RESEARCH ARTICLE

A New Cancer Cell Detection Method Using an Infectivity-enhanced Adenoviral Vector

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Abstract

Cytological examination is widely used as a diagnostic tool because of the ease of collecting cells from the involved area. However, the diagnostic yield of cytological examination is unsatisfactory; the reasons include sampling error, poorly prepared samples, small numbers of malignant cells, and low grades of cellular atypia. In this study, we focused on the high infectivity of adenovirus towards epithelial cells and applied the luciferase-expressing adenoviral vector to a new cancer cell detection tool. In addition, adenoviral infectivity was enhanced by modifying viral fiber proteins. The sensitivity of the diagnostic tool was tested using the NCI-H1299 lung cancer cell line, and validated in body fluid samples from cancer patients with a variety of etiology. Results showed that the adenovirus efficiently transfected NCI-H1299 with high sensitivity. Only 10 cancer cells were sufficient for detection of luciferase signals. In body fluid samples, the adenovirus confirmed the diagnosis for malignant and benign cancer, but not in non-epithelial cell derived samples. This study provides proof-of-concept for a more reliable and sensitive diagnostic tool for epithelium-derived cancer.

Keywords: Adenoviral vector - cytology - luciferase assay - malignant effusion

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Introduction

The diagnosis of malignant disease is made by cytological or histological examination of clinical samples obtained from patients. Histological examination requires a certain amount of tissue, and using an invasive procedure to obtain this type of sample is inevitable. In contrast, cytological examination only requires small clinical samples collected by non-invasive techniques, including body fluids, lavage of targeted area, and needle aspiration of lymph nodes. This approach is commonly used in the case of poorly conditioned patients unable to tolerate invasive procedures. Clinical samples suitable for cytological examination include urine, sputa, pleural effusion, ascites, fine needle aspirations of lymph nodes or lesions, and lavage fluid from involved areas, all of which can be obtained by minimally invasive procedures. The malignant pleural effusion (MPE) defines effusion from direct infiltration of the pleura by cancer cells. The annual incidence of MPE in the United States is about 150,000. More than 75% of MPEs are caused by neoplasms of the lung, breast, or ovary or by lymphoma (Hausheer and Yarbro, 1987; Henschke et al., 1991; Martinez-Moragon et al., 1998; Antunes and Neville, 2000; Heffner and Klein, 2008). However, the sensitivity of cytological examination in the detection of cancer cells is only approximately 65% (Nance et al., 1991; Starr and Sherman, 1991; Woenckhaus et al., 2005; Benlloch et al., 2006; Sriram et al., 2011). Additional studies could complement standard cytology. Combinations of tumor markers, however, could help select patients with negative pleural effusion cytologic results for additional diagnostic studies. Moreover, additional immunostaining or other specific staining improves the diagnostic yield to some extent, though the promptness of diagnosis is adversely impaired (Kuenen-Boumeester et al., 1996 ; Porcel et al., 2004; Lee and Chang, 2005; Shitrit et al., 2005; Westfall et al., 2010 ; Su et al., 2011). Methods such as fluorescence in situ hybridization analysis, image analysis cytometry, and PCR, are more sensitive than standard cytologic studies (Fieglure, 2005; Holloway et al., 2006; Sriram et al., 2011). Investigations are underway to determine if the detection of aneuploidy adds diagnostic value and meaningful therapeutic consequences to standard effusion analysis for the detection of MPE (Fieglure, 2005; Osterheld et al., 2005; Northup et al., 2007; Sriram et al., 2011). In this study, we focused on effective gene transduction into cancer cells by an adenoviral vector and conceived of

