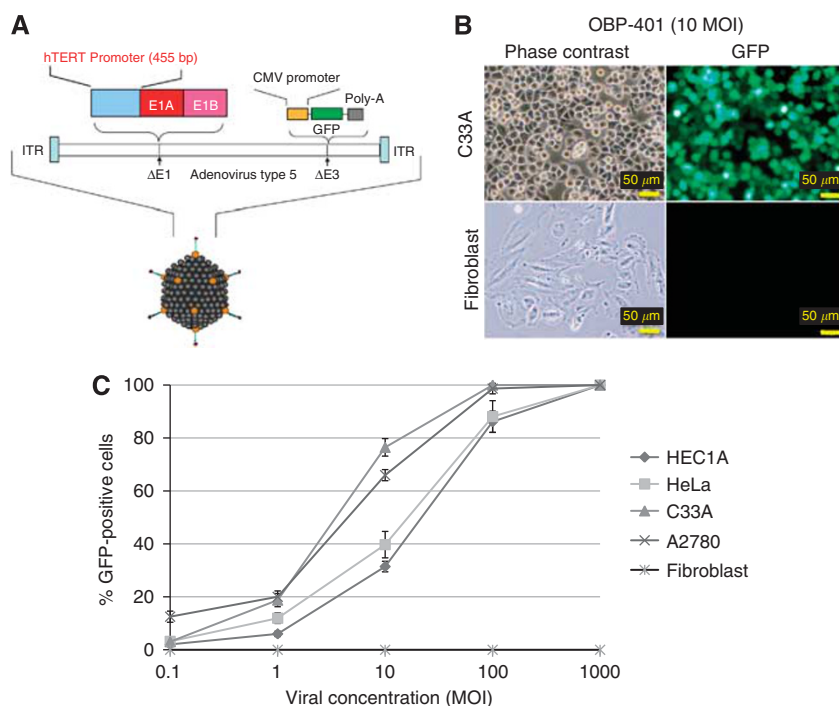
**Figure I** Selective GFP expression in human cancer cell lines by OBP-401 infection *in vitro*. (**A**) The schematic structure of OBP-401 is shown. (**B**) The human cervical cancer cell line C33A and normal human fibroblasts were infected with OBP-401 at a MOI of 10 and GFP expression was assessed using a fluorescence microscope 24 h after infection. (**C**) The human endometrial cancer cell line HEC1A, cervical cancer cell lines HeLa and C33A, ovarian cancer cell line A2780, and normal human fibroblasts were infected with OBP-401 at the indicated MOI. The ratios of GFP-expressing cells were counted 24 h after infection. Bars indicate s.e.

living CTCs, minimising the false-positive detection of healthy circulating epithelia. However, some technical problems remain, such as false-positive signals caused by white blood cells (WBCs) that cannot be excluded, because numerous WBCs contained in the clinical blood samples generate nonspecific infection of virus and telomerase is known to be activated in specific groups of blood cells (Matsumura-Arioka *et al*, 2005; Ge *et al*, 2006). This study focused on this point and developed a novel strategy to overcome these problems.

## MATERIALS AND METHODS

### Cell culture

The human cervical cancer cell line C33A and ovarian cancer cell line A2780 were cultured at 37 °C under 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum (Sigma-Aldrich, St. Louis, MO, USA), 100 $\mu$g ml$^{-1}$ streptomycin, and 100 IU ml$^{-1}$ penicillin.

### Viruses

OBP-401 (Telomescan) is a telomerase-specific replication-competent adenovirus, in which the *hTERT* gene promoter has been inserted upstream of the *E1A* and *E1B* genes linked with an internal ribosome entry site in the adenovirus type 5 genome, and the *GFP* gene is inserted under the cytomegalovirus promoter into the E3 region (Figure 1A; Fujiwara *et al*, 2006; Kishimoto *et al*, 2006; Watanabe *et al*, 2006). The construction and features of these viruses have been described in detail in our previous studies (Umeoka *et al*, 2004; Fujiwara *et al*, 2006). The viruses were purified by $CsCl_2$ linear gradient ultracentrifugation. The viral titres were determined by a plaque-forming assay using 293 cells and the virus sample was stored at −80 °C.

