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KRAS and BRAF mutations in serum exosomes from patients with colorectal cancer in a Chinese population

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Abstract. The efficacy of epidermal growth factor receptor-targeted therapy is significantly associated with Kirsten rat sarcoma viral oncogene homolog (KRAS) and B-raf serine/threonine kinase proto-oncogene (BRAF) mutation in patients with colorectal cancer (CRC), for which the standard gene testing is currently performed using tumor tissue DNA. The aim of the present study was to compare the presence of KRAS and BRAF mutations in the serum exosome and primary tumor tissue from patients with CRC. Genomic DNA were extracted from the tumor tissues of 35 patients with histologically-confirmed CRC and exosomal mRNA were obtained from peripheral blood, which were collected from the corresponding patients prior to surgery. Three mutations in the KRAS gene (codons 12, 13 and 61) and a mutation in the BRAF gene (codon 600) were detected using a polymerase chain reaction-based sequencing method and their presence were compared between tumor tissues and the matched serum exosomes. The KRAS mutation rates in tumor tissues and the matched serum exosomes were 57.6 and 42.4%, respectively, which was not significantly different (P=0.063). The detection rate of the BRAF mutation was 24.2 and 18.2% in tumor tissues and the matched serum exosomes, respectively, and there was no significant difference (P=0.500). The patients with CRC that had a KRAS mutation of codon 12 in exon 2 in their tumor tissues and serum exosomes were significantly older compared with those without this mutation (tumor tissue, P=0.002; serum exosome, P=0.022). The sensitivity of KRAS and BRAF mutation detection using exosomal mRNA was 73.7 and 75%, respectively. The specificity of the detected mutations exhibited an efficiency of 100%, and the total consistency rate was 94.9 and 93.9% for KRAS and BRAF mutations, respectively. These results suggested that serum exosomal mRNA may be used as a novel source for the rapid and non-invasive genotyping of patients with CRC.

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Introduction

Colorectal cancer (CRC) is one of the four most prevalent solid tumors that cause cancer-associated mortality globally. Early detection of CRC improves the 5-year survival rate from 12-13% in stage IV metastatic disease to 90% in stage I-II early-stage disease (1). Fecal occult blood test is typically used for CRC screening. Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) have been used for diagnosis and for disease monitoring following treatment. Although CEA and CA19-9 have exhibited a certain level of sensitivity, their sensitivity remains low. The pathological testing of tumor tissue is the optimal method for histological diagnosis, and the detection of mutations in rat sarcoma viral oncogene homolog [RAS; Kirsten RAS (KRAS) and neuroblastoma RAS (NRAS)] gene and B-raf serine/threonine kinase proto-oncogene (BRAF) gene in tumor tissue are used as predictive biomarkers aiding in the selection of targeted drug treatments (2). Colonoscopy of the primary tumor and needle biopsy of metastatic tumors are the techniques used for histological and genomic diagnosis; however, these methods are invasive, uncomfortable and costly (3-5). Thus, novel non-invasive methods for diagnosis and the detection of mutations are required.

Exosomes are small stable vesicles of 30-100 nm in diameter in the circulating blood, in which microRNA (miRNA/miR), mRNA and DNA fragments are coated in numerous proteins and bioactive lipids (6-9). Previous studies have identified that exosomes may be directly released from cells through the outward budding of the plasma membrane in a calcium-dependent manner, and be shuttled from donor cells to recipient cells (10-14). The level of exosomes released from cancer cells has been demonstrated to be increased compared with normal cells, and exosomal RNAs and proteins may implicate the origin of the donor cells (15,16). Therefore,

exosomes may serve as a highly sensitive and specific diagnostic tool for the repetitive and non-invasive monitoring of patients with cancer, aiding clinicians in the diagnosis, classification and treatment of cancer (17).

To validate the potential of the serum exosome as a novel biomarker for the monitoring of cancer, the current study investigated whether established cancer-associated mutations could be amplified from mRNA in the serum exosome of patients with cancer. In the present study, KRAS and BRAF gene mutations were detected in patients with CRC, and the consistency of the detection of these mutations between primary tumor tissues and the matched serum exosomes were compared. The results of the current study indicated that serum exosomal mRNA had the potential to be used for gene mutation detection in patients with CRC.