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the application of the viral vector as a diagnostic tool in malignant diseases. Serotype 5 adenoviral vector is used in basic research as an effective tool for gene transduction into human cells. In clinical trials, the adenoviral vector containing various genes has been used for gene therapy of malignant disease. Because serotype 5 adenovirus infects human cells via the coxsackie-adenovirus receptor (CAR) and integrin on the cell membrane, infectivity depends mainly on the expression status of these molecules. The efficiency of Ad5 (serotype 5 adenovirus) gene transfer may closely correlate with the cell surface density of its primary receptor, coxsackie and adenovirus receptor (CAR) (Zabner et al., 1997; Kaner et al., 1999; Nalbantoglu et al., 2001). Unfortunately, the expression of CAR is highly variable, and is often low on lung and other primary cancer cells, which results in relative resistance to Ad5 infection (Hemmi et al., 1998; Miller et al., 1998; Takayama et al., 2003). To overcome this limitation, we have developed a new chimeric Ad5 vector, Ad5/3, which contains a chimeric fiber protein possessing a serotype 3 knob. In addition, our previous study has revealed that a distinct Ad3 (serotype 3 adenovirus) receptor exists in various cancer cells based on a novel knob binding assay, and that the Ad5/3 chimeric vector is retargeted to the Ad3 receptor with higher gene transfer efficiency than Ad5 (Kanerva et al., 2002 ;Kawakami et al., 2003). We also confirmed that this Ad5/3 showed higher infectivity toward various cancer cells, especially ovarian and lung cancer cells (Kanerva et al., 2002 ;Kawakami et al., 2003). The present study proposes a new approach to cancer detection based on gene transfer. Combining this new diagnostic tool with conventional cytology can considerably improve the diagnostic yield.

Materials and Methods

Cell culture

The NCI-H1299 lung cancer cell line and HEK293 adenoviral-transformed human embryonic kidney cell line were obtained from ATCC (American Type Culture Collection, Manassas, VA). Cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 5% fetal bovine serum and incubated at 37 °C in 5% CO2.

Artificial malignant fluid

NCI-H1299 cells were used to prepare the artificial malignant fluid sample because they were confirmed to be susceptible to adenoviral infection in our previous experiment because preliminary experiments showed that these cells are susceptible to adenoviral infection. The artificial malignant fluid was prepared by diluting human peripheral blood (collected from healthy donors) with culture medium (5% v/v) (A.P.Fishman et al., 2008), and a range of NCI-H1229 cells were added to it (10-1000 cells) to determine the cutoff point of diagnostic value.

Adenoviral vectors

Recombinant adenoviral vectors expressing firefly luciferase were constructed through homologous recombination in Escherichia coli using the AdEasy system (He et al., 1998). A cytomegalovirus immediate early promoter derived from plasmid pCEP4 (Invitrogen, Carlsbad, CA) was placed next to the firefly luciferase gene in an Ad E1 shuttle vector, recombined with the E1- and E3-deleted adenoviral backbone vector pAdEasy 1, and transfected into HEK293 cells by standard techniques to form Ad5CMVLuc (Figure 1) (He et al., 1998). The luciferase gene was derived from pGL3 Basic (Promega, Madison, WI). A modified adenoviral vector was also used for enhancement of infectivity to cancer cells in this experiment. In this modified adenovirus, only the knob domain of the conventional serotype 5 adenovirus was replaced with that of serotype 3 adenovirus as reported previously (Kawakami et al., 2003). The luciferase-expressing adenovirus based on this chimeric adenovirus (Ad5/3CMVLuc) was generated in the same fashion described above. The adenoviruses were propagated in the adenovirus-packaging cell line HEK293 and purified by double CsCl density gradient centrifugation, followed by dialysis against phosphate-buffered saline with 10% glycerol.

Luciferase assay

NCI-H1299 cells were plated in 12-well plates in triplicate at a density of 1 × 105 cells/well. After overnight culture, the cells were infected with Ad5CMVLuc or Ad5/3CMVLuc at 1 viral particle (vp)/cell or 10 vp/cell in DMEM with 2% FCS for 3 h and maintained in complete medium (Adachi et al., 2001). The infected cells were harvested and treated with 100 μL of lysis buffer (Promega, cat #E153A) after a 2-day culture. A luciferase assay (Luciferase Assay System, Promega) and a luminometer (GENE LIGHT 55A, Microtec Nition, Tokyo, Japan) were used for the evaluation of luciferase activity in the infected cells. Luciferase activity was normalized by protein concentration in the cell lysate (Bio-Rad DC Protein Assay Kit, Hercules, CA).

In the fluid samples, a fixed dose of 1 × 104 pfu of Ad5/3CMVLuc was mixed into the fluid with or without a red blood cell (RBC) lysis procedure, because RBCs can prevent infection by adenovirus (Lyons et al., 2006). The infected samples were cultured in the flask for 48 h. Collected cells were then applied to the luciferase assay in the same fashion described above. Luciferase activity was expressed as an arbitrary unit. To remove RBCs in the fluid, 10 mL of lysis buffer (RBC Lysis Buffer, eBioscience, San Diego, CA) was mixed in 10 mL of fluid sample and incubated for 10 min at room temperature. The lysis reaction was stopped by adding 30 mL of PBS. After the RBC lysis procedure, the cells in the fluid sample were spun down at 4 °C and applied to the luciferase assay.