### Blood sample preparation

Blood samples (5 ml) were drawn into heparinised tubes and incubated with lysis buffer containing ammonium chloride ($NH_4Cl$) for 15 min twice to remove erythrocytes. After centrifugation, the cell pellets were mixed with $10^5$ PFUs of OBP-401 and incubated at 37 °C for 24 h with gentle rotation. Following centrifugation, the cells were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde. Following washing with phosphate-buffered saline, the cells were incubated with 1/200 diluted mouse anti-human CD45 antibody (BioLegend, San Diego, CA, USA) for 20 min on ice. CD45 expression was detected by incubation with CF 555-labelled goat anti-mouse secondary antibody (Biotium, Hayward, CA, USA) for 20 min in the dark. Following washing with phosphate-buffered saline and centrifugation, cells were resuspended in 50 $\mu$l of mounting media (DAKO, Glostrup, Denmark) and placed onto slides under coverslips.

These studies were approved by the Medical Ethics Committee of Kanazawa University Graduate School of Medical Science. All patients and healthy volunteers gave written informed consent.

### *In vitro* fluorescence imaging

Fluorescence levels were assessed with excitation/emission at 485 nm/505 nm for GFP and 550 nm/570 nm for CF 555 using a BX53 fluorescent microscope with 100-w mercury lamp unit (Olympus, Tokyo, Japan), photographed (×400), and counted by viewing the monitor and evaluating cell shapes or CD45 staining. High-resolution image acquisition was accomplished using an EPSON personal computer (SEIKO EPSON, Suwa, Japan). Images were processed for contrast and brightness with the use of Adobe Photoshop CS2 software (Adobe, San Jose, CA, USA), and were observed by persons who did not know the clinical information of the patients.

### Patients

Patients with primary or recurrent gynaecological cancers before any treatment as well as healthy volunteers were eligible to participate in this study. Serial blood samplings were done before and after the treatments, including surgical reduction, chemotherapy, or their combination. Blood samples from healthy volunteers were also examined as negative controls. All experimental procedures including blood sampling, processing, and analysis were performed under the approval of the Medical Ethics Committee of Kanazawa University Graduate School of Medical Science. All patients and healthy volunteers gave written informed consent. The clinical characteristics of the patients are summarised in Supplementary Table 1.

### Statistical analysis

To assess the statistical significance of differences in the numbers of CTCs, the $\chi^2$ test or Mann–Whitney U test were performed using the Microsoft Excel 2010 software (Microsoft, Redmond, WA, USA). A *P*-value of <0.05 was considered to indicate statistical significance.

## RESULTS

### Optimising the condition of OBP-401 infection

OBP-401 was designed to replicate only in cells in which the *hTERT* promoter is activated. To confirm whether GFP expression by OBP-401 is specific in cancer cells, we first evaluated the GFP expression in various telomerase-positive gynaecological cancer cell lines and normal human fibroblasts infected by OBP-401 (Figure 1). The cells expressing GFP were counted under the fluorescent microscope 24 h after infection. No GFP-positive cells were observed in healthy fibroblasts at any viral concentration (Figure 1B). The GFP expression ratios varied among cell lines at an intermediate viral concentration (multiplicities of infection (MOI): 10). At higher viral concentrations over 100 MOI, most of the cancer cells expressed GFP, irrespective of the cell type (Figure 1C). Importantly, a cytotoxic effect was not observed within 24 h after infection, appearing at least 36 h after infection (data not shown).

