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Monitoring of Resistant-Related Genes to Cetuximab in Extracellular Vesicle-Derived DNA from Metastatic Colorectal Cancer Patients

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Keywords:

Extracellular vesicle-derived DNA; Gene mutation; Metastatic colorectal cancer; Cetuximab

1. Abstract

1.1. Background: Extracellular vesicle derived-DNA (EV DNA) have emerged as attractive diagnostic biomarkers in diseases diagnosis, while its role in metastatic colorectal cancer (mCRC) is unknown. This study aimed to explore the role of EV DNA in monitoring of gene mutations related to drug resistance during therapy with the anti-EGFR antibody cetuximab.

1.2. Methods: Tumor tissue and blood samples from 28 patients were collected. EV DNA was extracted from Extracellular vesicle and sequenced.

1.3. Results: Our work showed that EV DNA sequencing provided very high consistency with tumor tissue, which can genotype colorectal tumors and detect gene mutations related to drug resistance during therapy with cetuximab. Furthermore, use of EV DNA also demonstrates that TP53/KRAS co-mutation was significantly associated with poor overall survival.

1.4. Conclusion: Monitoring of cetuximab-resistant gene mutations in EV DNA could be attractive diagnostic methods and provide a comprehensive overview of the acquired resistance mutational landscape.

2. Introduction

Colorectal cancer is one of the leading causes of cancer-related mortality worldwide [1]. Despite improvements made in treatment strategies, in particular of the introduction of agents targeting EGFR, such as cetuximab, many tumors eventually manifest acquired resistance to treatment for mCRC patients [2]. Clonal evolution associated with acquired resistance presents a critical therapeutic challenge [3]. Definition of the molecular changes underlying acquired resistance to anti-EGFR antibodies is needed to improve clinical benefit and devise further lines of treatment [4]. Mutations in KRAS are considered to be the main drivers of acquired resistance to cetuximab in mCRC [5]. Other escape routes include mutations in the EGFR signaling pathway and its intracellular signal transduction intermediates, such as NRAS, BRAF, PIK3CA, ERBB2, and PTEN mutations, as well as members of the platelet-derived growth factor receptor α signaling pathway [2, 6].

Acquired resistance mechanisms have been conventionally identified by obtaining individual resistant tumor lesions for molecular analysis. However, tumor tissue genotyping has inherent limitations for dynamic monitoring of disease progression and response to therapy: tumor spatial and temporal heterogeneity, difficulties to obtain repeated tissue samples [7]. Liquid biopsy has been proposed as a

way to overcome the restrictions of tumor tissue genotyping, which can be collected with minimal invasiveness and permit following the disease over time [8]. Several studies have exploited circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) to track clonal evolution during the treatment of cetuximab [9]. As the important liquid biopsy targets, EVs can be released from tumor cells to extracellular space and biological fluids, carrying biological molecules including DNA, RNA, and proteins, which can domesticate recipient cells and become potential biomarkers for cancer diagnostics and prognosis [10]. EVs are more abundant in quantity than CTC, and EV DNA is more stable than ctDNA for being surrounded by a lipid bilayer membrane, which is the premise that EV DNA could serve as minimally invasive liquid biopsies for longitudinal sampling to follow disease progression. EV DNA has been demonstrated valuable in detecting cancer-associated mutations in many kinds of tumors [11]. However, little evidence supports the clinical relevance of EV DNA in mCRC patients. Our previous study has demonstrated the stability of EV DNA in mCRC and high consistent of genomic variants between EV DNA and tissue samples [12]. Herein, EV DNA from dynamic blood samples of mCRC patients treated with cetuximab was detected by second-generation sequencing to assess the potential role of EV DNA as a biomarker for cetuximab resistance in mCRC.