Body fluid samples

Body fluid samples consisting of 15 pleural effusions and 10 ascites samples were obtained from 25 patients at Kyushu University Hospital. All patients provided consent (one sample was used as a negative control) for cytological examination of their samples at the central clinical laboratory. Cytology results were classified

into positive, negative, or inconclusive. In addition to cytological evaluation, the pleural effusion and ascites samples were clinically evaluated for malignancy. For example, some cytologically negative pleural or peritoneal effusions were found to be malignant effusions based on the following findings: detection of malignant cells after repeated cytological examination, increasing amounts of effusion in the clinical course with intrapleural or intraperitoneal disseminated lesions confirmed by imaging studies, or decreasing amounts of effusion following anticancer chemotherapy.

Statistical methods

Data represent mean values from three separate readings with the error bars showing standard deviation. Data shown was consistent for two or more repeat studies performed on different days. Continuous measures were compared between groups using two-sample unpaired t tests.

Results

Infectivity of Ad5 and Ad5/3 in NCI-H1299 cells

NCI-H1299 cells derived from human lung cancer were infected by Ad5CMVLuc or Ad5/3CMVLuc at various m.o.i. Approximately 48 h after infection, cells were collected and applied to the luciferase assay as described in Materials and Methods. As shown in Figure 2, the cells infected by Ad5/3CMVLuc exhibited higher luciferase activity compared with those infected by Ad5CMVLuc. We have observed that ovarian and lung cancer cells were susceptible to Ad5/3 infection which is in agreement with published literature (Kanerva et al., 2002). Based on these results, NCI-H1299 cells were used in an artificial effusion in the next step.

Luciferase activity in artificial effusions containing cancer cells

Luciferase activities in artificial malignant effusions containing various numbers of cancer cells are depicted graphically in Figure 3. First, artificial effusions were applied to luciferase assays without RBC lysis procedures. The luciferase activity in the sample mixed with 10, 30, and 100 cancer cells showed a similar value and no significant difference compared with that the negative control (sample not containing any cancer cells). The sample containing 300 or more cancer cells expressed significantly higher luciferase activity. The cutoff point was between 100 and 300 cancer cells in the artificial effusion. Because RBCs have a negative charge on the cell surface, they may nonspecifically interfere with viral infection of cancer cells. In the next step, the RBC lysis procedure was performed prior to adenoviral infection. After the removal of RBCs, adenovirus efficiently infected cancer cells and expressed a 1- to 2-log unit higher luciferase activity in each sample, as shown in Figure 3. The luciferase activity also correlated with the number of cancer cells mixed in the artificial effusion. The high sensitivity of this diagnostic technique is evidenced by the significant luciferase signal detected with only 10 cancer cells in RBC-free samples. The sample containing only 10 cancer cells showed significant luciferase activity when compared with the control.

Luciferase activity in clinical samples

This new cancer cell detection tool, which uses artificial effusions, showed a promising result that urged us to check its utility in the clinical setting. A similar experiment was performed with body fluid samples obtained from cancer patients. Table 1 shows the clinical background of each sample, including the primary malignant disease, pleural effusion or ascites, results

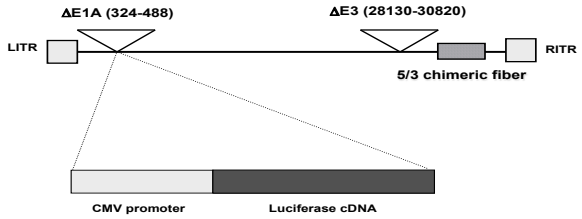

Figure 1. Schematic Diagram of 5/3 Chimeric Adenovirus Construction. This vector was constructed from an E3 region-deleted Ad5 backbone, does not contain the Ad E1A promoter region (324 bp–488 bp of the Ad genome), and has modified fiber genes that contain an Ad5 shaft region and Ad3 knob region (647 bp–1208 bp of accession no. X01998 M12411).