We next performed the preparatory experiments to evaluate the efficiency of OBP-401 in marking the cancer cells in peripheral blood: a total of 100 C33A human cervical cancer cells were placed in 5-ml blood samples from healthy volunteers. Red blood cells were lysed in $NH_4Cl$ buffer; subsequently, the cell pellets were recovered and infected with OBP-401 at the indicated concentrations. Green fluorescent protein-expressing cells were counted under the fluorescent microscope. In general, GFP-expressing C33A cells were observed large and strong GFP fluorescence, whereas GFP-expressing WBCs were apparently small with weaker fluorescence (Figure 2A). However, we sometimes had difficulty distinguishing these GFP signals, because we detected some large GFP-positive cells in negative control bloods. The numbers of GFP-expressing cells counted in a 5-ml blood sample containing 100 C33A cells are shown in Figure 2B. In negative control samples
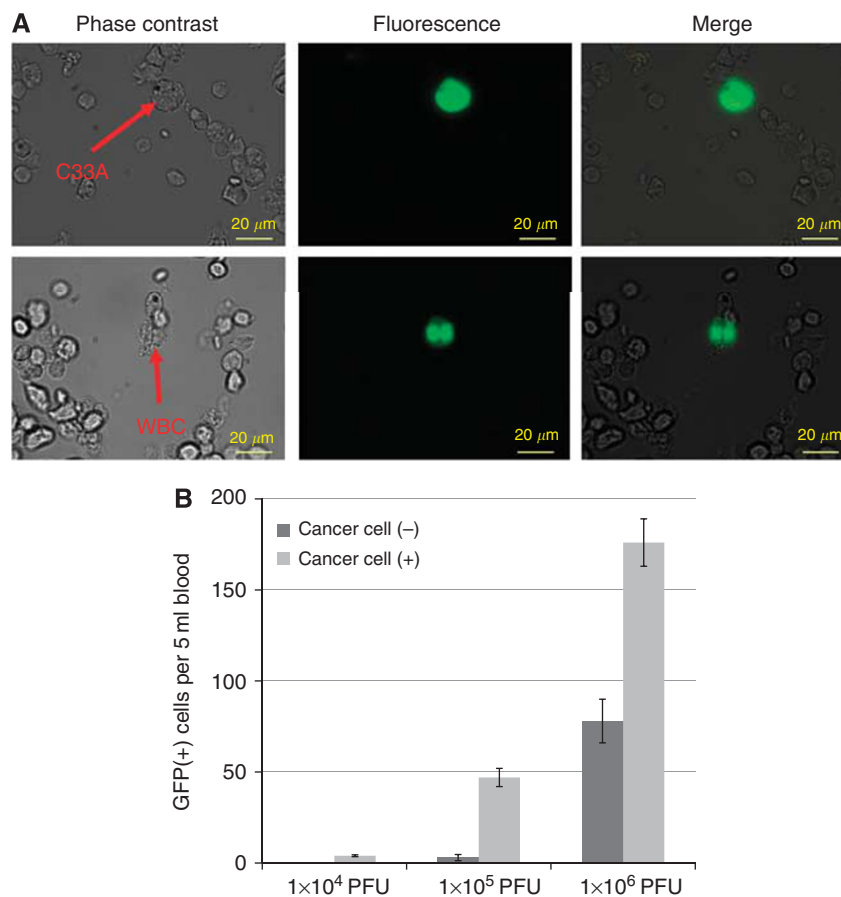