Materials and methods

Clinical samples. The current study consisted of 35 patients (age, 40-75 years; mean ± standard deviation, 60.0±9.5 years) from The First Affiliated Hospital of The People's Liberation Army General Hospital (Beijing, China). Patients underwent tumor resection surgery between July 2013 and December 2013 with histologically confirmed colorectal adenocarcinoma prior to the surgery. Colorectal primary tumor tissue samples were obtained from the surgical specimens and the matched blood samples were obtained from the patients prior to the surgery. Detailed information of the patients were presented in Table I. The classification of tumor differentiation and stage were assessed according to the 2000 World Health Organization (WHO) classification system for tumors of digestive system and the American Joint Committee on Cancer (AJCC) staging system, respectively (18). The present study obtained ethical approval from the Ethics Committees of The First Affiliated Hospital of The People's Liberation Army General Hospital (no. 2013067) and informed written consents were obtained for all patients.

Exosomes were obtained from blood serum. Exosomes were prepared using the differential ultra-centrifugation method, as previously described (19). Blood serum (5 ml) was centrifuged at 500 x *g* for 10 min, at 2,000 x *g* for 20 min and at 10,000 x *g* for 10 min, all at 4˚C. The supernatant was filtered through 0.22 μm disposable filter units, and transferred to an Amicon® Stirred Ultrafiltration Cell (Model 8050) with a 100,000 KDa molecular weight cutoff ultrafiltration membrane (all EMD Millipore, Billerica, MA, USA) at a nitrogen gas pressure of <75 psi (5.3 kg/cm^2). The samples were stirred and the rate of stirring was adjusted so that the vortex created was 1/3 the depth of the liquid volume. Following the supernatant ultrafiltration, 10 ml PBS was added and ultra-filtrated three times. Following washing twice, 0.1 ml PBS was added to suspend the exosomes. Subsequently, exosomes were isolated using ultra-centrifugation at 120,000 x g for 1 h at 4˚C and stored at -80˚C. The purified exosomes from the microcapsule membrane structure (30-80 nm) were observed using a Hitachi H-7500 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan; Fig. 1A). For immunoelectron microscopy, exosomes were incubated with a rabbit anti-human cluster of differentiation (CD)63 monoclonal antibody (dilution, 1:1,000; #EXOAB-CD63A-1; System Biosciences Inc., Palo Alto, CA, USA) for 1 h, followed by 20 μl Staphylococcal protein A Immunogold (dilution, 1:15; Meridian Life Science, Inc., Memphis, TN, USA) for 30 min at room temperature. Samples incubated with PBS were used as the blank control. The positively labeled exosomes were confirmed as vesicles containing black colloidal gold particles using a transmission electron microscope (Fig. 1B).

cDNA synthesized from exosomal mRNA. Total RNA were extracted from serum exosomes using the RNeasy Mini Spin kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. A total of 1 μl Oligo dT (5'-d(TTTTTT TTTTTTTTTTTT)-3') and 1 μl random sequence (Takara Bio, Inc. Otsu, Japan) were added into the 10 μl of RNA elution fluids, incubated at 70˚C for 10 min and then on ice for 2 min. For cDNA synthesis, reagents were added to the 12 μl RNA mixture described above, resulting in final concentrations (in 20 μl) of 200 U RNase M-MLV (RNase H-), 40 U ribonuclease inhibitor, 10 mM of each dNTP, 4 μl 5X M-MLV buffer (all from Takara Bio, Inc.) and RNase-free distilled H_2O. This reaction was incubated at 42˚C for 1 h, 70˚C for 15 min and then placed on ice. The cDNA products were stored at -20˚C.

Polymerase chain reaction (PCR) of KRAS and BRAF. Genomic DNA were extracted from 100 mg of tumor tissue using a standard protein kinase K procedure (20) and observed through separation on a 0.5% agarose gel and visualized by ethidium bromide (DL2000 DNA Marker, Takara Bio, Inc.). A total of 2 μl DNA isolated from tumor tissue or 2 μl cDNA from serum exosomes were added to result in the following final concentrations in a 20 μl reaction: 5 units Ex Taq (Takara Bio, Inc.); 0.2 μM of forward primer; 0.2 μM of reverse primer. The primers used in the PCR are presented in Table II. These reactions were incubated at 95˚C for 40 sec, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 20 sec. PCR products were separated on a 2% agarose gel (Fig. 2) and the target products were purified using UNIQ-10 Column DNA Gel Extraction kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's protocol. The remaining elution containing the purified DNA products was stored at -20˚C.

