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Utility of exosomal DNA as a biomarker for anti-cancer drug response using continuous blood sample of colorectal cancer patients

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Utility of exosomal DNA as a biomarker for anti-cancer drug response using continuous blood sample of colorectal cancer patients

Directed by Professor Han Sang Kim

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Hoyun Cho

December 2021

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<TABLE OF CONTENTS>

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LIST OF FIGURES

LIST OF TABLES

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ABSTRACT

Utility of exosomal DNA as a biomarker for anti-cancer drug response using continuous blood sample of colorectal cancer patients

Hoyun Cho

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(Directed by Professor Han Sang Kim)

Background: Cell-free DNA in body fluids of cancer patients is a promising practice to assess noninvasively mutations from tumor. And exosomes originated from viable cancer cells may reflect tumor biology. The comparison between relatively intact exosome-derived DNA (exoDNA) and fragmented cfDNA in mutation detection among patients with CRC deserves investigation.

Patients and methods: ExoDNA and cfDNA were obtained from 32 CRC patients with known *KRAS* mutation. The respective *KRAS* G12D, G13D mutation status was interrogated with droplet digital PCR assays (ddPCR).

Results: We have demonstrated the feasibility of using exoDNA in detecting the *KRAS* mutation of CRC and the correlation of mutant detection rate with carcinoembryonic antigen (CEA) and overall survival.

Conclusions: Our data suggested that mutational status might be reflected in exosome rather than cfDNA alone exosome rather than cfDNA alone. Finally, in a prospective cohort of CRC patients, we showed that using exosome and cfDNA

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liquid biopsy provides clinical information relevant to therapeutic stratification.

Key words: Cancer, exosome, exosomal DNA, liquid biopsy, colorectal cancer, cell-free DNA

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Utility of exosomal DNA as a biomarker for anti-cancer drug response using continuous blood sample of colorectal cancer patients

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I. INTRODUCTION

Liquid biopsy refers to the analysis of any tumor-derived material circulating in one's blood or any other body fluid. This concept is relevant in colorectal cancer as the tumor is often difficult to reach and require invasive and potentially harmful procedure.[1] Advantages of liquid biopsy have led to easier patient management and treatment[2]. Liquid biopsy can non-invasively detect any targetable genomic alteration and guide corresponding targeted therapy, allow monitoring of response to treatment and explore genetic changes that confer resistance, overcoming spatial and temporal heterogeneity.[1] Those materials can be found in a range of components in body fluids, such as circulating tumor cells (CTCs)[3], the tumor-derived cell-free DNA (cfDNA)[4], extracellular vesicles [5], tumor educated platelets[6].

Cell-free DNA (cfDNA) is originated from the hematopoietic system [7], organ

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[8], and tumor[9]. It has been studied as one of the most significant cancer biomarkers among the liquid biopsies. Because several studies have shown the reason that cfDNA tends to represent somatic mutations of the tumor genome and provided an alternative non-invasive method to enable the detection of cancer[9,10]. Abnormalities in cancer patients were observed that such individuals have higher amounts of cfDNA [11] [12]. In particular, quantitative analysis of cfDNA levels has been shown to yielded important prognostic value, e.g., an increase of cfDNA concentration correlated with tumor stages and overall survival in colorectal cancer (CRC) patients[13]. However, cfDNA has limitations. Most of the cfDNA is unstable, most of it is derived from leukocytes, and difficult to detect early-stages of cancers [14-16].

Other reservoirs of proteins, DNA, and RNA have been identified in the form of microvesicles termed 'exosomes'[17]. Exosomes, extracellular vesicles 50-150nm in size, have the some components of their cell of origin including nucleotides [18], and proteins[19], and transfer those materials to other cells, thus acting as packages in intracellular communications[20]. Importantly, exosomes tend to be secreted more from tumor cells than normal cells[21] although the mechanisms have not been fully understood yet. The DNA of exosomes (exoDNA) has a large size, stably at > 3kb, making this source advantageous for detection of mutations, as it consists of both double-stranded DNA and single-stranded DNA[22]. Moreover, patient-derived-exoDNA contains genomic DNA that covers all chromosomes including mutations of the tumor[23].

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We are actively involved in developing methods to detect point mutations in liquid biopsy. The assessment of the biomarker source cfDNA and exoDNA could be of great importance in diagnostics. To shed light on this topic, we investigated exoDNA of colorectal cancer (CRC) with *KRAS* mutation and compared the results with those obtained analyzing cfDNA in the same samples.[24]

In this study, we aimed to investigate the utility of the exosomes for clinical use with collected CRC patients' blood with *KRAS* G12D, G13D mutations. By running ddPCR with the isolated exoDNA and cfDNA, we compared both with matching clinical data to determine if they can be used as another method for prognosis.

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II. MATERIALS AND METHODS

1. Cell culture and collecting cultured media.

Human colorectal cancer cell (HCT-116, LS 174T) were purchased from ATCC (USA). The cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin. Cells were cultured in 37℃ and 5% CO_2.

To harvest exosome from cell culture, HCT-116, LS 174t cells were plated in 100 mm culture dishes at 80% confluence and then treated with exo-free DMEM medium containing 10% exo-free FBS and 1X P/S for 72 hr. After then, cultured media were collected for isolating exosome. Cultured medium was centrifuged at 500 x g for 10 min, 3000 x g for 20 min.

2. Patient's sample collection

All patients with all stages of colorectal cancers were included in our study, and each consented following institutional review board of Severance Hospital approval (4-2019-0811). Three bottles of ten milliliters of whole blood were collected *via* blood draw and (plasma was isolated) for isolation of cfDNA and exosomes. Three blood bottles were centrifuged at 1900 x *g* 15 min, then one of the bottles was centrifuged at 1900 x *g*, 15 min for cfDNA and the other two bottles were centrifuged first at 500 x *g* 10 min, then 3000 x *g* 20 min for exosome. The centrifuged plasma samples were stored at -80℃ in a deep freezer until exosome or cfDNA isolation since 2019.