**Figure 2** Preparatory experiments using human blood samples containing cancer cells. (**A**) Phase contrast and fluorescent images of peripheral blood cells containing C33A cervical cancer cells. A total of 100 C33A cells were mixed with 5-ml whole blood samples from healthy volunteers and treated with haemolysis buffer, followed by the infection with $1 \times 10^5$ PFU of OBP-401. Green fluorescent protein-expressing cells were analysed 24 h after infection. Green fluorescent protein was mainly expressed in C33A cells (upper lane), but some WBCs expressed GFP (lower lane). (**B**) The number of the GFP-expressing cells in 5 ml blood with or without 100 C33A cells is shown. The samples were infected with the indicated amount of OBP-401. Bars indicate s.e.
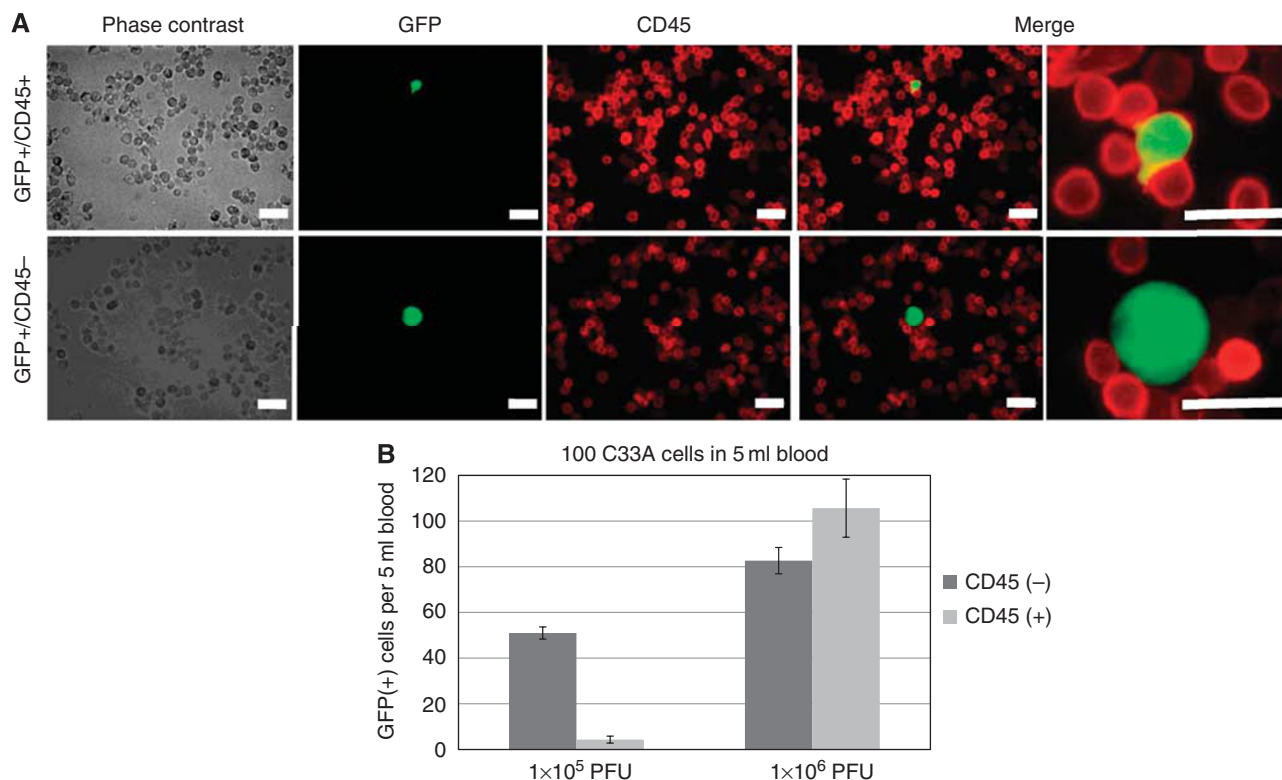
**Figure 3** Application of CD45 staining to distinguish CTCs from GFP-expressing WBCs. (**A**) Phase contrast and fluorescent images of peripheral blood cells mixed with C33A cervical cancer cells. OBP-401-infected samples were fixed and stained with anti-CD45 antibody and CF555-labelled secondary antibody. The overlap of green (GFP) and red (CD45) fluorescence is displayed as yellow. Bar, 20 $\mu$m. (**B**) The number of the GFP-expressing cells with/without CD45 fluorescence in 5 ml blood mixed with 100 C33A cells is shown. The samples were infected with the indicated amount of OBP-401. Bars indicate s.e.