3. Material and Methods

3.1. Patients and Blood Samples

This prospective, single-center study (conducted from May 2015 to July 2017) included patients with mCRC who received chemotherapy with molecular targeted drugs cetuximab in the Fifth Medical Center of General Hospital of PLA. The main inclusion criteria were histologically confirmed TNM stage IV colorectal adenocarcinoma; older than 18 years of age; patients with wild-type RAS (including KRAS and NRAS on exon 2, 3, 4) and wild-type BRAF sequences, confirmed by sequencing; any previous treatment with the exception of cetuximab; Eastern Cooperative Oncology Group performance status of 0 to 1; measurable metastatic disease according to the Response Evaluation Criteria in Solid Tumors, version 1.1. Exclusion criteria were age less than 18 years, secondary primary malignant tumor, palliative or emergency surgery needed. This study only involved an observational protocol, and did not affect the patients' treatment. This study protocol was approved by the Ethics Committee of the Fifth Medical Center of PLA General Hospital (No. KY-2011-8-3), and was conducted in accordance with International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). Written informed consent was obtained from each patient. Computed tomography (CT) scans were performed every 6 to 8 weeks to evaluate clinical response using the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1. Serial peripheral blood was sampled from each patient at baseline and during the treatment. Blood samples (6 ml) were centrifuged at 3,000 *g* for 15 min at 4°C for plasma isolation and then stored at -80°C until the time of EV isolation. From May 2015 to May 2019, 123 longitudinal blood samples from 28 patients were collected, including 28 pretreatment samples and 26 samples at disease progression (PD), 67 samples during treatment and 2 samples at the last blood collection.

3.2 Statistical Analyses

Descriptive comparisons of study variables used the Fisher's exact test for categorical data. Survival curves were generated using the Kaplan–Meier method, and log-rank tests were used to compare survival curves. Multivariate Cox proportional hazards analysis were performed to examine potential clinical and molecular factors contributing to survival. All statistical analyses were performed with SPSS (v.21.0; STATA, College Station, TX, USA) or GraphPad Prism (version 6.0; GraphPad Software, La Jolla, CA, USA) software. Statistical significance was defined as a two-sided *P*-value of <0.05.

4. Results

4.1. Patient Characteristics and Clinical Therapeutic Response

In total, 28 patients were included from May 2015 to July 2017, and patient characteristics are described in Table I. No mutations in KRAS, NRAS, and BRAF were detected in pretreatment tumor tissue in 28 patients. Patients received cetuximab combined with chemotherapy, including irinotecan-based chemotherapy (8, 28%), oxaliplatin-based chemotherapy (10, 36%), and fluorouracil drugs (10, 36%). The median progression free survival (PFS) of the whole cohort was 9.27 months (95% CI 8.56–20.24), and median overall survival (OS) was 21.4 months (95% CI 18.7–32.7). At the time of analyses (December 2019), two patients did not progress and 25 (85.3%) had died. 26 patients with resistance to cetuximab were divided into two groups based on the response evaluation at week 12. We defined primary resistance as PFS < 12 weeks and acquired resistance as PFS ≥ 12 weeks. Of all, 2 patients were primary resistant to cetuximab and 24 patients developed acquired resistance.

4.2. Identification of EVs

The EVs were extracted from the plasma of all mCRC patients by ultracentrifugation, and EVs were evaluated by TEM and NTA to analyze the morphology and sizes distribution. TEM and NTA analysis showed that EVs were bowl-shaped with a size range mainly between 75 nm to 200 nm (Figure 1A and B, Supplementary Table 1).

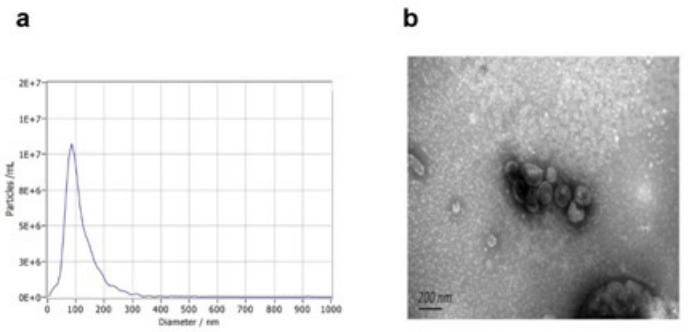

Figure 1: Isolated EVs from patients' plasma. (A). NTA results suggested that EVs enriched from plasma were about 75-200 nm in diameter. (B). TEM images showed that EVs were oval or bowl-shaped capsules without the nucleus.

