

Quantitative Analysis of Exosomes From Murine Lung Cancer Cells by Flow Cytometry

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In vivo studies regarding biochemical, molecular biological, and histopathological changes in cancer tissues have been widely performed by the administration of carcinogens in rodents. In these established methods, dissection of the animal following sacrifice must be carried out. Exosomes are cell-derived vesicles that are present in all body fluids and these vesicles have specific roles within cells. Thus, much attention is given to the clinical application of exosomes that can possibly be used for prediction and therapy and as biomarkers related to cancer. To develop a new tool for monitoring in vivo genetic alterations, as a result of carcinogenesis, without the need for frequent euthanasia, we performed quantitative measurement of exosomes in Mlg2908 murine lung fibroblasts and LA-4 and KLN 205 murine lung cancer cells using fluorescence-activated cell sorting. We detected an increase in CD63-specific exosomes in LA-4 lung cancer cells. This result is able to be applied to the classification of cancer-specific proteins and miRNA as diagnostic markers.

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Key Words: Quantitative, Analysis, Exosomes, Flow cytometry, Lung

INTRODUCTION

Tumor growth and metastasis are dependent on a complex interplay between tumor cells and tumor stroma, as well as tumor-tumor interactions. This communication occurs through direct interaction, secreted factors, and microvesicles. The tumor microvesicles like exosomes carry a wide range of cargo, including oncogenic proteins, fragmented DNA, mRNA, and non-coding RNA, each with regulatory functions that can be transferred between different cells. Increasing attention has been focused on the role of exosomes in malignant progression and metastasis. Because the exosomes are cell-derived vesicles displaying various proteins on their membrane surfaces, they are readily available in blood samples where they constitute potential cancer biomarkers.¹

Extracellular vesicles (EVs) are small membranous structures

with variable sizes (from 30 nm to a few μm) and released from any type of cell into the extracellular space.² EVs are detectable in body fluids and responsible to several physiological and pathological processes like inflammation and cancer.³ The term "exosome" was coined by Trams et al. in 1981⁴ for "exfoliated membrane vesicles with 5'-nucleotidase activity." This term originated from the discovery of the secretion of neoplastic cell line-derived exfoliated vesicles (within larger vesicles), which mirrored the 5'-nucleotidase activity of the parent cells⁵ and rat reticulocytes.⁶ Following purification by ultracentrifugation, the sedimented microvesicles were found to contain the transferrin receptor in native reticulocytes.⁷ The exosomes can be taken up by different types of cancer cells; however, the potential functional effects of mast cell exosomes on tumor cells remain unknown.⁸ EVs are identified as useful tools for cellular communication in various diseases. Their formation and miRNA

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content have the ability to transfer biological information to other cancer metastasis-related cells.⁹ miRNAs harvested from body fluids allows for the development of liquid biopsies that miRNAs as circulating biomarkers can be analyzed *ex vivo*.¹⁰ However, it has limitations in the methods for obtaining high yields of pure exosomes from body fluids.¹¹

In the last few years, the scientific community has started to use the term "liquid biopsy". A liquid biopsy is a liquid biomarker that can be easily isolated from many body fluids (blood, saliva, urine, ascites, and pleural effusion).^{12,13} In this study, we were interested in circulating tumor cells, circulating tumor DNA, exosomes, with the aim of demonstrating their usefulness and potential application for lung cancer diagnosis and monitoring of treatment responses.

To perform exosomal analysis, it is necessary to develop useful methods by which extract and analyze exosomes from target biofluids without damaging the internal contents.¹⁴ We attempted to develop new protocols with which to quantify exosomes, and thus detect carcinogenesis *in vitro* via measurement using fluorescence-activated cell sorting (FACS). FACS detects the fluorescence emitted from flowing samples and can be used to measure a variety of information related to each cell. It can quickly detect the fluid state of cells as they pass through the detection area (sensing point) and measure simultaneously several features of the cells, including size, intracellular composition, and function. In addition, it can separate only a certain cell type.