without C33A cells, we also counted GFP-expressing cells that gave false-positive signals. Approximately 180 GFP-positive signals were counted from the infection of $1 \times 10^6$ PFU of OBP-401, in which about 80 GFP-positive cells were considered to be false-positives by WBCs, based on the count in the negative control samples. Conversely, no false-positive signals were detected from the infection of $1 \times 10^4$ PFU of OBP-401, but the sensitivity for detecting GFP-expressing C33A cells was too low (less than 5%) in this condition. We found that $1 \times 10^5$ PFU of OBP-401 could confer optimal results, detecting ∼50% of C33A cells while minimising false-positive signals at <3% (Figure 2B). These findings indicated that increasing viral load improved the sensitivity for detecting cancer cells, while it decreased specificity, thereby suggesting its importance to effectively eliminate false-positive signals.

## Application of CD45 staining to distinguish GFP signals between CTCs and WBCs

To distinguish GFP expression in CTCs from that in WBCs, we attempted co-immunostaining of CD45 after OBP-401 infection (Figure 3A). As CD45 expression is broadly observed at the surface of the WBCs, GFP-expressing WBCs should be recognised with CD45 signals (red fluorescent) at the membrane and should be distinguishable from CTCs lacking these signals in merged pictures. Preparatory experiments using artificial samples in which 100 C33A cells were added to 5-ml blood samples from healthy volunteers showed that about 50 CD45-negative/GFP-positive C33A cells were counted at a viral dose of $1 \times 10^5$ PFU and about 80 of these C33A cells were counted at a viral dose of $1 \times 10^6$ PFU (Figure 3B), whereas

4 CD45-positive/GFP-positive WBCs were detected at a viral dose of $1 \times 10^5$ PFU and about 100 of these WBCs were detected at a viral dose of $1 \times 10^6$ PFU. On the basis of these experiments, we considered the viral concentration of $1 \times 10^5$ PFU to be optimal for detecting CTCs in clinical samples. No CD45-negative/GFP-positive cell was observed in negative control samples, meaning that the specificity is extremely high (almost 100%) in this system.

## Detection of CTCs in patients with gynaecological cancers

We analysed fresh blood samples collected from 53 patients with both primary and recurrent gynaecological cancers as well as from 22 healthy volunteers (14 male and 8 female, mean age 31.4; s.d. 6.6). Using fluorescence microscopy, we counted GFP-expressing cells and those with CD45 immunofluorescence were judged to be WBCs. We first confirmed that no CTCs were detected in samples from healthy volunteers, but the number of GFP-positive, CD45-positive cells (WBCs) ranged from 1 to 64 in 5-ml blood samples (mean 11.5; s.d. 13.9), indicating a relatively high frequency of nonspecific signals.