3. Exosome isolation

Then plasma samples and cell cultured medium were centrifuged at 12,000 x

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g for 20 min by using the differential ultra-centrifugation (Beckman Coulter Optima X100, Brea, California, USA). Sequentially, the supernatants were centrifuged at 100,000 x g for 70 min at 10°C in a fixed-angle rotor in a Beckman Coulter ultracentrifuge. Exosome pellets were resuspended in 200 μL of PBS and stored at -80°C. The enrichment of exosomes in our preparations was verified using nanoparticle tracking analysis (Malvern panalytical, Malvern, U.K) and manufacturer's software (v3.1 with camera level set to 10 for exosome from plasma and cell line, and detection threshold to 3).

4. DNA preparation

(A) ***Isolation of cell-free DNA (cfDNA)***

Circulating cell-free DNA was extracted from 2 mL plasma with Bio Scientific NextPrep-Mag cfDNA Isolation Kit (PerkinElmer, Austin, Texas, USA) under the manufacturer's instruction. Briefly, in 50 mL conical tube, 2 mL plasma with 2.5 mL binding solution, 48 μL proteinase K, and 32 μL magnetic beads were added, vortexed, and incubated for 15 minutes at 55°C. The tube was placed on a magnetic stand until the beads were completed attracted and the supernatant was discarded. 1.5 mL of Wash buffer 1 was added to resuspend beads and the slurry was transferred to a 2 mL low binding e-tube. The 2 mL e-tube was placed on a magnetic stand, the supernatant was discarded, and the wash was repeated in the same tube. 1.5 mL of wash buffer 2 was added, the tube was placed on the magnetic stand, and the supernatant was discarded. This step was repeated one more time and a 24 μL of elution solution was added before incubation for 5 min

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at 55°C. The cfDNA elute was transferred to a new 1.5 mL e-tube, and the concentration was measured using Qubit™ dsDNA High Sensitivity assay kit (Invitrogen, Waltham, Massachusetts, USA) with Qubit®2.0 fluorometer (Invitrogen, Waltham, Massachusetts, USA) and stored at -80°C.

(B) ***Isolation of exoDNA***

ExoDNA was extracted from nanoparticles using the AMPure XP beads (Beckman coulter, Brea, California, USA) following the manufacturer's protocol. Exosomes suspended in 200 uL of PBS were added to equal volumes of AL buffer and 20 μg/ml of protease K (Qiagen, Hilden, Germany) for lysis, then incubated at 56°C for 10 min. 1 volume of AMPure XP beads (Beckman coulter, Brea, California, USA), isopropanol-2, PEG solution was added to exosome lysate and gently mixed for 5 min. The DNA binding with bead eluted by magnetic bar in 22 uL of nuclease-free water after twice of washing and dry step using 80% ethanol. The concentration was measured using Qubit™ dsDNA High Sensitivity assay kit (Invitrogen, Waltham, Massachusetts, USA) with Qubit®2.0 fluorometer (Invitrogen, Waltham, Massachusetts, USA) and stored at -80°C.

(C) ***Analysis of exosome DNA by Agilent Bioanalyzer***

Exosome DNA was extracted from blood as described above. DNA extracted from exosomes analysed using Agilent Bioanalyzer 2100 instrument and Agilent High Sensitivity DNA Kit (Agilent technologies, Santa Clara, California, USA) following manufacturer's recommended protocol. The Agilent 2100 Expert software analyses DNA profile of each sample automatically and displays

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electropherogram for each sample.

5. Western blot analysis

Exosomes were lysed with RIPA buffer (Thermo fisher, Waltham, Massachusetts, USA) and 1X protease cocktails (Sigma-Aldrich, Saint Louis, Missouri, USA), and Phenylmethylsulfonyl fluoride (Sigma-aldrich, Saint Louis, Missouri, USA). Sample loading was normalized according to Bradford protein quantification with Bradford reagents (Biorad, Hercules, California, USA). The proteins (30 μg) were mixed with NuPAGE LDS sample buffer (4X) (Invitrogen Waltham, Massachusetts, USA) including 5% of β-mercaptoethanol, then heated at 95°C for 5 min. Proteins were electrophoresed on loaded to Bis-Tris premade gel (Invitrogen Waltham, Massachusetts, USA), electroblotted on a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 hr at room temperature with 3% BSA solutions in PBS/0.05% Tween and incubated overnight at 4°C with the following primary antibodies: 1:1000 anti-CD9 (Cell signaling, Danvers, Massachusetts), 1:1000 anti-CD81 (Novus biologicals, Centennial, Colorado), 1:1000 anti-flotillin-1 (Cell signaling Danvers, Massachusetts) and 1:2000 anti-β-actin (Santa cruz, Dallas, Texas, USA). After overnight incubation, the membranes were washed four times with PBS 0.05% Tween20. Immunoblots were visualized by SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo fisher,) using ImageQuant™ LAS4000 mini (General electric health care, Boston, Massachusetts, USA).

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6. Exosomes analysis with ExoView

Exosomes were detected using the ExoView® Tetraspanin chip (Nanoview Biosciences, Brighton, Massachusetts, USA) arrayed with antibodies against proteins CD81, CD63, and CD9. Mouse IgG1 was used as a negative control. Plasma derived exosome was diluted to a ratio of 1:1000. 35 μL of diluted exosome was dropped onto the chip surface and incubated overnight. After three times of washing, the chips were treated with ExoView Tetraspanin Labelling ABs (EV-TC-AB-01), including anti-CD9/ALEXA-488, anti-CD81/ALEXA-555, and anti-CD63/ALEXA-647, for co-localization tests in order to characterize the sub-populations on the surface of exosomes. The chips were incubated with 250 μL of the labelling solution for 2 hr. The chips were then imaged with the ExoView R100 reader through the Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS) technology using the ExoScan v0.998 acquisition software. The data was analyzed using ExoViewer v0.998 with sizing thresholds set at 50-200 nm diameter.[25,26]

7. ExoDNA amplification

Whole genome amplification was performed using the REPLI-G Single cell kit (Qiagen, Hilden, Germany) for exoDNA according to the manufacturer's instructions. The 2.5 μL of exoDNA was mixed with an equal volume of 2.5 μL of a 1:8 dilution of Solution A (0.4 M KOH, 10 mM EDTA) was added and incubated for 3 min at room temperature. Stop Solution and REPLI-g polymerase were added to the reaction. This solution (40 μL) was added to 5 μL exoDNA of

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buffer DLB. The solutions were gently mixed and incubated at 30°C for 16 hr and heat inactivated at 65°C for 10 min, and the DNA was purified.