MATERIALS AND METHODS

1. Cell lines and culture

Murine Mlg 2908 lung fibroblasts and KLN 205 lung squamous cell carcinoma cells were purchased from American Type Culture Collection (Manassas, VA, USA). LA-4 murine lung adenoma cells were obtained from Korean Cell Line Bank (Seoul, Korea). Mlg 2908 and KLN 205 cells were cultured Eagle's minimum essential medium supplemented with 10% FBS (HyClone Laboratories, South Logan, UT, USA), and LA-4 cells were cultured in Ham's F12K medium (Life Technologies, Grand Island, NY, USA). For exosome isolation, exosome free FBS were used.

2. Exosome isolation and purification

Exosomes were isolated and purified with exosome isolation reagent (ExoQuick-TC; System Biosciences, Palo Alto, CA, USA) and exosome purification kit (Exo-FLOW; System Biosciences) according to the manufacturer's guide (System Biosciences).¹⁵

The cultured cells were centrifuged at $3,000 \times g$ for 15 minutes to discard floating matter. Ten millimeters of supernatant was mixed with 2 mL exosome precipitation solution and incubated overnight with refrigeration. The exosome isolation reagent were added to cell culture media and the mixture was centrifuged at $1,500 \times g$ for 30 minutes to produce a white pellet of exosomes, followed by further centrifugation at $1,500 \times g$ for 5 minutes to remove the residual exosome isolation reagent. The exosome pellet was subsequently dissolved in 500 μ L buffer.

3. Preparation of magnetic beads and antibody immobilization on the beads

The bead slurry (40 μ L) was added to a 1.5-mL tube, incubated on a magnetic stand for 2 minutes, and then washed twice. The tube with bead slurry was removed from the magnetic stand and 10 μ L biotinylated capture antibody (CD9 and CD63) was added. After mixing, the tube was incubated on ice for 2 hours with gentle mixing by flicking every 30 minutes. Two hundred micrometers of wash buffer was added and gently flicked to mix. Following sample incubation on the magnetic stand for 2 minutes, the supernatant was discarded gently so as not to scatter the pellet. After removal of the sample from the magnetic stand, 500 μ L wash buffer was added and overturned several times. The sample was then washed 3 times in place on the magnetic stand, followed by the final addition of 400 μ L wash buffer to the capture antibody-beads.

4. Exosome capture

The separated exosomes were added to bead samples to a final total volume of 500 μ L and placed on a rotating shelf at 4°C overnight to capture. The tube was placed on a magnetic stand for 2 minutes and the supernatant was discarded gently so as not to scatter the pellet. After removal of the sample from the magnetic stand, 500 μ L wash buffer was added and the tube was gently flicked 2 to 3 times to mix. The samples were placed back on the magnetic stand and washed twice.

5. Exosome staining

Two-hundred forty micrometers of exosome stain buffer and 10 μ L Exo-fluorescein isothiocyanate (FITC) exosome staining solution were added to the beads and incubated on ice for 2 hours with gently flicking every 30 minutes to mix. After the sample incubated in magnetic stand for 2 minutes, the supernatant was discarded gently so as not to scatter the pellet. After removal of the sample from the magnetic stand, 500 μ L wash buffer was added and gently flicked to mix. The sample was washed 3 times

in place on the magnetic stand, followed by the addition of 300 µL wash buffer for FACS analysis, without vortexing.

6. Fluorescence-activated cell sorting analysis

The analyses were performed with FACS Canto II and FACS Diva software (ver. 8.0; BD Biosciences, Franklin Lakes, NJ, USA). The analytical conditions were: forward-scattered light (FSC), 500 nm; scattered light (SSC), 300 nm; and FITC, 420 nm; and more than 5,000 cells were counted.

RESULTS

1. Exosome quantification using fluorescence-activated cell sorting

Normal or cancer cell lines used in the present study are shown in Table 1. Counting the number of each vesicle made it possible to elucidate the proportion of certain vesicles among the total vesicles, and thus the amount of cells that we needed to obtain. Unusually, there were more exosomes secreted in normal cells (Mlg 2908) compared with two cancer cell lines. According to the average FITC-A, 1,670 exosomes were secreted in normal cells (Mlg 2908) compared with the two cancer cell lines (852 in KLN 205 and 1,046 in LA-4) in the case of CD9 antibody binding. With CD63 antibody binding, 1,784 exosomes were secreted in normal cells compared with the two cancer cell lines (845 in KLN 205 and 1,084 in LA-4) (Table 2). FITC indicated the increased exosome-specificity with CD63 in both Mlg 2908 and LA-4 cells with comparison of SSC and FSC (Fig. 1). SSC represents the degree of particle scattering in the cell, which is the complexity of the cytosol. FSC represents the degree of scattering by the outside of

the cell (size), and FITC represents the fluorescence signal relating to either CD9 or CD63 binding to detect a certain vesicle marker.

