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Detection of extracellular RNAs in cancer and viral infection via tethered cationic lipoplex nanoparticles containing molecular beacons

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Supporting Information

Materials and methods, design of molecular beacons, additional CryoTEM images, detailed image analysis and additional fluorescent microscope images of samples. This material is available free of charge at <http://pubs.acs.org>.

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Abstract

Non-invasive early detection methods have the potential to reduce mortality rates of both cancer and infectious diseases. Here, we present a novel assay by which tethered cationic lipoplex nanoparticles containing molecular beacons (MBs) can capture cancer cell-derived exosomes or viruses, and identify encapsulated RNAs in a single step. A series of ultracentrifugation and Exoquick™ isolation kit were first used to isolate exosomes from the cell culture medium and human serum respectively. Cationic lipoplex nanoparticles linked onto the surface of a thin glass plate capture negatively charged viruses or cell-secreted exosomes by electrostatic interactions to form larger nanoscale complexes. Lipoplex/virus or lipoplex/exosome fusion leads to the mixing of viral/exosomal RNAs and MBs within the lipoplexes. After the target RNAs specially bind to the MBs, exosomes enriched in target RNAs are readily identified by the fluorescence signals of MBs. The *in situ* detection of target extracellular RNAs without diluting the samples leads to high detection sensitivity not achievable by existing methods, e.g. qRT-PCR. Here we demonstrate this concept using lentivirus and serum from lung cancer patients.

Keywords

lipoplex nanoparticles; exosome; extracellular RNA; cancer detection; viral infection

Introduction

Given their important role in regulating gene expression and the recognition that their dysfunction plays a casual role in human cancers, messenger RNAs (mRNAs) and microRNAs (miRNAs) have emerged as potential biomarkers for cancer detection.¹⁻⁴ The stability of extracellular RNAs in blood and other bodily fluids is partially attributable to their encapsulation in cell-secreted vesicles, so-called extracellular vesicles (EVs) that are consisted of exosomes and microvesicles.⁵⁻⁸ Thus, capturing these EVs and quantifying the encapsulated miRNAs and mRNAs has become a promising approach to achieving non-invasive detection of cancer biomarkers. Although miRNAs and mRNAs have been quantitatively measured in human serum by qRT-PCR⁹, existing approaches to EV capture and RNA isolation/concentration have proven to be expensive and time consuming¹⁰. More importantly, these approaches quantify target RNAs from EVs secreted by all mammalian cells. Since cancer cell-derived EVs represent only a small fraction of the EV population in circulation, these approaches lack sensitivity for biomarker detection.

Many infectious diseases and some cancers have been linked to viral infections.^{11, 12} Current methods for detecting viral infections, which rely on antibodies against the virus or

the presence of viral genetic material, are tedious.^{13, 14} It may also take several days for those antibodies to appear.¹⁵ Thus, the development of a simple detection method for capturing and identifying virus for early warning of infection is a desirable clinical goal.

Here, we describe a new technology termed tethered cationic lipoplex nanoparticle (tCLN) biochip and demonstrate the simultaneous capture of exosomes and quantification of target miRNAs and mRNAs in the serum of lung cancer patients and lentivirus. Fig. 1 shows the concept. Serum can be isolated from the whole blood and then applied directly on the tCLN biochip. Molecular detection probes, such as molecular beacons (MBs), are encapsulated in the cationic lipoplex nanoparticles which can capture negatively charged exosomes secreted by cells via electric static interactions to form a larger nanoscale complex. This lipoplex-exosome fusion leads to mixing of exosomal RNAs and MBs within the nanoscale confinement near the biochip interface. Fluorescence signals of MBs after their binding to target RNAs are observed by the total internal reflection fluorescence (TIRF) microscopy, which is capable of detecting a single biomolecule within <300 nm distance from the interface. Lung cancer is the number one cause of cancer related deaths.¹⁶ In lung cancer, elevated circulating levels of miR-21 have been shown to distinguish patients with malignant solitary pulmonary nodules from those with benign lesions^{17, 18}, while Thyroid Transcription Factor-1 (TTF-1) is a well-recognized biomarker in lung cancer^{19, 20}. Therefore, we selected miR-21 and TTF-1 mRNA as model biomarkers to test our novel assay compared to widely used qRT-PCR method in lung cancer detection. We also demonstrate the feasibility of tCLN device in detecting viral RNAs using lentivirus as the model virus.