Cloning of the PCR products and gene sequencing. For the ligation of the PCR products and vector, 500 ng of PCR product was added to a total reaction volume of 10 μl that contained 0.1 μg T-Vector, 2 μl T4 DNA Ligase buffer (10X), 0.5 μl T4 DNA ligase (all from Takara Bio, Inc.) and distilled H_2O. These reactions were incubated at 22˚C for 2 h. DNA ligation product (5 μl) was added to 100 μl of competent DH5α *Escherichia coli* cells (Takara Bio, Inc.), mixed and incubated on ice for 30 min. The mixture was subjected to heat shock at 42˚C for 90 sec and subsequently incubated on ice for 2 min. A total of 900 μl super optimal broth with catabolite repression (SOC, Sangon Biotech Co., Ltd.) was added and the mixture was agitated at 37˚C for 1 h at 150 rpm. Subsequently, 100 μl or 900 μl of DH5α cells were spread onto ampicillin-containing Luria broth plates and incubated overnight at 37˚C. Plasmid extraction was performed using a TIANprep Mini Plasmid kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. A total of 30 μl of each elution

Table I. Clinicopathological characteristics of 35 CRC patients.

Clinicopathological characteristic	No. of patients (%)
Gender	
Male	22 (62.9)
Female	13 (37.1)
Age, years	
>65	10 (28.6)
≤65	25 (71.4)
Tumor site	
Colon	21 (60)
Rectum	14 (40)
Tumor differentiation	
G1	4 (11.4)
G2	20 (57.2)
G3	11 (31.4)
Tumor stage	
I	4 (11.4)
II	17 (48.6)
III	11 (31.4)
IV	3 (8.6)

G1, well differentiated; G2, moderate differentiation; G3, poor differentiation.

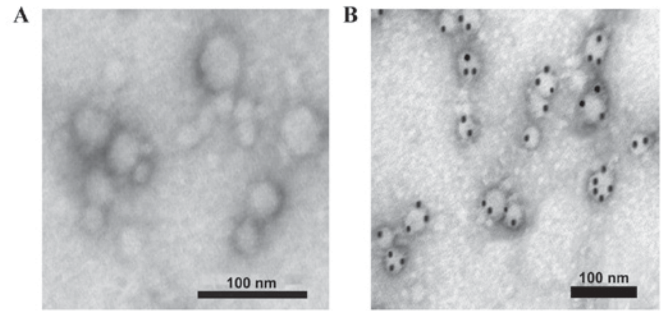

Figure 1. Exosomes obtained from the peripheral blood serum of patients with colorectal cancer. (A) Transmission electron microscopy images of exosomes collected. (B) The presence of serum exosomes expressing cluster of differentiation 63 were confirmed using immunoelectron microscopy (diameter, 30-80 nm).

sample was sent to Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for gene sequencing. The gene mutations identified in the samples are presented in Fig. 3.

Statistical analysis. Statistical analysis was performed using SPSS software (version 22.0; IBM SPSS, Armonk, NY, USA). The McNemar test was used to compare the distribution differences of KRAS and BRAF gene mutations between primary tumor tissues and matched serum exosomes in patients with CRC. The kappa statistic was used to assess the reliability of mutation detection in the tumor tissue and serum exosome samples. The consistency rate was the proportion of the samples with same mutation in the matched serum exosome and tumor tissue of total samples. The gene mutations distribution according to the clinicopathological characteristics of patients were analyzed using the χ^2 test. The significance of association between gene mutations and the age of patients were performed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