DISCUSSION

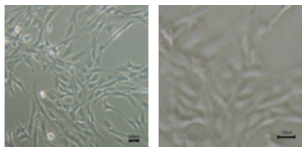
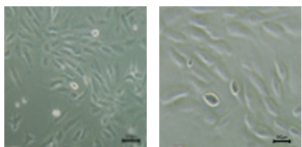
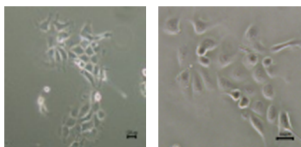
Since lung cancer is the main human malignancy and causes poor survival in patients with late diagnosis and resistance to classic chemotherapy, it is necessary to identify novel biomarkers for early detection. microRNAs (miRNAs) are considered to show

Table 2. Exosome quantification by FACS

Sample	#Events (n)	Parent (%)	FITC-A mean (n)
Negative control	8,237	15.2	611
CD9 Ab			
F-12K	4,989	25.4	708
MEM	5,029	20.0	753
Mlg 2908	5,072	12.6	1,670
KLN 205	5,016	20.5	852
LA-4	5,000	24.6	1,046
CD63 Ab			
F-12K	5,000	16.2	684
MEM	5,000	17.5	705
Mlg 2908	5,000	17.9	1,784
KLN 205	5,000	23.3	845
LA-4	4,813	10.4	1,084

The analyses were performed with a fluorescence-activated cell sorting (FACS) Canto II and FACSDiva ver. 8.0. The analytical conditions were: FSC, forward-scattered light (500 nm); SSC, scattered light (300 nm); FITC, fluorescein isothiocyanate (420 nm); and we attempted to count more than 5,000 cells. FITC represents the fluorescence signal relating to either CD9 or CD63 binding to detect a certain vesicle marker.

Table 1. Cell lines used in the present study

Variable	Name		
	Mlg 2908	LA-4	KLN 205
Mouse strain	ddY	A/He	DBA/2
Tissue	Lung	Lung	Lung
Disease	Normal	Adenoma (urethane induced)	Squamous cell carcinoma
Morphology ^a	Fibroblast	Epithelial	
			
Growth properties	Adherent	Adherent	Adherent
Cytogenetic analysis	Modal number = 64; range = 51 to 76	Unstable karyotype, range = 38 to 256	-

^aScale bar = 100 µm.