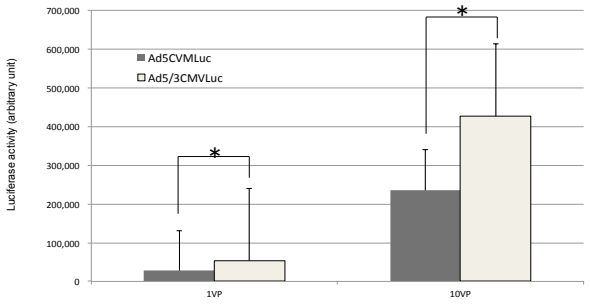

Figure 2. Luciferase Assay after Infection by Ad5 and Ad5/3 Viruses. NCI-H1299 cells were infected with equal amounts (1 vp/cell or 10 vp/cell) of Ad5Luc or Ad5/3Luc. Approximately 48 h later, luciferase activity was measured. The graph represents the average of triplicate samples. The average background luciferase activity was subtracted from all experimental values. Difference was assessed using unpaired t test; *P < 0.05

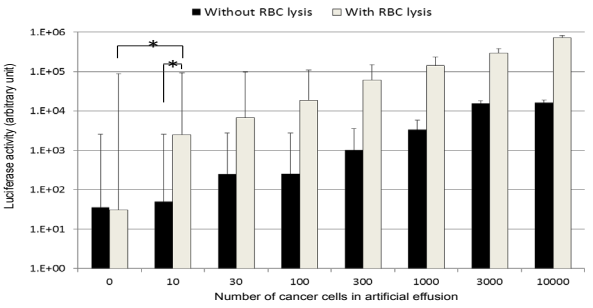

Figure 3. Luciferase Activities in Artificial Malignant Effusions Containing Various Numbers of Cancer Cells. After the removal of RBCs, adenovirus efficiently infected cancer cells and expressed 1- to 2-log unit higher luciferase activity in each sample. Difference was assessed using unpaired t test; **P <0.05, *P < 0.001

Table 1. Luciferase Activities in Body Fluid Samples and their Clinical ackground

Sample#	Primary disease	Effusion	Cytological diagnosis	Clinical diagnosis	Luciferase activity
1	Ovarian cancer	Ascites	Positive	Malignant	9691850
2	Ovarian cyst	Ascites	Negative	Benign	8392006
3	Esophageal cancer	Ascites	Positive	Malignant	4973694
4	Lung cancer	Pleural effusion	Positive	Malignant	3367039
5	Hepatic caner	Ascites	Negative	Malignant	1781689
6	Colon cancer	Ascites	Negative	Malignant	1502799
7	Ovarian cancer	Pleural effusion	Negative	Malignant	345152
8	Lung cancer	Pleural effusion	Negative	Malignant	323605
9	Breast cancer	Pleural effusion	Positive	Malignant	100029
10	Dermoid cyst	Ascites	Negative	Benign	30406
11	Lung cancer	Pleural effusion	Positive	Malignant	3636
12	CML	Pleural effusion	Positive	Malignant	1330
13	Ovarian cyst	Ascites	Negative	Benign	666
14	Pnemonia	Pleural effusion	Negative	Benign	524
15	Colon cancer	Ascites	Negative	Benign	488
16	Pulmonary tuberculosis	Pleural effusion	Negative	Benign	235
17	Heart Failure	Pleural effusion	Negative	Benign	166
18	Lung cancer	Pleural effusion	Suspicious	Benign	65
19	Heart Failure	Pleural effusion	Negative	Benign	63
20	Liver chirosis	Ascites	Negative	Benign	56
21	Renal Failure	Pleural effusion	Negative	Benign	52
22	Burkitt lymphoma	Pleural effusion	Positive	Malignant	45
23	Malignant mesothelioma	Pleural effusion	Negative	Malignant	37
24	Rectal cancer	Ascites	Negative	Benign	30
25	Pneumonia	Pleural effusion	Negative	Benign	23

of cytological examination, malignant or benign status based on clinical decision, and luciferase activity. Twelve samples were found to be clinically malignant, while 5 were negative. Of the 12 clinically malignant samples, 7 were cytologically positive, in which showed a sensitivity of 58% with the conventional method. However, 9 samples were found to be positive by using the new method, when 2,474 arbitrary units in the luciferase activity was set as the cutoff point based on the result obtained with artificial effusions. Out of the 13 clinically negative samples, 12 samples were cytologically negative and 1 was unclear (pseudo-positive). By using the new method, 11 samples were found to be negative while 2 were positive. The sensitivity and specificity of the new method was 75% and 79% (p=0.006: chi-square test) respectively, which was better than that of the conventional cytological examination. Therefore, this new method showed better sensitivity and exhibits some improvement in specificity compared to conventional cytological examination.