Table 1: Clinical Characteristics of Patients with mCRC

Patient Characteristics	**N =28**
Median age, years (range)	55 (25–78)
Gender, n (%)	
Male	17 (61)
Female	11 (39)
ECOG PS, n (%)	
0	6 (21)
1	22 (79)
Tumor Differentiation, n (%)	
Well/Moderate	69 (79)
Poor	18 (21)
Primary Tumor Location, n (%)	
Right-sided	8 (29)
Left-sided	20 (71)
Synchronous metastases, n (%)	12 (43)
Metastatic sites, n (%)	
Liver	17 (61)
Lung	6 (21)
Lymph nodes	6 (21)
Other	3 (7)
Line of prior therapy, n (%)	
< 2 lines	17 (61)
≥ 2 lines	11 (39)
Regimen, n (%)	
Irinotecan-based chemo + Cet	8 (28)
Oxaliplatin-based chemo + Cet	10 (36)
Fluorouracil drugs + Cet	10 (36)
Best response, n (%)	
CR	1 (4)
PR	14 (50)
SD	11 (39)
PD	2 (7)

Abbreviation: PS, performance status; chemo, chemotherapy; Cet, cetuximab.

Supplementary Table 1: The data of nanoparticle tracking analysis for EVs.

Diameter / nm	Particles/mL	FWHM / nm	Percentage
88.0	1.0E+7	62.4	96.0
300.6	1.9E+5	82.6	1.1
363	5.4E+4	19.4	0.2
514.9	3.7E+4	21.0	0.2
404.0	3.1E+4	11.6	0.2

NOTE: FWHM: full width at half maximum.

4.3. Pretreatment EV DNA Mutations

A total of 28 pretreatment EV DNA were sequenced. 46% (13/28) of EV DNA were detected to harbor mutations in designed targeted regions. The most frequent mutant genes were TP53 (29%, 8/28), EGFR (11%, 3/28) (Fig. 2A). Sequencing results of baseline EV DNA obtained prior to start of cetuximab therapy were compared to the mutational status found in routinely tested tumor tissue of which KRAS, NRAS and BRAF had been detected. The results revealed an almost perfect consistency (Kappa 0.964-1.000) between EV DNA and tissue in detecting the mutation of RAS and BRAF genes (Supplementary Table 2).

Three cases were detected to harbor mutations in EV DNA, while no mutation in tumor tissue. Of them, two patients (No. 18 and No. 19) primary resistant to cetuximab, were detected to harbor mutation in BRAF or EGFR in baseline EV DNA. BRAF p. L588P mutation was confirmed in EV DNA for patient No.19, and EGFR p.L453P mutation was confirmed in EV DNA for patient No.18, both were not detected in tumor tissue. Furthermore, a mutation in codon 12 of KRAS was found in pretreatment EV DNA in one patient (No. 2), which were not detected in tumor tissue. Further validation of this clonal mutation in EV DNA was still clustered during the disease progression. This patient received FOLFIRI-cetuximab as the first line treatment, maintained a disease-stable state for only 4.1 months and then progressed. This likely reflects what we already have known about sampling bias of selected tissue specimens that confound resolution of the clonal status of mutations and illustrate the problems of using tissue alone as the gold standard.

Supplementary Table 2: The consistency of tumor tissue and EV DNA in detecting pretreatment mutation genes.

	EV DNA	Tumor tissue		Total	Kappa	95%CI
		Wild	Mutant			
KRAS	Wild	27	0	27	0.964	1.033-0.895
	Mutant	1	0	1		
	Total	28	0	28		
NRAS	Wild	28	0	28	1.000	1.000-1.000
	Mutant	0	0	0		
	Total	28	0	28		
BRAF	Wild	27	0	27	0.964	1.033-0.895
	Mutant	1	0	1		
	Total	28	0	28		
PIK3CA	Wild	27	1	28	0.929	0.879-0.979
	Mutant	0	0	0		
	Total	27	1	28		
EGFR	Wild	24	1	25	0.627	0.385-0.869
	Mutant	1	2	3		
	Total	25	3	28		
TP53	Wild	20	1	21	0.579	0.393-0.765
	Mutant	3	4	7		
	Total	23	5	28		