Experimental Section

Materials

1,2-Di-O-octadecenyl-3-trimethylammonium propane (chloride salt) (DOTMA) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[biotinyl (polyethylene glycol)-2000] (ammonium salt) (Biotin-DSPE-PEG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol, β -Mercaptoethanol (β ME) and 3-mercaptopropyl-trimethoxysilane (MPTMS) were purchased from Sigma-Aldrich (St. Louis, MO). Synthesis of the lipidic anchor molecule WC14 (20-tetradecyloxy-3,6,9,12,15,18,22-heptaohexatricontane-1-thiol) was described previously.²¹ The biotinylated alkane thiol (biotin-SH), 5-[2-oxohexahydro-1H-thieno [3,4]imidazol-4-yl]-*N*-(29-mercaptop-3,6,9,12,15,18-hexaoxonacosyl) pentanamide, was purchased from Nanoscience Instruments (Phoenix, AZ). Molecular beacons (MBs) were custom synthesized by Sigma-Aldrich (St. Louis, MO) and TIB MOLBIOL, LLC (Adelphia, NJ). See Supporting Information (SI)-A for details.

Prepare cationic lipoplex nanoparticles containing MBs

Cationic lipoplex nanoparticles containing MBs were prepared by injecting MBs/lipids mixture to PBS. See SI-A for details.

Fabrication of tCLN biochip

Mixed thiol self-assembled monolayers (SAMs) as an anchoring membrane were formed on Au layers as described previously.²² Then the glass slide was incubated with an avidin derivative (NeutrAvidin, Thermo Scientific, Waltham, MA) at room temperature for 5 min. Cationic lipoplex nanoparticles containing MBs were tethered onto the glass slide surface by biotin-avidin linkage. See SI-A for details.

Cell culture

Non-small cell lung cancer A549 cells and normal human bronchial epithelial cells (HBEC) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in RPMI 1640 media (Invitrogen, 11875119, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, 16000, Carlsbad, CA). HBEC cells were cultured in 1% gelatin coated 10 cm petri-dish containing 10 mL Keratinocyte-SFM media (Invitrogen, 17005-042, Carlsbad, CA).

Isolation of exosomes secreted by A549 and HBEC cells

A549 and HBEC cells were cultured in cell culture medium containing serum until they reached 80% confluence. The cell culture medium containing serum was removed. The cells were washed with PBS once and then cultured in cell culture medium containing no serum for 48 hours. The exosomes were recovered in a series of centrifugation/ultracentrifugation as described in detail elsewhere.¹⁰ See SI-A for details.

Isolation of exosomes from human serum

ExoQuick™ exosome precipitation solution (System Biosciences, EXOQ5A-1) was used to isolate exosomes from human serum samples. See SI-A for details.

Asymmetric Flow Field Flow Fractionation and Dynamic Light Scattering

Size distributions of cell-secreted exosome were determined using asymmetric flow field flow fractionation coupled with multi-angle static light scattering (A4F-MASLS, Wyatt Technology Corporation Santa Barbara, CA) and Nano Zetasizer Zen3600 dynamic light scattering (DLS, Malvern Instruments Ltd., Worcestershire, United Kingdom). Absolute numbers of exosomes and larger microvesicles secreted per cell were quantified by A4F-MASLS. See SI-A for details.

Cryo-Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM (FEI Tecnai G2 F20 ST TEM, Hillsboro, OR) was used to characterize CLNs, exosomes from cell lines and patient samples, and complexes of CLNs fused with A549 exosomes. See SI-A for details.

Biological Atomic Force Microscopy (Bio-AFM)

Bio-AFM images were acquired in the PBS buffer solution using a MFP-3D-Bio-AFM (Asylum Research, Santa Barbara, CA) with an iDrive Magnetic Actuated Cantilever holder. See SI-A for details.