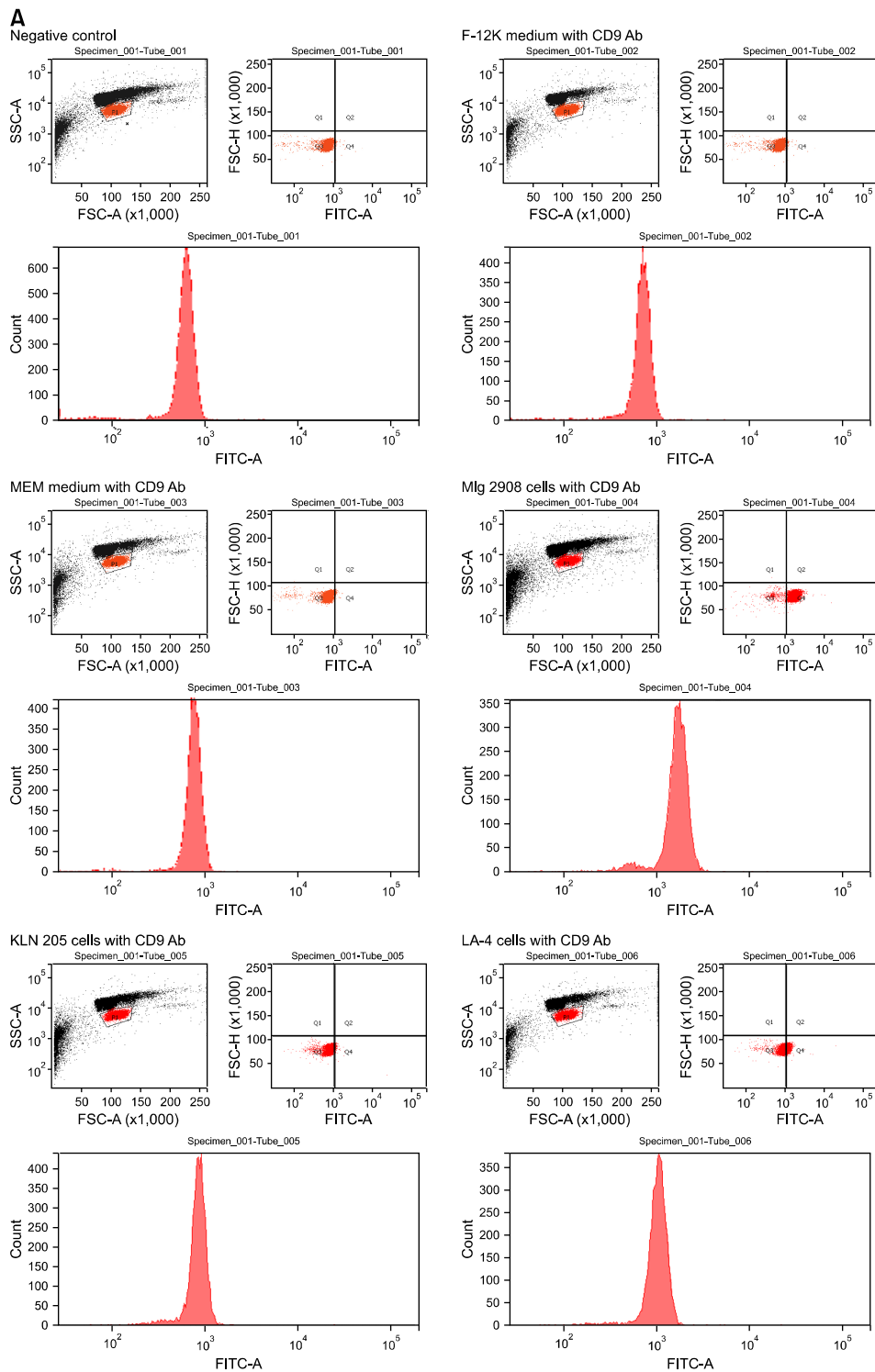


Figure 1. Two-dimensional plots of the exosome fluorescence-activated cell sorting analysis. Exosomes were isolated and purified from murine Mlg 2908 lung fibroblasts, KLN 205 lung squamous cell carcinoma cells, and LA-4 murine lung adenoma cells with (A) CD9 antibody and (B) CD63 antibody. Purified exosomes were analyzed using fluorescence-activated cell sorting (FACS). SSC, scattered light; FSC, forward-scattered light; FITC, fluorescein isothiocyanate.