4.4. Multiple Resistant-Related Genes to Cetuximab Co-Mutations in EV DNA

Of 24 acquired cetuximab-resistance patients, 13 (54.2%, 13/24) patients were mutation-positive in known resistant-related gene, including KRAS (69%, 9/13), PIK3CA (54%, 7/13), NRAS (8%, 1/13), BRAF (8%, 1/13), PTEN (8%, 1/13), EGFR (23%, 3/13) and PDGFRA (8%, 1/13) (Fig. 2B). Interestingly, we found multiple pathways were simultaneously activated in the same patients. 8 (62%, 8/13) cases were detected to harbor mutations in multiple resistant-related genes (Supplementary Table 3). For example, clone temporal evolution of serial EV DNA samples during treatment of patient (No. 20) showed an increase in the abundance of BRAF p.E586G, which was a potential functional mutation in the RAS/RAF pathway as disease progressed. Meanwhile, a new PIK3CA clone, PIK3CA p.K944N, emerged in EV DNA, which was reported to be related to cetuximab resistance by activation of the PI3K-Akt-mTOR signaling pathway [13]. The abundance of subclonal EGFR p.L862R and subclonal PDGFRA p.V982A mutation increased in serial EV DNA samples concurrently. These observations may reflect heterogeneity in the mechanisms of cetuximab resistance in mCRC.

Supplementary Table 3: Multiple gene mutations in EV DNA at PD.

Case No.	Gene	Transcript Accession	Exon	Nucleotide(cDNA)	Amino acid(protein)	AF(%)	P Value
9	KRAS	NM_004985	2	c.35G>A	G12D	7.65	<0.0001
9	KRAS	NM_004985	2	c.38G>A	G13D	6.08	<0.0001
9	PIK3CA	NM_006218	19	c.2832A>T	K944N	2.63	<0.0001
11	PIK3CA	NM_006218	19	c.2900G>T	G967V	2.2	<0.001
11	BRAF	NM_004333	15	c.1757A>G	E586G	2.91	<0.0001
11	EGFR	NM_005228	21	c.2585T>G	L862R	3.99	<0.0001
11	PDGFRA	NM_006206	22	c.2945T>C	V982A	2.71	<0.0001
11	PDGFRA	NM_006206	22	c.3002A>G	K1001R	2.73	<0.0001
13	EGFR	NM_005228	21	c.2585T>G	L862R	4.70	<0.0001
13	PIK3CA	NM_006218	19	c.2791C>A	H931N	2.03	<0.01
16	KRAS	NM_004985	2	c.35G>A	G12D	2.29	<0.001
16	KRAS	NM_004985	3	c.183A>C	Q61H	3.15	<0.0001
16	EGFR	NM_005228	21	c.2585T>G	L862R	4.74	<0.0001
17	EGFR	NM_005228	21	c.2585T>G	L862R	4.33	<0.0001
17	KRAS	NM_004985	2	c.14_15insG	L6Tfs*28	5.04	<0.0001
17	NRAS	NM_002524	3	c.185A>G	E62G	2.86	<0.001
17	PTEN	NM_000314	8	c.758T>C	I253T	2.35	<0.001
20	KRAS	NM_004985	2	c.35G>A	G12D	2.63	<0.001
20	PIK3CA	NM_006218	19	c.2816A>G	D939G	2.41	<0.001
24	KRAS	NM_004985	2	c.35G>A	G12D	2.13	<0.001
24	PIK3CA	NM_006218	19	c.2791C>A	H931N	2.03	<0.01
27	KRAS	NM_004985	2	c.38G>A	G13D	3.62	<0.001
27	PIK3CA	NM_006218	19	c.2816A>G	D939G	2.03	<0.01

NOTE: P value: The differences of the identified mutant allelic fraction from the background non-reference allelic fractions were calculated using Student t test.

Abbreviation: AF, mutant allelic fraction of the last plasma sample; PD, progressive disease.