Total Internal Reflection Fluorescence (TIRF) Microscopy

Samples were added on tCLN biochip and incubated at 37°C for 2 h. TIRF microscopy (Nikon Eclipse Ti Inverted Microscope System) was used to detect the miR-21 and TTF-1 fluorescence signals from the samples. See SI-A for details.

Image analysis

Image J and MATLAB software was used to statistically analyze the images by generating a binary mask to remove the background and measure the sum intensity of each image. In this study, PBS controls and scramble MB controls were used to define the background. Any fluorescence signals from the samples that were equal or lower than the signals observed in PBS controls and scramble MB controls were defined as background in the image analysis. See SI-A for more details.

qRT-PCR measurement of target RNA expression

The expression of miR-21 and TTF-1 mRNA in exosomes was measured using qRT-PCR following the previous published protocol.⁹ See SI-A for details.

Preparation of microRNA-181a encoding lentivirus and negative control virus

The lentivirus construction was previous described.²³ See SI-A for details.

Results

Overview of tCLN technology

The tCLN technology allows for the simultaneous capture of EVs and the *in situ* analysis of encapsulated RNA targets in a single step without pre- or post-treatment of the samples. As shown in Figure 1a, the serum can be isolated from the whole blood and then applied directly on the tCLN biochip (Figure 1b), which is placed on a Total Internal Reflection Fluorescence (TIRF) microscope. Molecular beacons (MBs) are encapsulated in the CLN, which capture the negatively charged exosomes via electrostatic interactions to form larger complexes. Lipoplex- exosome fusion (Figure 1c) leads to the mixing of exosomal RNAs and MBs within the nanoscale confinement of the complexes near the biochip interface. Given its high sensitivity and near-interface (~300 nm) detection, TIRF microscopy coupled with our tCLN technology is an ideal modality for detecting RNAs or other genetic materials within the tethered nanoparticles.

We have developed a simple method for preparing the tCLN biochip (Figure 1d). The CLN containing MBs were tethered on the substrate surface through biotin-avidin interactions. AFM image shows that the mean diameter of CLN is ~100 nm. MBs are oligonucleotide hybridization probes that can identify the presence of specific nucleic acids. To achieve high stability, locked nucleic acid (LNA) enhanced MBs and nuclease resistant MBs were used to detect specific miRNAs and mRNAs, respectively. (See SI-A and Figure S1 for the design of MBs and Figure S7 for the signal to noise ratio analysis of MBs.)

Characterization of exosome secretion and fusion with tCLN

We used TIRF microscopy to visualize the secretion of exosomes by A549 lung cancer cells and their capture by tCLN in a live-cell imaging assay (Figure 2a). We observed fluorescent signals from the miR-21-specific MBs from the exosomes released by A549 cells. We also observed fluorescent signals from the miR-21-specific MBs inside the A549 cell cytoplasm (Figure 2b), suggesting that the tCLN biochip can detect miR-21 in both exosomes and cells. After CLN are internalized by the cells, the subsequent release of the MBs leads to the detection of target intracellular RNAs. In normal HBEC cells, we mainly observed green fluorescent signals from miR-21-specific MBs inside the cells. Compared with A549 cells, HBEC cells secreted much fewer miR-21 containing exosomes.

Using A4F-MASLS and DLS, we measured the number and size distribution of exosomes and larger microvesicles secreted by the two cell types (Figures 2c and 2d). A4F7 MASLS measurements indicate that A549 cells secrete 30–50 times more exosomes and 2–4 times more microvesicles compared to HBEC cells over 48 h, while the mean diameter by number of A549 exosomes is smaller than that of HBEC.