8. Broad PCR and nestsed PCR

To enrich for *KRAS* region, broad PCR reactions were performed in a 50 μL volume with 35 μL of WGA exoDNA following Takara ExTaq (Takara, Kyoto, Japan) manufacturer's manual, including 500 nM of forward primer (5'-AAAGGTACTGGTGGAGTATTTG-3') and reverse primer (5'-CCTGCACCAGTAA TATGCATA-3') of final concentration. To capture the amplified DNA, we added a 5-biotinylated probe (biotin-5'-TTATAAGGCCT GCTGAAAATGA-3', biotin-5'-TGTTGGATCATATTCGTC CA C-3'; Bionics) to the amplified genomic DNA and proceeded with nested PCR. The PCR cycling was performed on a SimpliAmp™ Thermal Cycler (Thermo) machine as follows: 95 °C for 120 s, 30 cycles of 95 °C for 15 s and 60 °C for 30 s, followed by DNA melting from 60 °C to 95 °C at a temperature ramping rate of 0.2 °C/s.

9. Amplicon capture

The 10 μL of Dynabeads M-270 Streptavidin (Invitrogen) was washed three times with 1X binding/washing buffer (5 mmol/L Tris-HCl, pH 7.5, 0.5 mmol/L EDTA, and 1.0 mol/L NaCl) and resuspended in 40 μL of 2X binding/washing buffer. The 80 μL of hybridization mixture was captured by mixing with the 10 μL of processed Dynabeads and incubating the mixture on a shaker for 30 min at room temperature. The beads were washed three times with 1X binding and washing buffer supplemented with 0.05% (v/v) Tween 20, twice with 1X binding

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and washing buffer. Finally, the beads were resuspended in 20 μL water, denatured at 95°C for 2 min, and placed immediately on DynaMag magnets (Invitrogen). The suspension was recovered for further analysis.

10. Droplet digital PCR

Droplet digital PCR (ddPCR; QX200, BioRad) was used for the highly sensitive detection of genetic mutations. This assay is a multiplex ddPCR assay designed to recognize specific mutations of codon 12 and 13 of the *KRAS* gene: G12D, G13D, which together account for the majority of all *KRAS* mutations present in CRC. Blue dots represent any one of 7-FAM-labeled probes detecting *KRAS* mutation; green dots represent wild type detection in HEX channel. Interpretation of the digital PCR results was in accordance with BioRad Rare Mutation Detection Best Practice Guidelines for ddPCR. The presence of *KRAS* mutant DNA was observed in the exosomes of each patient. The fractional abundance is calculated as follows: absolute quantification of mutant clone/ (absolute quantification of mutant + wild type clones). Based on our prior experience using cell line gDNA, exosome DNA, patient exoDNA, a set cutoff threshold of more than 10 000 total droplets, 5 or more positive droplets, and FA of 0.1% or more was used to determine positivity.

11. Statistical Analyses

Statistical analyses were performed using Graphpad Prism version 8.0 software. Normality was assessed by D'Agostino & Pearson test, Shapiro-Wilk tests and Kolmogorov-Smirnov test. Differences in continuous variables among three or

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more groups were assessed with Kruskal-Wallis for non-normally distributed variables. The Mann–Whitney U test was applied to assess the association among the clinical characteristics, *KRAS* status of DNA and amount of cfDNA and exosomes. $P<0.05$ was considered as significant. Survival curves were generated using the KaplanMeier method and compared using log-rank test by R software, version 4.1.2, with RStudio, (R Foundation for Statistical Computing).

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3. RESULTS

1. Cohort characteristics

In total, 32 CRC patients with *KRAS* mutation and 10 CRC patients with *KRAS* wild type were included in this study. All blood samples were collected by needle, following tissue biopsies. qPCR test was implemented prior to ddPCR to compare with liquid biopsy data. Samples from patients with *KRAS* wild type were used for setting cut off. The characteristics of patients are reported in **Table.1** Among patients with *KRAS* mutations, *KRAS* G13D accounted for 37.5% (n = 12) and the majority were diagnosed with G12D 67.5% (n = 20). The 12.5% (n = 4) of patients were classified as stage I, 18.8% (n = 6) as stage II, 15.6% (n = 5) as stage III, and 53.1% (n = 17) as stage IV. Just over half of the blood of baseline (53%) was extracted ≤30 days before the first chemotherapy dosing. Among them, seven patients did not receive chemotherapy. This division was supported by the clinical characteristics recorded for each patient. The median of cfDNA and exoDNA level is 1.020 ng and 0.254 ng, respectively ($p < 0.0001$).

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Table 1. Characteristics of cohort.

Table 1.	Characteristics of patients	
Total	32	
Sex	Male	22
	Female	10
Age	(41~87)	
Stage	I	4
	II	6
	III	5
	IV	17
KRAS mutation	G13D	12
	G12D	20
cfDNA, median (range) (ng)	1.020, (0.140 - 16.600)	$p < 0.0001$
exoDNA, median (range) (ng)	0.254, (low - 2.480)	
CEA, median (range) (ng/ml)	4.99 (0.61 – 14682)	

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2. Exosome characterization

To isolate the exosomes from plasma and cell line, we used serial ultracentrifuge then validated our method**.** The individual size and concentrations of isolated exosomes were confirmed by Nanosight tracking analysis (NTA), and the size of cell line derived exosomes is between 50–150 nm and the size of plasma exosomes also have been showed similar size distribution with the exception of some large particles **(**Fig.1A).