KRAS mutation detection in tumor tissues and matched serum exosomes. The mutation status of the KRAS gene was analyzed in 35 tumor tissues and the matched serum exosomes. The spectrum of these mutations is presented in Table III. The KRAS gene was not detected in 2 serum exosome samples. For the other 33 tumor tissue-serum exosome matched samples, 19 (57.6%) tumor tissues had a mutation in exon 2 or exon 3, of which 12 (36.4%) were at codon 12 of exon 2, 5 (15.2%) at codon 13 of exon 2 and 2 (6.1%) at codon 61 of exon 3. The most frequent mutation was G12D (42.1%) followed by G13D (26.3%) of all KRAS mutations in tumor tissues. A total of 14 (42.4%) matched serum exosomes contained a mutation in exon 2 or exon 3, of which there were 8 (24.2%) had a mutation at codon 12 of exon 2, 4 (12.1%) at codon 13 of exon 2 and 2 (6.1%) at codon 61 of exon 3. The most frequent mutation in serum exosomes was also G12D followed by G13D, which represented 35.7 and 28.6% of all mutations, respectively. There was no significant difference found in the distribution of detected KRAS mutations between tumor tissues and the serum exosomes of patients with CRC (P=0.063).

The sensitivity, consistency rate and κ score of KRAS gene mutation detection in the serum exosome of patients was calculated (Table III). Comparison of the KRAS mutation status between tumor tissue and the matched serum exosome identified that 84.8% (28/33) of patients with CRC had the same three KRAS mutations (codon 12, 13 and 61; data not shown). At codon 12 of exon 2, 8 (24.2%) patients with CRC were positive for a KRAS mutation in their tumor tissue and 5 (15.1%) in the serum exosome, giving a total consistency rate of 87.9%. At codon 13 of exon 2, 4 (12.1%) patients with CRC were identified to have mutant KRAS in their tumor tissue and serum exosome, with 1 (3.0%) patient positive for a KRAS mutation in their tumor tissue only, giving a total consistency rate of 97.0%. The KRAS mutation status was identical in tumor tissue-serum exosome matched samples at codon 61 of exon 3 and therefore the consistency rate was 100%. Overall, the sensitivity of exosomal mRNA KRAS mutation detection was 73.7%, the specificity was 100% and the total consistency rate was 94.9% (κ=0.819).

There was no significant association between KRAS mutations and the gender, age, tumor site, tumor differentiation and tumor stage of the patients, analyzed using the χ^2 test (P>0.05; Table IV). However, using the Student's t-test, the patients with a KRAS gene mutation at codon 12 of exon 2 were found to be significantly older compared with those patients without this mutation (tumor tissue, P=0.002; serum exosome, P=0.022).

BRAF mutation detection in tumor tissues and matched serum exosomes. BRAF mutations were detected in tumor tissues and matched serum exosomes (Table III). For the 33 tumor tissue-serum exosome matched samples, a BRAF mutation at

Table II. Primers used for polymerase chain reaction amplification of the KRAS and BRAF genes.

Gene target	Primer sequence (5'-3')	Predicted product size (bp)
KRAS-exon 2	F: TACTGGTGGAGTATTTGATAG	243
	R: TCCTGCACCAGTAATATGCATAT	
KRAS-exon 3	F: AAGTAAAAGGTGCACTGTAATAA	235
	R: AACCCACCTATAATGGTGAATATCT	
BRAF-exon 15	F: TTCATAATGCTTGCTCTGATAG	243
	R: AACTCAGCAGCATCTCAGGGCCAA	
KRAS-CDS	F: ATGACTGAATATAAACTTGT	230
	R: AGTCCTCATGTACTGGTCCCTC	
BRAF-CDS	F: TGGATTACTTACACGCCAAGTCA	238
	R: AATGCATATACATCTGACTGAAAGC	

KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, B-raf serine/threonine kinase proto-oncogene; CDS, cDNA fragment; F, forward; R, reverse.