Bio-AFM measurements of nanoparticle sizes before and after exosomes fusion with CLN (Figure 2e) shows the number of nanoparticles remained unchanged, but the average diameter of the fused complexes was 2.5 times that of the CLN before fusion, suggesting that each CLN captured >10 exosomes. Since the multi-layered CLN contain more cationic lipids (with high zeta potential) than the phospholipids comprising the exosomes, a single lipoplex nanoparticle is able to capture many exosomes.

tCLN detection of miRNA and mRNA in cell culture medium

Exosomes collected from A549 and HBEC cell culture medium were applied to the tCLN biochip containing both miR-21-specific and TTF-1 mRNA-specific MBs. After incubation at 37°C for 2 h, TIRF microscope was used to take images. As shown in Figure 3a, the A549 exosomes revealed much higher miR-21 and TTF-1 fluorescence signals compared to those for HBEC exosomes. Image analysis of fluorescence intensity distributions shows that more A549 exosomes have higher miR-21 and TTF-1 abundances than HBEC exosomes (Figures 3b and 3c, see SI-A Figure S6 for detailed image analysis). The sum of fluorescence intensity in A549 exosomes relative to HBEC exosomes (Figures 3d and 3e) confirms that tCLN and qRT-PCR provide comparable results for miR-21 detection. TTF-1 mRNA in the exosomes was not detected by qRT-PCR (Figure 3e), but was clearly detected using the tCLN biochip. Since the population of exosomes secreted from a cell line is very uniform in fluorescence intensity, a single $80\ \mu\text{m} \times 80\ \mu\text{m}$ image containing $\sim 10^5$ CLN ($\sim 10^6$ exosomes) is sufficient to provide a consistent result. Figure 3d shows that the variation among 100 images is small (more images in SI-B Figure S7).

tCLN detection of miRNA and mRNA in lung cancer patient serum and virus

We then tested the tCLN biochip using serum samples from 2 normal donors and 7 lung cancer patients (See SI-A Table S1). Exosomes isolated from the serum samples using ExoQuick™ exosome precipitation solution were applied on the tCLN biochip and incubated at 37°C for 2 h.

As shown in Figure 4a, the miR-21 and TTF-1 fluorescence signals were stronger in the patient samples compared to normal donors (See SI-C Figures S8-11 for more images). The fluorescence intensity distributions also indicated that patient samples have more exosomes with higher miR-21 and TTF-1 abundance than samples from normal donors (Figure 4b). Generally, patients diagnosed with late stage lung cancer, such as patient 9P (stage IV), showed higher expression of miR-21 and TTF-1 mRNA than patients diagnosed with early stage lung cancer, such as patient 3P (stage IB) (Figure 4c). Detailed patient information is given in Supporting Information Table S1. Selected serum samples were also directly applied on the tCLN biochip without an exosome isolation step. Higher miR-21 and TTF-1 abundance in the patient serum samples was similarly expressed without exosome isolation (See SI-A, Figure S4).

Exosomes in human serum samples come from various cell types. Consequently, more images (i.e. more exosomes) are required to provide a meaningful average signal (Figure 4c). However, the trend between patient and normal donor samples remained the same when we used fewer images for the analysis (see SI-A and Figure S4). In qRT-PCR, total RNA is isolated from all exosomes in a sample. Thus, RNA from disease-specific exosomes are mixed with exosomes from a myriad of other cell sources, and diluted in the RNA isolation steps, thereby diminishing the sensitivity of target RNA detection (Figure 4d).

The tCLN biochip was also demonstrated for lentivirus detection. MiR-181a MBs were encapsulated in tCLN to detect miR-181a encoding mRNA in lentivirus. The miR-181a encoding lentivirus was applied on the biochip and incubated at 37°C for 2 h. Green fluorescence from miR-181a MBs demonstrated the successful capture and detection of this specific virus (Figure 4e), while the negative control virus showed little fluorescent signals, demonstrating for the first time, the direct capture and characterization of virus in one step.

Detection sensitivity comparison between tCLN and qRT-PCR

To compare the detection sensitivity of tCLN assay and qRT-PCR, we spiked exosomes secreted from A549 cells into exosomes isolated from the normal donor serum. As shown in Figure 5, qRT-PCR measures miR-21 expression in exosomes secreted by all types of cells, not limited to cancer cells, therefore it is insensitive until exosomes from 10^7 tumor cells were spiked in normal donor's exosomes. The tCLN assay can detect the spiked exosomes secreted from $<2 \times 10^4$ tumor cells when high cut off level was used in image analysis in order to collect miR-21 signals only from cancer cell secreted exosomes. When low cut off level was used in image analysis to collect the miR-21 signal from all types of cells, we got similar results as qRT-PCR. Comparing Figures 4c and 5, we speculate that the miR-21 and TTF-1 signals observed in lung cancer patients #3 (early stage, stage IB) and #9 (late stage, stage IV) come from the exosomes secreted by $\sim 2 \times 10^4$ and $\sim 10^7$ cancer cells respectively.