Supplementary Table 4: Primers used in PCR reactions.

Gene	Forward primer	Reverse primer
KRAS G12 G13	5'-CCTGCTGAAAATGACTGAATATA-3'	5‘TCTATTGTTGGATCATATTCGTC3’
KRAS Q61	5'-AATTGATGGAGAAACCTGTCTCTT-3'	5‘TTATGGCAAATACACAAAGAAAGC3’
TP53 R175-R179	5'-ATCTACAAGCAGTCACAGCACAT-3'	5‘CTAAGAGCAATCAGTGAGGAATC3’
TP53 R213	5'-ATTTGCGTGTGGAGTATTTGGA-3'	5‘TAACCCCTCCTCCCAGAGAC3’
TP53 G245-R248	5'-TTGGGCCTGTGTTATCTCCTAG-3'	5‘TCTTCCAGTGTGATGATGGTGAG3’
TP53 R273-R282	5'-TGCCTCTTGCTTCTCTTTTCC-3'	5‘TTGCGGAGAATTCTCTTCCTCTG3’
PTEN R233	5'-TGAAGATATATTCCTCCAATTCAG-3'	5‘TGTTTGTGGAAGAACTCTACTTTG3’
NRAS Q61	5'-TGAAGATATATTCCTCCAATTCAG-3'	5‘ATTATTGATGGCAAATACACAGAG3’
PIK3CA E542-E545	5'-AGCTAGAGACAATGAATTAAGGGA-3'	5‘TAGCACTTACCTGTGACTCCATAG3’
PIK3CA H1047	5'-ATTCGAAAGACCCTAGCCTTAGAT-3'	5‘CCATTTTTGTTGTCCAGCCAC3’
PIK3CA Exon 19	5'-CATAATTTCTTATTTTTGAAAGCTG -3'	5‘TCTCTTGTCTTTGTGCATTCTTGC3’
EGFR E746 P753	5'-CTCTCTCTGTCATAGGGACTCTG-3'	5‘AGCAAAGCAGAAACTCACATC3’
EGFR L833 H835 L858	5'-TGAACTACTTGGAGGACCGTCG-3'	5‘GTATTCTTTCTCTTCCGCACCC3’
EGFR G719	5'-CACCCAGTGGAGAAGCTCCCA-3'	5‘TTATACACCGTGCCAACGCA3’
ERBB2 L755	5'-TCACTCACATCCTCCTCTTT-3'	5‘CTAAGATTTCTTTGTTGGCTTTG3’
PDGFRA R981	5'-TATGAAAAATTCACCTGGACTTC-3'	5‘AGTCTCTGCTCATCCAGACCAC3’
BRAF V600	5'-TCTTCATAATGCTTGCTCTGATAG-3'	5‘TCGAGATTTCACTGTAGCTAGACC3’

4.5. Monitored EV DNA for prediction of progressive disease

We further monitored EV DNA abundance during patient received cetuximab treatment. EV DNA abundance increased when disease progressed. For example, Patient No.13 achieved a 50% reduction in the size of liver metastases during the patient's initial eight weeks on FOLFOX-cetuximab treatment. In the following two months, the patient was treated with FOLFOX-cetuximab, and maintained partial response. Subsequent plasma analyses revealed a gradual increase in the proportion of PIK3CA p.H931N EV DNA alleles from 0.81% to 2.03% and a rapid increase in the proportion of EGFR G12C ctDNA alleles from 0.71% to 4.7% that anteceded disease progression (Fig. 3). We found EV DNA abundance increased at or before PD in 13 patients. The mean fold change of increasing EV DNA was 2.63 (ranged from 0.14 to 11.89) and showed a leading time of 8.9 weeks (ranged from 4 to 16 weeks) before imaging method.