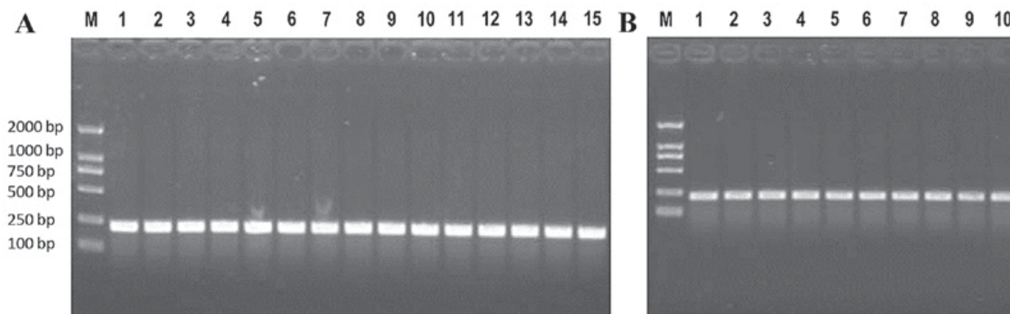

Figure 2. PCR products of KRAS and BRAF gene amplification from patients with CRC. (A) PCR products of KRAS and BRAF gene amplification in tumor tissues were separated using an agarose gel. Lanes 1-5, 243 bp fragment of KRAS-exon 2; lanes 6-10, 235 bp fragment of KRAS-exon 3; lanes 11-15, 243 bp fragment of BRAF-exon 15. (B) PCR products of KRAS and BRAF gene amplification from the matched serum exosomes were separated using an agarose gel. Lanes 1-5: 230 bp fragment of KRAS; lanes 6-10, 238 bp fragment of BRAF. Lane M, DNA marker. PCR, polymerase chain reaction; KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, B-raf serine/threonine kinase proto-oncogene; CRC, colorectal cancer.

codon 600 in exon 15 was detected in 8 (24.2%) of the tumor tissues and 6 (18.2%) of the serum exosomes. Gene detection was not possible in 2 serum exosomes, potentially due to a low level of exosomes in the serum. A total of 93.9% (31/33) of patients with CRC had the same BRAF mutation in their tumor tissue and serum exosome matched samples (data not shown), and therefore there was no significant difference in BRAF mutation distribution (P=0.500; Table III). In 6 (18.2%) patients with CRC, a mutated BRAF gene was detected in the tumor tissue and serum exosome, and in 2 (6.1%) patients with CRC the BRAF mutation was detected in their tumor tissue only. The sensitivity of BRAF gene mutation detection in the serum exosome was 75%, the specificity was 100% and the total consistency rate was 93.9% (κ=0.820). There was no significant association between BRAF mutations and the clinicopathological characteristics of the patients through the χ^2 test or the Student's t-test (all P>0.05; Table IV).

Discussion

Colorectal cancer is becoming the fourth most common malignant carcinoma in recent years (2). Early diagnosis and effective treatment may significantly reduce the mortality rate of this disease and these factors rely on accurate diagnosis, precise tumor staging and gene mutation status analysis. Aside from the traditional chemotherapy recommended by pathological diagnosis, epidermal growth factor receptor (EGFR) -targeted therapy may be administered according to the KRAS and BRAF gene mutation status of patients with CRC (5). Gene evaluation of the tumor tissue is the optimal standard for assessing mutation status, but it is typically performed a single time as it is invasive and costly. However, genomic alterations may differ in primary and metastatic tumor tissues as the disease progresses, and monitoring this requires repetitive genotyping. Other techniques that are in clinical use or are the focus of previous studies are not consistently successful; therefore novel methods allowing repetitive monitoring of these genetic events are being investigated (21-23).

It has been established that cancer initiation and progression are associated with numerous genetic and epigenetic factors, which may be detected through gene alternations in the tumor tissue. DNA, mRNA and miRNA are released into the blood and other bodily fluids from tumor tissues, and may be used to identify tumor-associated genetic and

Table III. KRAS and BRAF gene mutations in tumor tissues and matched serum exosomes.

				Detection in exosome		
Gene	Tumor tissue, no. (%)	Exosome, no. (%)	McNemar test P-value	Sensitivity (%)	Total consistency rate (%)	κ score
KRAS			0.063	73.7	94.9	0.819
Exon2 (codon 12)			0.125	66.7	87.9	0.718
G12D	8 (24.2)	5 (15.1)				
G12V	3 (9.1)	3 (9.1)				
G12A	1 (3.0)	0 (0.0)				
Wild-type	21 (63.7)	25 (75.8)				
Exon2 (codon 13)			1.000	80.0	97.0	0.872
G13D	5 (15.2)	4 (12.1)				
Wild-type	28 (84.8)	29 (87.9)				
Exon3 (codon 61)			1.000	100.0	100.0	1.000
Q61L	2 (6.1)	2 (6.1)				
Wild-type	31 (93.9)	31 (93.9)				
BRAF						
Exon15 (codon 600)			0.500	75.0	93.9	0.820
V600E	8 (24.2)	6 (18.2)				
Wild-type	25 (75.8)	27 (81.8)				

KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, B-raf serine/threonine kinase proto-oncogene.