Discussion

We have developed a non-invasive and highly sensitive tCLN biochip that simultaneously captures exosomes/lentivirus and quantifies the encapsulated target miRNA and mRNA in a single step. In our tCLN biochip, the exosomes/lentiviruses are fused with CLN by electrostatic interactions, which in turn lead to the mixing of target miRNAs and mRNAs

with MBs, and thus produce fluorescent signals. (Figure 1a) Images from Cryo-TEM (Figures 1b and S2) and TIRF microscopy (Figure 2b) revealed that exosomes secreted by A549 cells and exosomes identified in lung cancer patient serum contain more material than most exosomes secreted by HBEC cells, suggesting that the over-representation of target RNAs in exosomes secreted from cancer cells represents a sensitive method for cancer detection.^{3, 4}

First, we have demonstrated the feasibility of the tCLN biochip by using exosomes secreted from both A549 lung cancer cells and normal HBEC cells. A549 cells produce more miR-21 rich exosomes than normal HBEC cells (Figure 2). Thus, the tCLN biochip confirmed much higher miR-21 fluorescence signal in A549 exosomes compared to HBEC exosomes, which is consistent with widely used qRT-PCR method (Figure 3). In addition, the tCLN biochip also showed higher TTF-1 mRNA expression in A549 exosomes compared to HBEC exosomes, demonstrating higher sensitivity than qRT-PCR, which was unable to detect TTF-1 mRNA.

The tCLN biochip provided even higher sensitivity in detecting miR-21 and TTF-1 mRNA in human serum samples. (Figures 4a–d) Unlike exosomes isolated from cell culture medium, where exosomes come from single source (i.e. A549 cells or HBEC cells), exosomes in serum samples are secreted by many types of cells. As shown in Figure 5, by using tCLN, the exosomal content is confined within the lipoplex-exosome complex, which provides a “focusing” effect and circumvents dilution. In qRT-PCR, however, the RNA extraction step breaks up all exosomes and therefore the target RNAs in cancer cell-derived exosomes are significantly diluted by RNAs from the exosomes secreted by other cells. In addition, RNA detection by tCLN biochip is based on analyzing images in which each exosome-lipoplex cluster can be quantitatively evaluated individually for target RNA abundance above a specified cutoff level. This focusing effect can better distinguish potential cancer cell-derived exosomes from other exosomes because the former is more likely to contain many target RNAs leading to a strong local fluorescence signal. To demonstrate this ‘focusing’ effect by tCLN, we spiked exosomes isolated from A549 cell culture medium into exosomes collected from a normal donor's serum. Both tCLN and qRT-PCR were then used to characterize the miR-21 and TTF-1 expression in the spiked samples. The tCLN biochip could detect the spiked exosomes secreted from $< 2 \times 10^4$ tumor cells when high cut off level was used in image analysis to collect signals only from cancer cell derived exosomes, while qRT-PCR was insensitive below 10^7 tumor cells. In addition, the tCLN biochip was also a flexible tool for identifying RNA targets in virus, which were demonstrated by the successful detection of miR-181a encoding RNA in lentivirus. (Figure 4e).

Conclusions

In summary, the tCLN biochip is a novel, non-invasive and highly sensitive method for detecting low-level RNA targets that are important for early diagnosis of cancer and other diseases. It can be extended to a multiplexing array design in which specific MB mixtures (miRNAs, mRNAs) can be spatially separated on the biochip to allow for the detection of multiple targets for RNA profiling. The tCLN biochip holds great potential as a tool for EV

detection and characterization and potentially assisting in biomarker development for cancer detection, treatment response and surveillance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. tCLN technology overview. **(a)** Exosomes in serum are captured on the tCLN biochip. Exosomal miR-21 in lung cancer patient serum is identified using TIRF microscopy. **(b)** A typical 4-well tCLN biochip for simultaneous detection of 4 samples. **(c)** Cryo-TEM images show typical structures of an exosome from lung cancer patient serum, a cationic lipoplex nanoparticle (CLN) and the fusion between an exosome and a CLN. (More images in SI-A Fig. S2). **(d)** Schematic diagram of tCLN biochip fabrication. The CLN containing MBs were tethered on the substrate surface through biotin-avidin interactions. The AFM image shows that the mean diameter of CLN is ~100 nm.