The 53 patients consisted of those with 18 cervical cancers, 17 endometrial cancers, 14 ovarian cancers, 3 vulvar cancers, and 1 vaginal cancer (Supplementary Table 1). All samples were collected before treatment. Serial evaluations were done during their treatment courses. One or more CTCs were identified from 10 of 18 (55.6%) patients with cervical cancer, 5 of 17 (29.4%) with endometrial cancer, 5 of 14 (35.7%) of ovarian cancer, and 1 of 4 (25.0%) with other cancers (Supplementary Table 2). Overall, 21 of 53 (39.6%) of patients with gynaecological cancers were CTC-positive. The number of CTCs observed in 5-ml blood samples
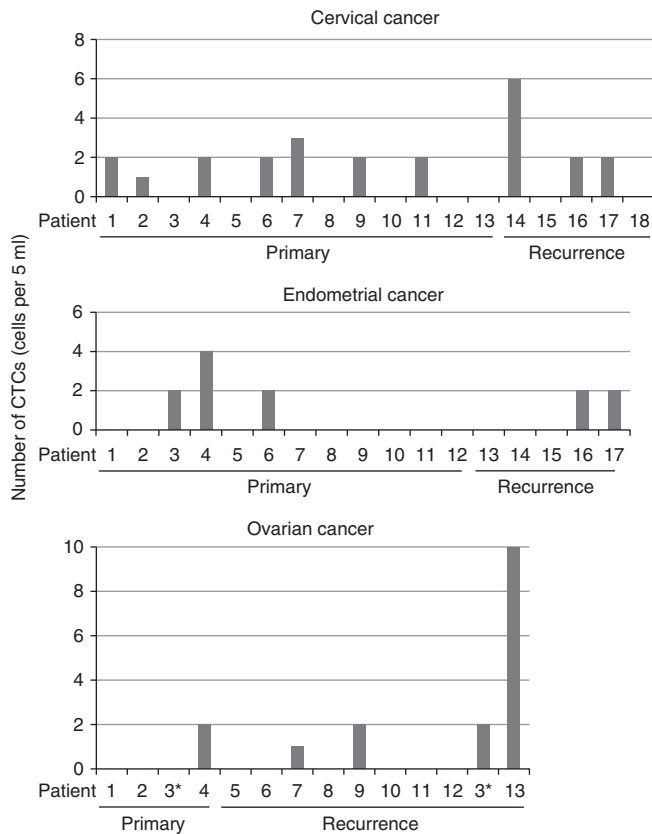
**Figure 4** Numbers of CTCs detected in patients by type of cancer. Comparison of the numbers of CTCs observed in each patient. Patient no. 3 with ovarian cancer was negative for CTCs at her initial examination. After 6 months, she relapsed, at which time CTCs were detected (indicated with asterisk).

ranged from 0 to 10 (Figure 4). There was no significant difference in the CTC-positive ratios among cancer types. No correlation was observed between CTCs and the clinicopathological characteristics of tumours (Supplementary Table 2).

Some doubt may arise regarding the reproducibility of this system owing to the small number of CTCs detected in a sample. To verify the reproducibility, the additional control experiments were performed, in which 50 C33A cells were spiked into 50 ml of control bloods from healthy donors, divided into 10 tubes and subjected to the counting. The average of observed C33A cells in each 5 ml blood was 2.2 (s.d. = 0.9). At least one cancer cell was observed in every sample (data not shown).

### Change in CTC counts correlates with clinical course in patients

Serial examination was performed during their treatment courses. The final evaluations of CTCs were done 4 weeks after the start of the treatments. Circulating tumour cells rapidly vanished after surgery or chemotherapy in most patients whose treatments were judged as effective including complete response and partial response by imaging diagnosis. Conversely, CTCs were sustained in patients in whom the treatments were not effective ( progressive disease). Representative cases are shown in supplementary figure 1. There was significant correlation between the outcome of the treatment and the presence of CTCs (Table 1). There was no patient whose CTC was negative at the initial evaluation but detected later during the treatment course.

**Table 1** Change in CTC count in patients with positive CTCs at initial evaluation

| Effect of treatment | CTCs no longer present | CTCs remained |
|---|---|---|
| Complete response/partial response | 15 | 1[#] |
| Progressive disease | 0 | 3[#] |

Abbreviation: CTC = circulating tumour cell. [#]$P < 0.005$.