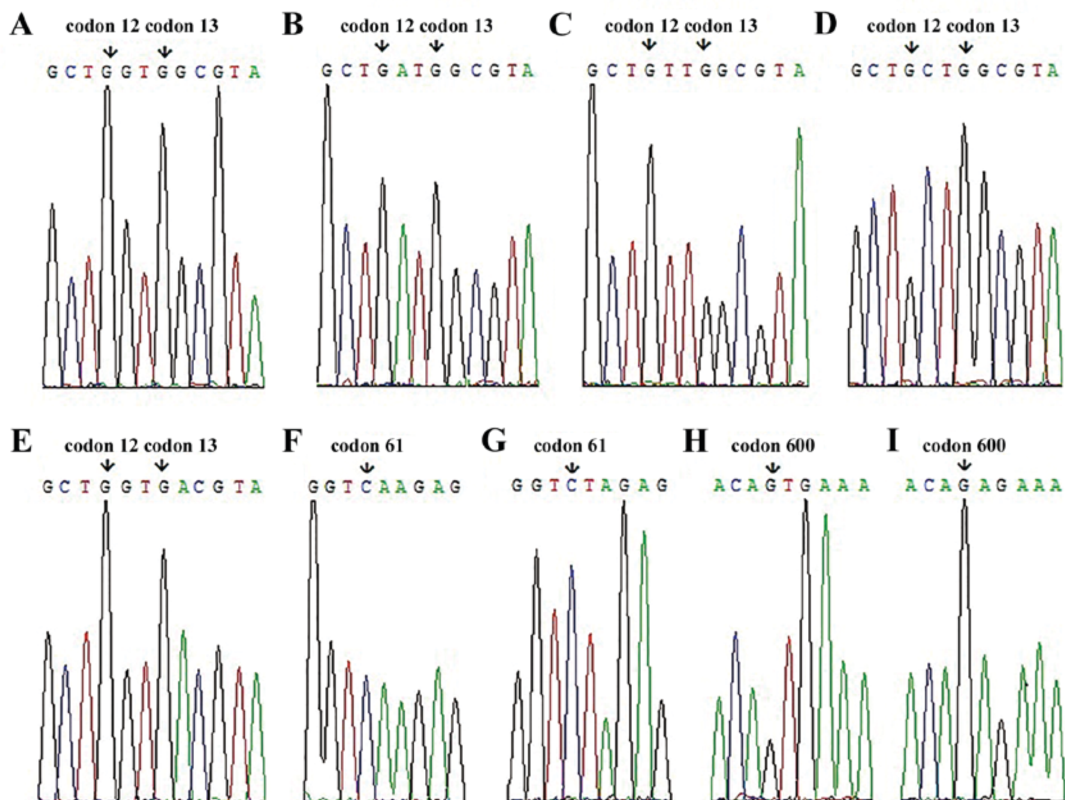

Figure 3. Mutations detected in the KRAS and BRAF genes in patients with colorectal cancer. (A) Wild-type KRAS gene, with arrows indicating codons 12 and 13. (B) G12D mutation of KRAS at codon 12 and wild-type codon 13. (C) G12V mutation of KRAS at codon 12 and wild-type codon 13. (D) G12A mutation of KRAS at codon 12 and wild-type codon 13. (E) Wild-type codon 12 and G13D mutation of KRAS at codon 13. (F) Wild-type codon 61 of KRAS. (G) Q61 L mutation of KRAS at codon 61. (H) Wild-type BRAF, with arrow indicating codon 600. (I) V600E mutation of BRAF at codon 600. KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, B-raf serine/threonine kinase proto-oncogene.

Table IV. Distribution of KRAS and BRAF mutations according to the clinicopathological characteristics of patients with colorectal cancer.