Then, we extracted protein and DNA from isolated exosomes to confirm characteristics of exosomal materials (Fig.1B)**.** The isolated exosome proteins were also analyzed using exosomal marker proteins, CD-9, CD-81, and Flotillin-1. The results showed the CD-9, CD-81, and Flotillin-1 in pellet after ultracentrifugation. The size distribution of exoDNA and cfDNA was determined by (B) bioanalyzer 2100 system. CfDNA have <300 bp size of fragment and the plasma derived exoDNA have 3-6 kb peak of fragment as other studies previously have shown[22]. (Fig.1C) While the ddPCR results of the cell line derived exoDNA demonstrated each fluorescence of numerous positive plot and FA, plasma exoDNA did not show any plot of the gene signal (Fig.E). This data prompted us to amplify the exoDNA, so we employed whole genome amplification and nested PCR. We amplified *KRAS* region of exoDNA from cell line and confirmed by using ddPCR. (suppl.1). Then, same method was proceeded to exoDNA from plasma. These results suggested that our methods of exosomes from patients and amplification of mutation are valid.

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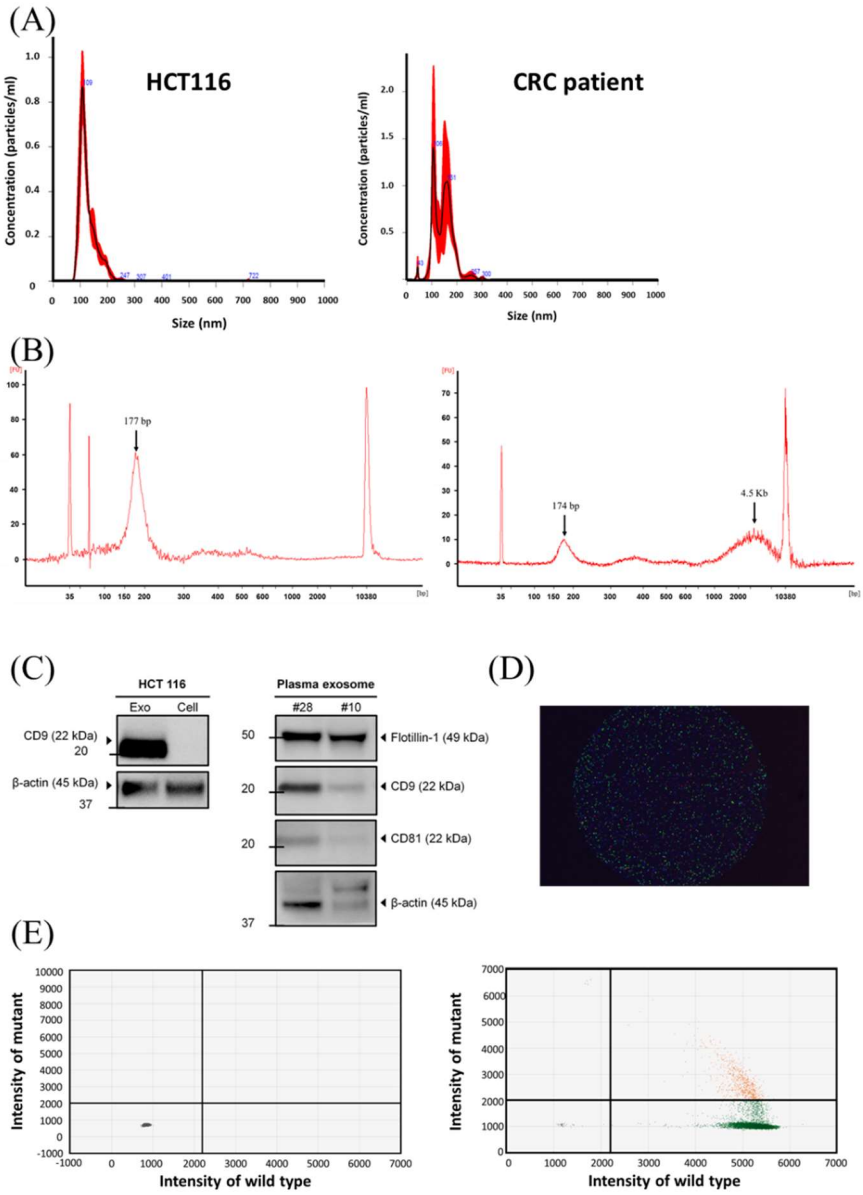

Figure 1. Exosome characterization and amplification of *KRAS* mutation in exoDNA

(A) Representative Nanosight tracking analysis (NTA) of exosomes isolated

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from HCT116 and plasma using ultracentrifugation. The presence and concentration of exosomes from HCT-116 cell line and human plasma samples from patients with colon cancer were determined by using a NanoSight. (B) Characterization of exosomes by western blot analysis. Common exosome marker (CD-9, CD-81 and Flotilin-1) in 30 μg from exosomal protein from HCT -116 cell line and 30 μg from CRC patients' plasma. (C) The presence of double stranded DNA of exosomes from CRC patient's plasma was confirmed by bioanalyzer 2100 system. (E) Representative 2D intensity scatter plot of wild-type and mutant amplicon for *KRAS* G13D in patient. The enrichment of *KRAS* G13D of exoDNA was confirmed using droplet digital PCR. Each plot of the four regions represents wild type (green), wild type and mutant co-existence (orange), mutant (blue) and no template (gray).