## DISCUSSION

Most of the detection systems for CTCs currently available rely on immunological recognition of epithelial-specific cell surface markers, such as EpCAM and cytokeratin. With regard to female cancers (cervical, endometrial, ovarian, and breast cancers), the detection rates of CTCs by such methods were reported as ranging from 14.4 to 90% (Judson et al, 2003; He et al, 2008; Behbakht et al, 2011; Poveda et al, 2011). Several critical problems in detecting CTCs using cell surface markers have been reported. First, there is no guarantee that the detected cells are real cancer cells; they may be merely healthy epithelia circulating in blood. Second, they may not be living cells because dead cells can still present surface antigens. Third, although the cascades of cancer metastasis formation are not fully understood, the epithelial-to-mesenchymal transition (EMT) process is believed to have a great role in these cascades (Klymkowsky and Savagner, 2009; Bonnomet et al, 2010; Iwatsuki et al, 2010) and the expression of epithelial markers is known to be downregulated during the process of EMT, probably leading to the false-negative findings (Mikolajczyk et al, 2011). The OBP-401-based method theoretically has a technical advantage for overcoming these issues, because OBP-401 infects and replicates only in the living cancer cells and the infectivity does not change during the process of EMT.

Our preparatory experiments showed that the positive ratio of GFP-positive cancer cells increased in a dose-dependent manner with OBP-401, but the ratio of GFP-positive WBCs also increased. There are some reports about the induction of hTERT expression in growth-stimulated healthy lymphocytes (Matsumura-Arioka et al, 2005; Ge et al, 2006). Although the frequency of GFP-positive WBCs was quite low compared with that of cancer cells, it is not ignorable because the number of WBCs contained in the blood sample is much larger than that of CTCs. These problems have been left unresolved in an OBP-401-mediated detection system. The unique technique applied in this study is the addition of a process of counterstaining with anti-CD45 antibody to mark WBCs. We could confirm that anti-CD45 staining did not interfere with the recognition of GFP-expressing cancer cells (data not shown). Merging GFP and CD45 fluorescences (red with CF555) allowed the discernment of cancer cells from WBCs (Figure 3). Therefore, we propose this step to be crucial in an OBP-401-mediated CTC detection system.

In this novel protocol, the CTC detection rate was ~ 40% in clinical samples from patients with gynaecological cancers. The reported rates varied widely among studies, ranging from 14.4 to 90%, and the clinical significance of the presence of CTCs are still under controversy (Judson et al, 2003; He et al, 2008; Behbakht et al, 2011; Poveda et al, 2011). Although our results were near the centre of this range, the number of CTCs detected in a sample was significantly lower than that in other reports. This might be due to the higher stringency of the system to minimise nonspecific WBC signals. The reproducibility of our system was confirmed by the additional experiments after examinations with clinical samples, in which 50 C33A cells were spiked into 50 ml of bloods from healthy donors, divided into 10 tubes. At least one cancer cell was observed in each tube (mean 2.2 ± 0.9 s.d.), supporting the reproducibility of our method.

There was no significant difference in the CTC counts among cancer types, clinical stage, or other clinicopathological findings. Notably, no statistically significant difference was observed between patients with hematogenous metastasis and those with non-hematogenous metastasis, suggesting that CTCs may appear in the early stage of cancers, irrespective of the cancer type. The persistence of CTCs after treatment, regardless of the type of treatment, was associated with poor response to the treatment, suggesting that CTCs may be a sensitive marker of treatment effect. However, it remains unclear whether the presence of CTCs can be used as a prognostic marker and we are currently analysing outcome data. Two of the important merits of our system are the cost effectiveness and technical feasibility. No special machine is required other than a fluorescent microscope, and it takes 15–20 min to test one sample. Furthermore, the CTCs can be captured under the microscope and then be used for further analysis such as whole-genome amplification and gene expression analysis. This might be attractive because we can obtain genome information from the cancers via CTCs without invasive procedures such as biopsy and surgery.

In conclusion, we developed a novel CTC detection system using a telomerase-specific replicative adenovirus combined with CD45 staining. Our system clearly resolved the technical defect of a viral-mediated CTC detection method, effectively eliminating false-positive signals by WBCs and maintaining sufficient sensitivity. The feasibility and superior cost-effectiveness of this method encourage further confirmation of the results with larger numbers of patients and longer follow-up periods to evaluate the prognostic impact.

## Conflict of interest

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

**Clinical Studies**

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