4.6. Acquired TP53/KRAS Co-Mutation Related to The Poor Outcome

Genomic aberrations changed with cancer evolution or drug selection pressure. In this study, after treatment, secondary TP53/KRAS co-mutation were detected in 5 pts, we further analyzed the prognostic value of co-mutation of TP53/KRAS for patients. TP53/KRAS co-mutation (18%, 5/28) was significantly associated with poor outcomes (median OS, 14.23 months, v.s. 29months; P =0.006; Fig. 4A), but no statistical difference in PFS (Figure 4B). TP53/KRAS co-mutation was also significantly associated with OS as determined by COX multivariate analysis (HR, 6.50 [95% CI, 1.27-33.40]; P=0.025) after adjusting for other clinical covariates, including tumor site, gender, age, and ECOG score.

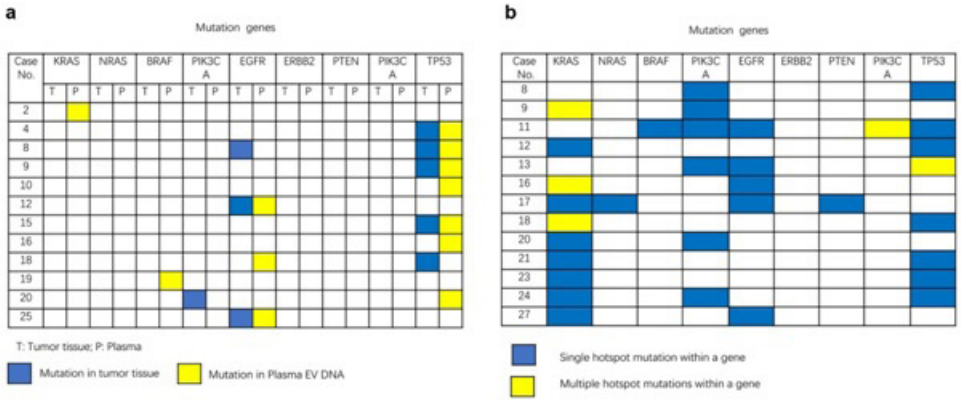

Figure 2: Genetic mutations detected in tumor tissues and in plasma EV DNA before and after treatment. (A). Genetic mutations detected in tumor tissue and in pretreatment plasma samples from individual patients. (B). Genetic mutations identified in EV DNA of individual patients that were associated with acquired resistance to cetuximab.

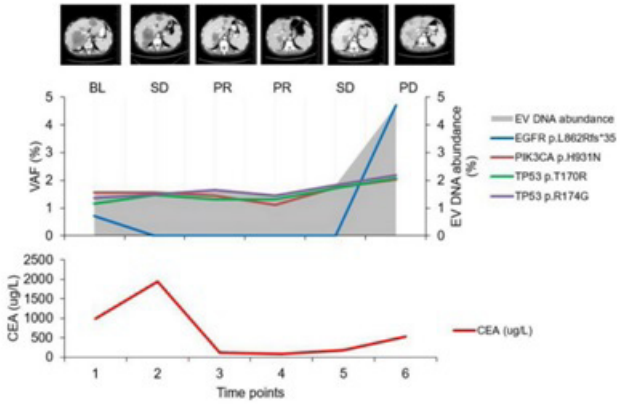

Figure 3: Treatment response and EV DNA status of patient No. 13 during FOLFOX-cetuximab treatment. Patient No. 13 presented a gradual increase of PIK3CA mutation and a rapid increase of EGFR mutation in EV DNA that anteceded disease progression. SD, stable disease; PR, partial response; PD, progressive disease.