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3. Comparison mutation detection between cfDNA and captured exoDNA

Overall, 77 blood samples from 42 CRC patients mutation were profiled with ddPCR. Among the samples from the patients with *KRAS* mutation, the thirty of baselines with cfDNA and thirty-two of captured exoDNA pairs profile showed *KRAS* fractional abundant (FA) range of 0% to 45.81 % and 0% to 75.68 % , respectively. For captured exoDNA, the mean of FA (32 samples; 5.167%) was significantly higher than the mean of FA of cfDNA (30 samples; 3.439%) (Fig.2A, $p < 0.05$). We then compared cfDNA with captured exoDNA from all blood samples including follow up; the mean of captured exoDNA showed significantly higher than the mean of FA for cfDNA. (Suppl.1, $p \leq 0.001$). To investigate sensitivity and specificity, same method was applied for 10 samples of *KRAS* wild type. The *KRAS* detection of cfDNA yielded 48.39% sensitivity and 100% specificity and captured exoDNA achieved 68.75% sensitivity and 100% specificity (Fig.2B). To determine if the prevalence of circulating mutational events changes depending on the stage of CRC, the 32 patients were grouped into according to their stage of cancer. Then we compared FA of cfDNA and captured exoDNA according to stage. The mean of FA for cfDNA in stage I is 0.188% (n = 6), 0% (n = 5) as stage II, 0.389% (n = 5) as stage III, and 6.277% (n = 17) as stage IV, respectively. In contrast, the mean of FA for captured exoDNA is 4.528% (n = 6), 0.778% (n = 5) as stage II, 2.806% (n = 5) as stage III, and 7.536% (n = 17) as stage IV. There was no significant difference between group of cfDNA and captured exoDNA, but there was mutation detection in stage

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I in captured exoDNA group (Fig.2C).

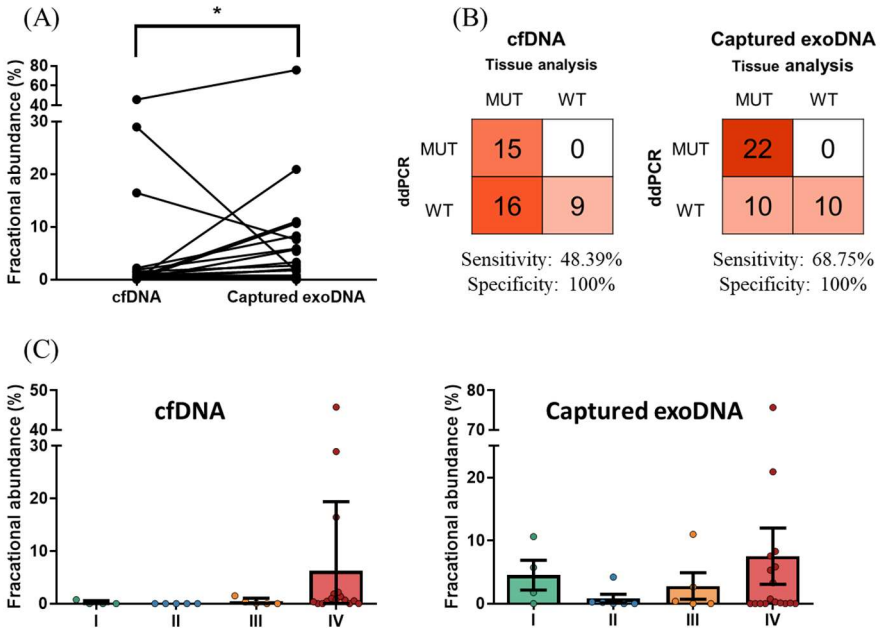

Figure 2. *KRAS* mutation in cfDNA and captured exoDNA from CRC patients.

Comparison of detection rate between cfDNA and captured exoDNA from blood of baseline; cfDNA (n=30) and captured exoDNA (n=32). Only paired samples show line in graph. (B) Classification error matrix for the *KRAS* detection of cfDNA (left) and captured exoDNA (right). The number of samples identified is noted in each box. (C) Detection rate for *KRAS* in different clinical stages between cfDNA (left) and captured exoDNA (right).

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4. Correlation between plasma cfDNA, exosomes, *KRAS* mutation levels and Carcinoembryonic antigens in patients

To identify correlation with clinical status and cfDNA or exosome, we analyzed with Carcinoembryonic antigen (CEA) level. The cut off value of CEA positivie was set 5 ng/μL. First, we separated the exosome concentration according to presence of CEA, but there was no difference (Fig.3A). Then, the amount of cfDNA and exoDNA was compared respectively with CEA value, there was no significant difference within group. These data suggest that quantities of tumor derived cfDNA and exosomes were little accounted of CEA secretion. Stratifying patients based on the expression of CEA, we found that the FA of cfDNA and captured exoDNA was greater in CEA positive patients compared to negative ones. ($p = 0.001$) (Fig.3C).

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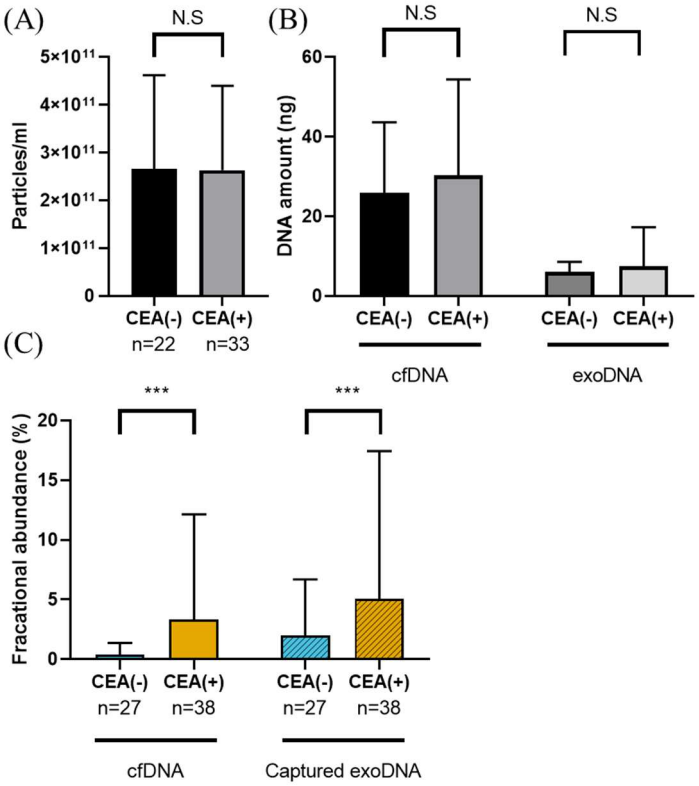

Figure 3 Evaluation of plasma components levels correlated with CEA of patients.