Clinicopathological characteristic	KRAS mutation				BRAF mutation			
	Tumor tissue		Exosome		Tumor tissue		Exosome	
	No. (%)	P-value[a]	No. (%)	P-value[a]	No. (%)	P-value[a]	No. (%)	P-value[a]
Gender								
Male	11/22 (50.0)	0.508	7/20 (35.0)	0.284	5/22 (22.7)	1.000	3/20 (15.0)	0.900
Female	8/13 (61.5)		7/13 (53.8)		3/13 (23.1)		3/13 (23.1)	
Age, years								
>65	8/10 (80.0)	0.120	6/10 (60.0)	0.335	3/10 (30.0)	0.849	3/10 (30.0)	0.503
≤65	11/25 (44.0)		8/23 (34.8)		5/25 (20.0)		3/23 (13.0)	
Tumor site								
Colon	14/21 (66.7)	0.072	9/20 (45.0)	0.710	5/21 (23.8)	1.000	4/20 (20.0)	1.000
Rectum	5/14 (35.7)		5/13 (38.5)		3/14 (21.4)		2/13 (15.4)	
Tumor differentiation								
G1	0/4 (0.0)	0.067	0/3 (0.0)	0.203	0/4 (0.0)	0.507	0/3 (0.0)	0.589
G2	12/20 (60.0)		10/19 (52.6)		5/20 (25.0)		3/19 (15.8)	
G3	7/11 (63.6)		4/11 (36.4)		3/11 (27.3)		3/11 (27.3)	
Tumor stage								
I-II	10/21 (47.6)	0.332	9/19 (47.4)	0.503	3/21 (14.3)	0.285	2/19 (10.5)	0.383
III-IV	9/14 (64.3)		5/14 (35.7)		5/14 (35.7)		4/14 (28.6)	

G1, well differentiated; G2, moderate differentiation; G3, poor differentiation; KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, B-raf serine/threonine kinase proto-oncogene. [a]P-value was calculated from the χ^2 test.

epigenetic alterations. These products may be more informative, specific and accurate compared with protein biomarkers. Sorenson *et al* (24) and Vasioukhin *et al* (25) identified that a RAS gene mutation may be detected in blood from cell-free DNA (cfDNA). Koyanagi *et al* (26) and Mori *et al* (27) identified that the association between circulating tumor cells and methylated cfDNA can aid in the assessment of disease severity and treatment efficacy in metastatic melanoma. Qiu *et al* (28) compared the diagnostic value of cfDNA and tumor tissue pathology, the current optimal standard, using a meta-analysis of EGFR mutations in non-small cell lung cancer, with the results suggesting that cfDNA is a highly specific biomarker, but has low sensitivity. However, other sources of cfDNA besides tumors exist and cfDNA is not stable for longer periods of time, therefore cfDNA has low sensitivity as a cancer biomarker (29-31). Previous studies have suggested that quantitatively assaying fecal DNA may provide a non-invasive method with improved sensitivity and specificity for the detection and monitoring of cancer (32,33). Ahlquist (34) identified mutated KRAS and tumor protein 53 (p53) genes in fecal samples from patients with CRC and associated these with the pathogenesis of the disease. However, fecal DNA testing is clinically challenging, as it is costly, time consuming and the results are variable due to DNA degradation (32,35). Despite the challenges, peripheral blood cfDNA and fecal DNA may provide the opportunity to repetitively monitor patients with cancer that are difficult to biopsy, but these methods have not yet been sufficiently successful.

In addition, RNAs are detectable in serum and other bodily fluids, and may also be a stable representation of exosomes (14,36-40). Exosomes are small membrane vesicles that are derived from the endosomal membrane compartment, following the fusion of multi-vesicular bodies with the plasma membrane, and have been found in a number of body fluids, including serum, malignant pleural effusion and urine (41-44). Previous studies have reported that exosomes released from a number of cell types, including immune, mesenchymal and cancer cells, contained identical proteins, mRNAs, miRNAs and DNA fragments (6-9). Ogata-Kawata *et al* (45) demonstrated that the serum exosome levels of seven miRNAs (let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223 and miR-23a) were significantly increased in patients with CRC compared with healthy controls, and significantly downregulated following surgical tumor resection. Furthermore, these miRNAs were also identified to be secreted at significantly higher levels in colon cancer cell lines compared with a normal colon-derived cell line (45). Skog *et al* (9) reported that serum exosomes were positive for the EGFR variant III mutation when the parental glioblastoma cells expressed the same mutation, and that the parental cells exhibited a lower rate of this mutation (28 vs. 47%). It has been reported that exosomes contain fragments of double-stranded genomic DNA of >10 kb, which spans all chromosomes, and that mutations in KRAS and p53 have been detected in pancreatic cancer cell lines and the serum from patients with pancreatic cancer (46). Although previous studies have reported that exosomes contain mitochondrial