Figure 2.

Characterization of exosome secretion and fusion with tCLN. **(a)** Schematic diagram shows the tCLN interactions with cells and cell secreted exosomes. **(b)** tCLN and TIRF microscopy detect the presence of miR-21 in A549 and HBEC cells, and their secreted exosomes 2 h after the cells were applied on the tCLN biochip containing miR-21-specific MBs. The red arrows point to miR-21 detected in exosomes, and the yellow arrows point to miR-21 detected in the cells. Clearly, there are more miR-21 rich exosomes secreted by A549 cells. **(c, d)** A4F-MASLS and DLS measurements show A549 cancer cells secrete more and smaller exosomes than normal HBEC cells. **(e)** Bio-AFM images show the average diameter

of the fused exosome-lipoplex particles is 2.5 times that of the original lipoplex nanoparticles before fusion.

Figure 3.

Comparison of tLCN and qRT-PCR for miRNA and mRNA detection in cell culture medium. **(a)** TIRF microscopy images of miR-21 and TTF-1 mRNA expression in A549 and HBEC exosomes. Minimal fluorescent signal was observed in scramble miR-21 MBs and scramble TTF-1 MBs as expected. **(b, c)** Fluorescence intensity distributions of both miR-21 and TTF-1 analyzed based on 100 images show that more A549 exosomes have higher miR-21 and TTF-1 expression than HBEC exosomes. **(d, e)** Using a low cutoff level, the tLCN biochip provided results similar to qRT-PCR for miR-21 detection but more sensitive in detecting TTF-1 mRNA. (See SI-A Fig. S3a for details) For tLCN, the fluorescence intensity of a single 80 μm × 80 μm image was summed and 100 images were used to calculate the average intensity and variation. (n=1, u=100n)

Figure 4.

Use of tCLN and qRT-PCR for miRNA and mRNA detection in human serum and virus. **(a)** A typical set of TIRF microscopy images of miR-21 and TTF-1 fluorescent signals from exosomes isolated from human serum samples. **(b)** Fluorescence intensity distributions of miR-21 and TTF-1 analyzed based on 1,024 images and a high cutoff level show that more exosomes isolated from patient serum samples have higher miR-21 and TTF-1 expression than normal donors. **(c)** Statistical results of tCLN experiments show higher expression of miR-21 and TTF-1 in lung cancer patient samples than in normal donor samples. The fluorescence intensity on 32 $80\text{ }\mu\text{m} \times 80\text{ }\mu\text{m}$ images is summed and averaged and 1,024 images (32 by 32) were used to calculate the average intensity and variation. (n=32, u=32n) **(d)** qRT-PCR was not sensitive enough to distinguish lung cancer patients from normal donors. For tCLN with a low cutoff level, similar results were observed. (See SI-A Fig. S3b) **(e)** tCLN detects miR-181a encoding mRNA in lentivirus, but not in negative control virus. (N: normal donors; P: patients)

Figure 5.

The tCLN biochip provided higher detection sensitivity than qRT-PCR. **(a)** In qRT-PCR, the RNA extraction step breaks up all exosomes and therefore the target RNAs in cancer derived exosomes are significantly diluted by RNAs from the exosomes secreted by other cells. **(b)** In tCLN the exosomal content is confined within the lipoplex-exosome complex, which provides a “focusing” effect and circumvents dilution. RNA detection by tCLN biochip is based on analyzing images in which each exosome-lipoplex cluster can be quantitatively evaluated individually for target RNA abundance above a specified cutoff level, which better distinguishes potential cancer cell-derived exosomes from other exosomes. The tCLN biochip could detect the spiked exosomes secreted from $< 2 \times 10^4$ tumor cells, while qRT-PCR was insensitive below 10^7 tumor cells.