Correlation between KRAS mutational status and CEA. (A) Exosomes levels were non siginificantly different according to CEA expression. (B) In addition, there is no correlation between amount of cfDNA or exoDNA and CEA expression. (C) CEA expression correlated with a higher fractional abundance in captured exoDNA and cfDNA ($p = 0.001$).

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5. *KRAS* mutations in exoDNA or cfDNA and survival

We analyzed whether the exoDNA or cfDNA of the latest plasma from *KRAS* mutant patients were associated with overall survival (OS) in 32 patients who received therapy. For cfDNA, the 21 patients with an FA lower than 1% was significantly longer than that of 9 patients with a higher FA (p <0.0001). For captured exoDNA, the 18 patients with an FA lower than 1% was significantly longer than that of 13 patients with an FA higher than or equal to FA 1%. Cox proportional hazard analyses were also performed and greater than a FA 1% was a significant risk factor impacting survival (respectively HR, 6.264, 47.35).

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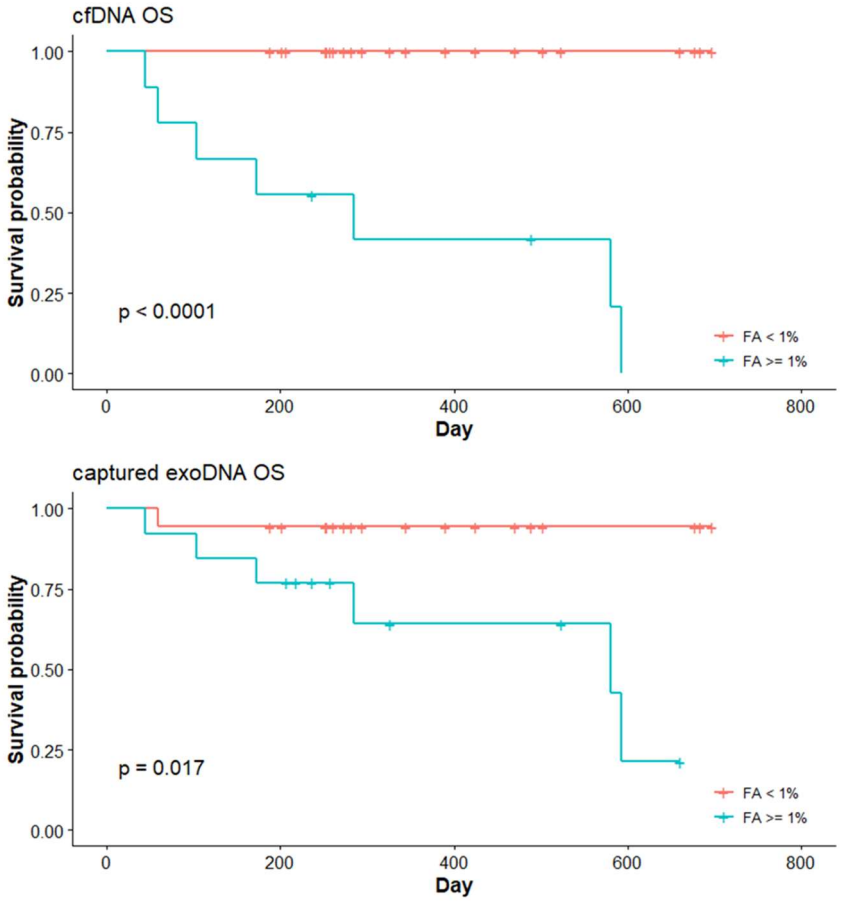

Figure 4. Overall survival and fractional abundance of *KRAS* mutations in plasma

Stratification of *KRAS* at a mutant allele frequency of 1% was associated with overall survival in patients at the time of first of blood draw. Patients with a low FA (pink line) in plasma cfDNA had a significantly longer median OS duration than patients with a high FA ($p < 0.0001$). And patients with a low FA (pink line) in plasma captured exoDNA had a significantly longer median OS than 20 patients with a high FA ($p = 0.017$)

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4. DISCUSSION

Liquid biopsy aims to provide information about solid tumors in a non-invasive manner. However, clinical application has been lagging due to the challenge of detecting tumor-derived material, particularly at early-stages of disease.

In recent years, many different studies used cell-free DNA (cfDNA) isolated from the blood of cancer patients to identify mutations. However, cfDNA originated from dying cells contributed to decreased sensitivity. In contrast, Exosome, comprising intact DNA, RNA and proteins, is secreted from live cells including tumor, thus it is studied as one of the significant liquid biopsies. Considering these, we analyzed exosomes from blood and compared with cfDNA.

First, we showed presence of exosomes with marker proteins and 50–150 nm of size. It suggested that ultracentrifugation is valid for exosome isolation from patients' plasma. Then, as other studies have shown[22,23], we found that exoDNA is large in size compared to cfDNA. Some studies treated exosomes with DNase I to reduce external contamination before DNA extraction. [18,23,27] However, as we treated DNase I to plasma derived exosomes, exoDNA were undetectable so we extracted exoDNA without DNase. Without Dnase I, as same size of cfDNA appeared in exoDNA, cfDNA may be present in exoDNA. We assumed that exoDNA and cfDNA may be complementary for detecting mutations in body fluids. However, we showed that the amount of exoDNA was significantly lower than the amount of cfDNA. In addition, earlier studies suggested that exosomes

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with gDNA preserved inside are account for <1% of total exosomes.[28] Consequently, we amplified exoDNA with WGA and PCR.