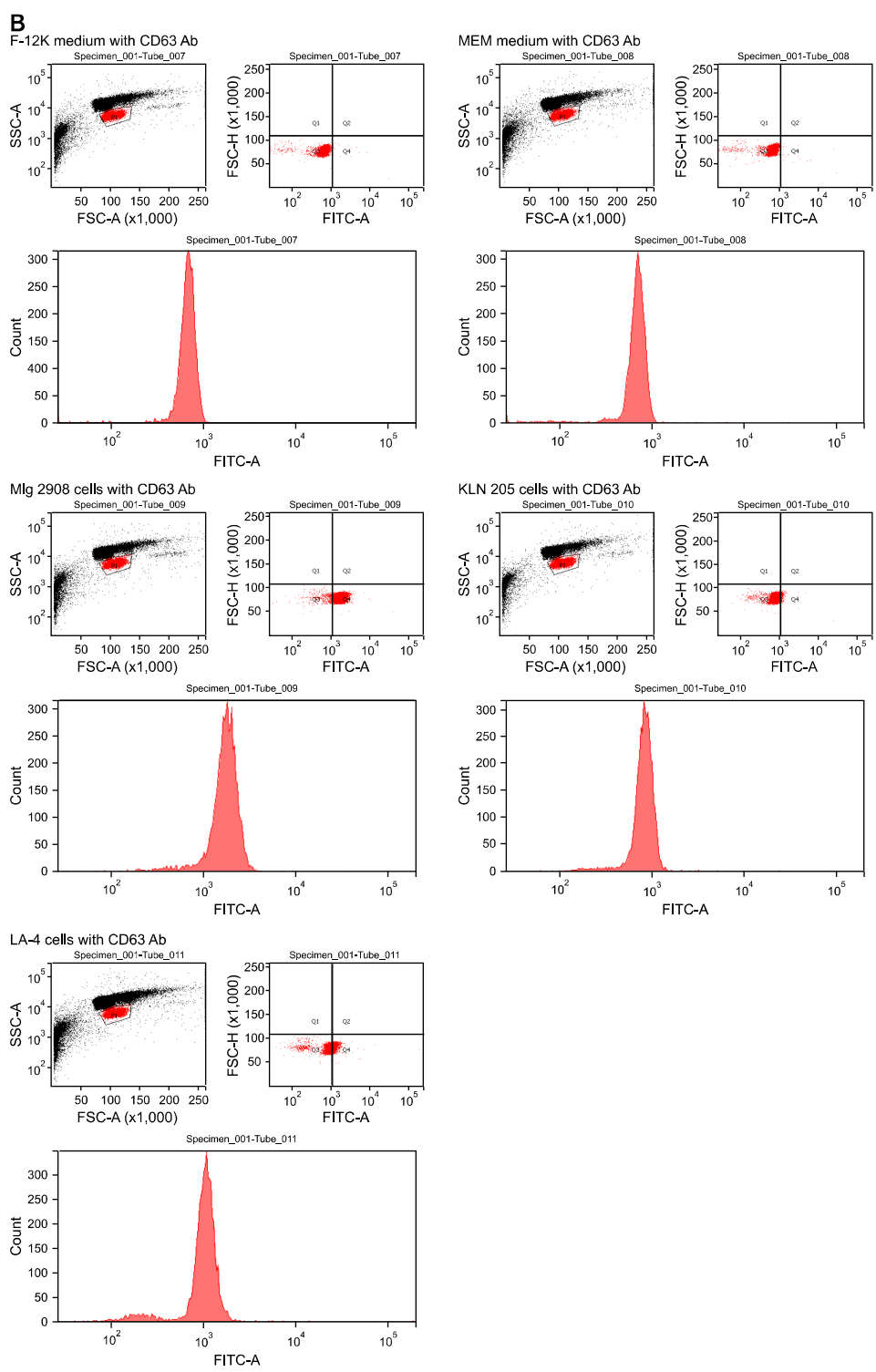


Figure 1. Continued.

the most remarkable specificity in body fluids. Interestingly, lung cancer patients have revealed high levels of miRNAs in tumor-specific exosomes, and some of these miRNAs are selectively detected in cultured lung cancer cells.¹⁶ It is confirmed that tumor

cells secrete miRNA-containing exosomes as a powerful cancer biomarker. Although all cell types may contribute to microvesicle shedding, their specific contributions to tumor progression have not established yet.¹⁷ Thus, quantification of miRNA in EVs is also

an exciting topic in cancer research due to the fact that the amount of extracellular miRNA appears to be upregulated in the plasma of patients bearing tumors.¹⁸ There is a growing interest in techniques that allow an absolute quantification of miRNAs, which could be useful for early diagnosis. Recently, digital PCR, mainly based on droplet generation, has emerged as an affordable technology for the precise and absolute quantification of nucleic acids.¹⁹