DNA, single-stranded DNA and double-stranded genomic DNA (13,46,47), no DNA was detected in exosomes derived from MC/9, BMMC or HMC-1 cells (14). Therefore, the presence of exosomal DNA is not consistent or it may be at low quantities that cannot be analyzed. In addition, compared with membrane-binding proteins, RNA/DNA-binding proteins and lipoprotein complexes, exosomes remain stable despite the presence of RNases, proteases and adverse physical conditions, and may be stored at 4˚C for 96 h, at -70˚C for long periods of time and endure multiple freeze-thaw cycles (14,48-51). Due to these characteristics, exosomal RNA has been considered as a potential novel biomarker for predictive analysis in patients with cancer.

According to previous studies, the KRAS and BRAF gene mutation rate of tumor tissue differs from 20-50%, but is considered to be 35-45% in patients with CRC (52-54). The fraction of plasma exosomes in patients with CRC has been reported to be statistically higher compared with healthy controls (55). However, there is a lack of previous studies investigating the association between gene mutations in serum exosome and tumor tissue in patients with CRC. In the present study, KRAS and BRAF gene mutations were detected and the consistency of detected gene mutations was compared between tumor tissues and matched serum exosomes from patients with CRC. The mutation status of tumor tissue served as the reference for detectable mutations and was therefore compared with that of the matched serum exosome. These results demonstrated that the KRAS mutation rate was 57.6 and 42.4% and BRAF mutation rate was 24.2 and 18.2%, in the tumor tissues and the matched serum exosomes, respectively. In serum exosomes, the sensitivity of KRAS and BRAF mutation detection was 73.7 and 75% with the total consistency rate of 94.9 and 93.9%, respectively, and the specificity of these two gene mutations were 100%. Previous studies have reported a detection rate of KRAS mutation in cfDNA of 3-50% in patients with CRC (21-23), thus the efficacy of cfDNA screening remains to be elucidated. The current study hypothesized that the detection rate of mutation in exosomal RNA is higher compared with that in cfDNA, as exosomal RNA were found to be enriched and stable (9). However, the results of the current study demonstrated that the KRAS mutation rate of serum exosomal RNA was similar to that of cfDNA. The similar detection rate may be due to the small sample size, the serum sample preparation, the exosome collection method, the RNA or DNA extraction method, or the sequencing method. In future, purifying the serum exosome RNA may increase the mutation detection rate.

Aging is a consequence of the accumulation of unrepaired naturally-occurring DNA damage. DNA damage typically causes errors in DNA replication or repair, and these errors are the primary source of mutations. Epigenetic alterations may also occur as a result of environmental exposure. The present study demonstrated that CRC patients with a KRAS mutation at codon 12 of exon 2 in their tumor tissue and serum exosome were significantly older compared with those without this mutation. It has also been established that KRAS mutation is significantly higher in CRC patients who are >50 years old in the Indian population (56). However, no statistically significant difference in the distribution of age was identified according to KRAS mutation status of patients with CRC in American (57) or Chinese (58) populations. Although the association between KRAS mutation and age has not yet been fully elucidated, it may be suggested that the KRAS gene mutation rate increases with age and other factors, including chemotherapy, radiotherapy and disease progression.

The present study identified that serum exosomal mRNA detection may be effective for the repetitive and non-invasive genotyping of patients with CRC, particularly in patients without the opportunity for a biopsy prior to treatment selection. However, the results of the current study were obtained from a small sample size, thus further studies with a larger sample size in multicenter settings are required to validate these results. The application of exosomes as a cancer biomarker is the focus of current studies, but not yet sufficiently optimized for clinical use. Whether the diagnostic and predictive value of exosomal RNA is similar to DNA from cancer tissues remains to be elucidated, but exosomes have the potential to replace tissue samples in certain situations. Whether exosomes may be used for clinical assessment, including overall survival and progression-free survival, also remains to be elucidated.

In conclusion, exosomal RNA has the potential to replace existing cancer tissue and blood biomarkers to provide information for diagnostic screens, personalized medicine and treatment efficacy.

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