In the cohort study, we only recruited CRC patients with *KRAS* G12D, G13D mutations for some reasons. First, *KRAS* mutations, which is one of the frequently mutated in CRC, targeted therapies failed to obtain clinically approved drugs. This is because anti-EGFR agents do not provide meaningful survival benefits versus anti-angiogenic/chemotherapy regimens in metastatic CRC patients whose tumors are not wild type (WT) with respect to RAS genes. Accurate detection of RAS mutations in these patients is therefore of high clinical importance for therapy selection. Second, *KRAS* G12D and G13D are the prevalent mutations in our cohort as other study showed.[29]

We assumed that intact exoDNA preserves mutation and would be more plausible than degraded cfDNA for mutation detection, especially in early-detection of CRC. Previous studies demonstrate similar findings that extracellular vesicle DNA is superior to ctDNA for mutation detection in early-stage[14]. We then compared captured exoDNA and cfDNA with detecting *KRAS* mutation of CRC patients. In the results of Fig.2B, although the difference is not statistically significant due to insufficient cases, captured exoDNA showed detecting *KRAS* in early-stage of cases unlike cfDNA. In our data, most of detection *KRAS* in cfDNA were appeared in stage IV similar to that of captured exoDNA. These results were predictable based on different mechanism of cfDNA and exoDNA biogenesis. cfDNA is mainly originated from cells undergoing necrosis or

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apoptosis, which is characteristics of later disease. In contrast, exosome, which preserves intact mutation information, is secreted from all kinds of cell including early-stages of tumor.

Carcinoembryonic antigen (CEA) has an established role as a biomarker in diagnosis, treatment and surveillance in CRC and elevated serum levels of CEA are associated with inferior prognosis. Correlation between CEA and cfDNA has been studied but there is few study of correlation of CEA and exosome[30]. Due to characteristics of exosome, status of patients might be reflected in exosomes and this study showed such potential of exosomes in liquid biopsy.

We also demonstrated that exoNA or cfDNA identified with ddPCR had a significantly longer survival than patients with lower FA exoDNA or cfDNA than higher FA patients. Other studies demonstrated that the amount of mutated exoDNA can be predictive survival in patients with cancer[17,31]. With these results, exoDNA could be used in observing serial response of therapy like CEA.

Our study is not without limitation. First, in the process for amplification of exoDNA with Repli-G whole genome amplification (WGA) and PCR, could induce biases. Although previous study which demonstrated WGA can enrich DNA with genome wide mutant allele frequency was conserved[32], bias might be occurred where be investigated. Since the amount of exoDNA was low, amplification of DNA or enrichment of exosome was required. The future study is needed to enrich the exoDNA without biases. Second, we only investigate two types of *KRAS* mutation, while there are tens of potential mutation loci in *KRAS*.

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Third, although we collected 66 blood samples from patients with *KRAS* mutation, the cohort was insufficient with early-stage of CRC to demonstrate mutant detection in early. Fourth, we were unable to keep tracking patients' blood constantly. Several cfDNA samples were exhausted before analysis.

In recent, many studies investigated importance of cfDNA in various physiological events, such as metastasis, epigenetics, etc. However, studies of exoDNA are insufficient to unveil its significance compared to studies of cfDNA. Because low amount of exoDNA is accounted for the lack of research, improvement of exosome and exoDNA isolation method will be required. Studies using improved methods will shed light on the characteristics of exoDNA and utility in liquid biopsy.

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V. CONCLUSION

In conclusion, Characterization of plasma derived exosome was performed by NTA, Western blot, Bioanalyzer, and Exoview®. Western blotting and Exoview® showed that exosomal marker CD-9, CD-81 and Flotilin-1 were expressed. The size distribution of exoDNA and cfDNA were found by bioanalyzer 2100 system. To detect *KRAS* mutant in exoDNA, we amplified and captured by WGA and nested PCR. Then we tested this method to plasma from CRC patients with *KRAS* mutation. The Fractional abundance (FA) of captured exoDNA was higher than that of cfDNA for baseline and overall samples. The FA of cfDNA and captured exoDNA was higher in CEA positive patients. Low FA of cfDNA and captured exoDNA are relevant to longer survival of *KRAS* mutant patients.

In this study, we have demonstrated the feasibility of using exoDNA in detecting the *KRAS* mutation of CRC. Our data suggested that mutational status might be reflected in exosome rather than cfDNA alone.

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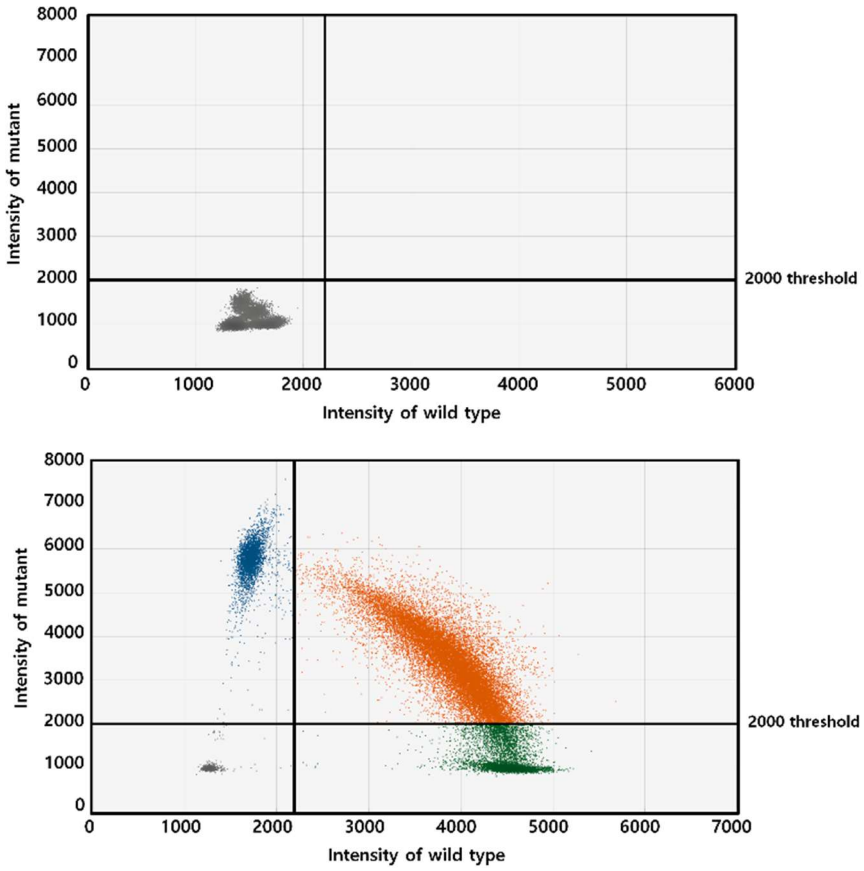
APPENDIX