Discussion

The diagnosis of a malignant disease is confirmed by pathological examination of clinical samples obtained from patients. Cytological examination is widely used because of the ease of collecting cells from the involved area. However, the diagnostic yield of cytological examination is unsatisfactory. Diagnostic accuracy of effusion cytology depends on the volume of liquid examined, the type of preparation and staining, the experience of the examiner, and the number of sufficient specimens investigated. However, the cytological interpretation of fluids can be challenging, and its diagnostic accuracy is limited (Garcia-Bonafe and Moragas, 1996). To supplement the morphological examination of doubtful cytological effusions, immunocytochemistry and a variety of molecular methods have frequently been applied (Motherby et al., 2002; Davidson, 2004; Sriram et al., 2011). However, the sensitivity and specificity of these techniques were not superior to current immunocytochemistry (Motherby et al., 1999; Sriram et al., 2011).

As expected, Ad5/3 chimeric vector showed good performance in cancer cell detection and better sensitivity compared to conventional cytological examination. This technique is highly reliable for body fluids and requires prior RBC lysis for blood samples. One of the limitations of this technique is decreased infectivity due to RBC interference, probably due to some interactions with RBC in the blood samples (Lyons et al., 2006). To avoid RBC interference, RBCs contained in samples must be lysed prior to viral infection. This additional procedure entails extra work for the cytologist. Another feature of this method is low infectivity towards malignant cells derived from non-epithelial tissue. As shown in Table 1, there were 3 pseudo-negative samples (No. 12, 22, and 23), showing very low luciferase activities despite the presence of many malignant cells in the samples. Interestingly, the primary diseases of these 3 pseudo-negative samples were leukemia, lymphoma, and mesothelioma, respectively. This finding may suggest a low infectivity of adenovirus in non-epithelial cells. Therefore, this method is suitable only for the detection of cancer cells derived from epithelial tissues.

Two samples that were found to be cytologically negative showed a high luciferase activity (pseudo-positive). This could be because of a dermoid cyst (Sample 10), which is a cystic teratoma that contains developmentally mature skin, complete with hair follicles

and sweat glands, sometimes clumps of long hair, and often pockets of sebum, blood, fat, bone, nails, teeth, eyes, cartilage, and thyroid tissue. We speculate that some epithelial cells exuded from the cyst into the ascites, and were infected by the adenovirus. In the case of sample 2, it is not clear that the ovarian cyst produced ascites; however, it could be similar to sample 10. Such pseudo-positive reaction to cystic tumor needs to be explored in our future studies. In the case of sample 11, the luciferase activity is low because the number of cancer cells in effusion is very low at the cytologic examination. In the current study, if a clinical condition suggested malignancy, we recommended a cytology re-examination. In addition, the low sample numbers used in this study may limit the validity of the results; a higher number of samples will be used for future studies.

In this method, the luciferase gene was driven by the CMV promoter, enabling nonspecific luciferase expression in normal as well as in malignant cells. Because floating cells in ascites and pleural effusions are mainly from blood or mesothelial tissues, there may be few epithelial cells in addition to cancer cells. Therefore, ascites and pleural effusion are suitable for this method. However, lavage fluid, such as bronchial washing solution, contains many normal epithelial cells. These normal cells deteriorate the signal-noise ratio by nonspecific gene expression. To improve expression in cancer cells specifically, tumor-specific promoters are preferred to the CMV promoter. As a part of our future studies, we will examine the specificity with the tumor-specific promoter.

In this study, we focused on efficient adenovirus-mediated gene transfer into epithelial cells and demonstrated the usefulness of the adenoviral vector as a cancer cell detection tool in malignant effusions. Although there is room for improvement of this method in the clinical setting, they are surmountable hurdles. In any case, the technique used in this study is definitely useful to detect malignancy in cytologically negative effusions. Tumor cell detection in effusions can be significantly improved by combining this technique with standard cytology. This finding should help to improve tumor diagnosis and staging.

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