In this study, we analyzed exosome quantification *in vitro* using FACS in order to replace the existing mouse culling method for the identification of cancer *in vivo*. It has the potential to be extended into *in vivo* studies with samples of many bodily fluids (blood, saliva, urine, ascites, and pleural effusion). Although flow cytometry is a powerful method and is widely used for high-throughput quantitative and qualitative analysis of cells, its straightforward applicability to particular exosomes is hampered by several challenges due to small size of these vesicles (exosomes: ~80-200 nm and microvesicles: ~200-1,000 nm) and its polydispersity and low refractive index. We performed quantification and characterization of exosomes isolated from cell culture media using a flow cytometer especially designed for small particles. Moreover, our protocol is compatible with antibody labeling using fluorescence-conjugated primary antibodies. Our methodology presents the possibility of routine quantification and characterization of exosomes from various sources. Currently, to facilitate flow cytometry analyses, exosomes are often bound to beads that provide a larger surface area and more scattered light. This detection is largely dependent on the abundance and availability of antigens on the exosomes, which are recognized by the antibody coupled to the beads, reflecting both pros and cons associated with the high specificity of antibodies.²⁰

Exosomal proteins have various functional groups, like CD9, CD63, CD81, heat shock proteins, membrane transporters, and lipid-bound proteins. Tetraspanins are common exosome specific markers and include CD9, CD63, and CD81. Especially, the CD63+ exosomes were increased in patients with melanoma²¹ and other cancers.²² In the present study, we firstly investigated the tetraspanin family of proteins, CD63 and CD9, as useful markers of exosomes derived from normal and two lung cancer cell lines. From the aspect of cancer diagnosis, circulating exosomes are ideal resources of biomarkers since they carry molecular features of tumor cells. We constructed anti-CD9 and anti-CD63 antibodies that allowed automated rapid and reproducible exosome extraction from cultured cells. Our simple device can promote not only biomarker discovery but also other researches concerning exosomes.

Exosomes are secreted by cells in both normal and pathological conditions and contain various membrane and cytosolic proteins. Thus, exosomal proteins can be potentially used for clinical diagnostics.²³ Cancer cell-derived EVs have essential roles in tumor progression and microenvironment. Noncancerous cell-derived EVs demonstrate protective functions against damage and repair for maintenance of homeostasis.²⁴ In addition, exosomes derived from fibroblasts have been shown to play a role in the migration of breast cancer cells²⁵ and are protumorigenic with respect to the transfer of mRNA and proangiogenic proteins, contributing to the horizontal transfer of oncogenes such as EGFRvIII.²⁶ A myriad of biological processes in the target cells are affected by exosomes, which induce proliferation and have the potential to convert normal cells into tumor cells. This study was conducted to determine the involvement of exosomes in cancer by the quantitative measurement of one normal (Mlg 2908) and two lung cancer (LA-4 and KLN 205) cell lines. As a notable result, more exosomes were secreted in normal fibroblast (Mlg 2908) cells compared with the two cancer cell lines, according to average FITC-A. The growth of tumors depends upon the balance between proliferation and cell death processes, which are regulated by multiple molecular mechanisms. Since the type of normal cells we used were lung fibroblasts, this is thought to be the reason why they secrete more exosomes compared with cancer cells. Therefore, studies may be necessary to further quantify the exosome secretion by culturing normal cells, fibroblasts, and cancer cells simultaneously.

New protocols for isolation do not distinguish the exosomes from ectosomes since the physical properties have not yet been fully characterized and specific markers are still insufficient. According to the average FITC-A, 1,670 exosomes were secreted in normal (Mlg 2908) cells compared with the two cancer cell lines (852 in KLN 205 and 1,046 in LA-4) in the case of CD9 antibody binding. With CD63 antibody binding, 1,784 exosomes were secreted in normal cells compared with the two cancer cell lines (845 and 1,084, respectively), indicating the detection of the increased exosomes with CD63 specificity in Mlg 2908 normal fibroblasts and LA-4 lung cancer cells with both CD9 and CD63 antibody binding, compared with SSC and FSC.

The significance and clinical value of exosomes remain still unknown, but current technical challenges have promoted in the rapid isolation and analysis of them. Although the detailed mechanism of resistance remains unclear, inhibition of exosome secretion may present a novel strategy for lung cancer treatment in the future.²⁷ Since the isolation of circulating exosomes is a minimally invasive procedure, this technique presents possi-

bilities for novel diagnostic applications. Our results may contribute the classification of cancer-specific proteins and miRNA as diagnostic markers.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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