Supplementary figure 1.

Amplification of exoDNA from cell line.



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Supplementary table.1

Sample No.	*KRAS* mutation	Follow up	Fractional abundance (%)	
			cfDNA	**captured exoDNA**
1	G13D (GGC>GAC)	1	1.52	2.64
		2	1.73	2.33
		3	0	1.35
		4	0.18	4.03
		5	0.06	0.9
2	G12D (GGT>GAT)	1	0	0
		2	0.48	1.5
		3	0.7	0
		4	0.24	2.3
		5	0.21	0.6
3	G13D (GGC>GAC)	1	0.39	11
		2	1.15	6.5
		3	0	7.8
		4	0	0.4
		5	1.06	1.1
4	G13D (GGC>GAC)	1	1.83	5.85
5	G13D (GGC>GAC)	1	0.7	3.3
		2	0.38	3.74
		3	1.24	3.36
		4	0.51	0.14
		5	0	4.58
		6	1.3	1.24
6	G13D (GGC>GAC)	1	0.46	0
		2	0.93	0
		3	1.58	0
		4	0.39	0
		5	0	0
		6	0	0
		7	0	0
		8	0	0
7	G13D (GGC>GAC)	1	45.81	75.68
8	G12D (GGT>GAT)	1	0	20.9
		2	0.64	2.1
		3	0.68	0
		4	0.36	5.4
9	G12D (GGT>GAT)	1	0.75	1.75
10	G12D (GGT>GAT)	1	0	0.2
11	G13D (GGC>GAC)	1	1.01	0
12	G13D (GGC>GAC)	1	0.76	0.75
		2	1.66	6.81
		3	6.51	18.62
		4	4.01	2.25
13	G12D (GGT>GAT)	1	0.08	0
14	G12D (GGT>GAT)	1	0	0
15	G13D (GGC>GAC)	1	16.42	7.55
		2	4.41	4.85
		3	4.92	9.45
16	G12D (GGT>GAT)	1	28.95	1.58
17	G12D (GGT>GAT)	1	0	0
		2	0	0
		3	0	0
18	G12D (GGT>GAT)	1	1.38	0.03
19	G12D (GGT>GAT)	1	0	0.08
20	G12D (GGT>GAT)	1	0	2.01
21	G13D (GGC>GAC)	1	0	10.64
22	G12D (GGT>GAT)	1	0	0
23	G12D (GGT>GAT)	1	0	0.37
24	G12D (GGT>GAT)	1	0	0.54
		2	0	0.54
25	G12D (GGT>GAT)	1	0	0.27
		2	0	0.41
26	G12D (GGT>GAT)	1	0	5.72
27	G12D (GGT>GAT)	1	0	0.39
28	G12D (GGT>GAT)	1	0.56	0.27
29	G13D (GGC>GAC)	1	2.19	8.28
30	G13D (GGC>GAC)	1	N/A	5.31
31	G12D (GGT>GAT)	1	0.36	0.09
32	G12D (GGT>GAT)	1	0	0.13

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ABSTRACT (IN KOREAN)

대장암 환자의 연속적 혈액 검체를 활용한 항암제 치료 반응 지표로써 엑소좀 디엔에이의 유용성 평가

<지도교수 김한상 >

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조호연

배경: 암 환자의 혈장에서 순환하는 세포유리 DNA (cfDNA)를 평가하는 것이 고형 종양의 체세포 돌연변이를 비침습적으로 평가하는 유망한 방법이라는 것은 널리 받아들여지고 있다. 최근 세포 외 엑소좀의 분리는 전이 환자의 혈장에서 돌연변이 DNA의 검출을 향상시키는 것으로 보고되었다. CRC 환자의 돌연변이 검출에서 비교적 온전한 엑소좀 유래 DNA (exoDNA)와 절편화된 cfDNA 간의 비교는 조사할 가치가 있다.

환자 및 방법: *KRAS* 돌연변이 상태가 알려진 32명의 CRC 환자로부터 exoDNA와 cfDNA를 모두 얻었다. 각각의 *KRAS* G12D, G13D 돌연변이 상태를 droplet digital PCR 분석(ddPCR)으로 조사를 진행했다.

결과: ddPCR을 사용하여 captured 엑소좀 DNA 및 세포유리 DNA에 존재하는 *KRAS* 돌연변이를 검출했으며 이를 바탕으로 암배아항원(CEA) 및 생존률과 짝을 이루어 분석하였다.

결론: exoDNA를 cfDNA와 같이 *KRAS* mutation을 이용해 혈액속의 돌연변이 검출이 가능하다는 것을 보여주었음. 또한 검출 결과를 환자의 임상데이터와 비교하여 exoDNA가 cfDNA와 같이 임상적으로 쓰일 가능성을 보여주었다.

핵심되는 말 : 암, 엑소좀, 엑소좀 DNA, 액체생검, 대장암, 세포유리 DNA