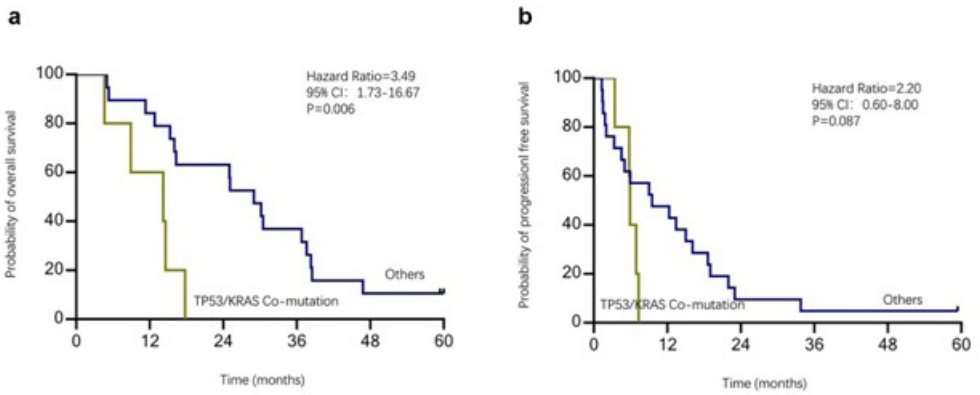

Figure 4: The prognosis of patients with secondary TP53/KRAS co-mutations in post-treatment EV DNA. (A). Overall survival between patients with and without acquired TP53/KRAS co-mutations. (B). Progression free survival between patients with and without acquired TP53/KRAS co-mutations.

5. Discussion

The present study summarizes the results of a prospective study of EV DNA performed on RAS wild-type mCRC patients who were treated with cetuximab. In recent years, EVs have been regarded as carriers of molecular biomarkers and mediators of intercellular communication. Although most studies have focused on the protein, lipid, and RNA components of EVs, EV DNA remains largely unknown [14]. EV DNA has potential functions in many pathological states, including malignancies and autoimmune diseases. Moreover, some studies have shown that EV DNA is valuable in detecting cancer-related mutations [11, 15]. However, whether EV DNA could represent a resource for dynamic monitoring of genetic mutations was rarely reported. Herein, our results support concordance between the results of mutation analyses performed using EV DNA and those that use tumor tissue biopsies (the current standard approach used at most institutions). The results of the present study showed that EV DNA sequencing provided very high consistency with tumor tissue when used for tumor genotyping.

EV DNA is a valuable non-invasive method for tumor mutation genotyping. A total of 28 pretreatment EV DNA were sequenced, although covering small target regions of several genes, we still find genomic alterations in EV DNA detected in 46% of cases. The spectrum and frequency of valuable alterations identified in EV DNA demonstrate a similarity to three colorectal cancer sequencing studies, including two tissue and one cell free DNA (cfDNA) cohorts [16-18]. These results support the feasibility and validity genomic analysis of EV DNA, at least in clinically valuable genes testing.

In addition to assess the prevalence of mutations detectable in EV DNA, we also explored mutations related to cetuximab resistance in colorectal cancer. Because of the limited insights available based on tumor-tissue datasets, we focused on the dynamic changes in EV DNA samples to find mutations attributable to cetuximab resistance. Mutations in the EGFR pathway significantly influence the efficacy of cetuximab treatment, including mutations in RAS, BRAF, PIK-3CA, PTEN and etc [4, 19-23]. Here, patients who eventually exhibited cetuximab resistance showed changes in their molecular profiles as positive including KRAS, NRAS, BRAF, PIK3CA, EGFR, PTEN, and PDGFRA mutations during anti-EGFR therapy. Therefore, EV DNA sequencing is a noninvasive and convenient means to identify resistance-associated mutations.

Our result also revealed the role of heterogeneity of cetuximab resistance mechanisms in patients with mCRC. Heterogeneous resistance mutations were identified in the EV DNA of individual patients, as demonstrated by the existence of multiple tumor resistance mechanisms in a given individual [24, 25]. Indeed, we observed up to four different mutations associated with cetuximab resistance in a single patient. The co-occurrence of multiple cetuximab resistance mechanisms suggests that profound and complicated molecular mechanisms occur in the course of cetuximab to facilitate resistance. This finding also highlights the difficulty to overcome extensive resistance mechanisms using a single therapeutic method, especially with respect to those mutations that often affect multiple functionally distinct targets in a single patient. Furthermore, polyclonal nature is very common whether in primary or acquired resistance to EGFR antibody and the dynamics can be discovered in EV DNA, allowing prediction of the expected time to therapeutic failure in individual patients. In this study, the emergence of resistant mutations was detectable in EV DNA from patients with anti-EGFR therapy about 4 to 16 weeks before radiographic evidence of disease progression.

Previous study showed EV released from tumor cells could result in cetuximab resistance by downing PTEN and increasing phosphorylated Akt levels [26]. Our study demonstrated serial monitoring of EV DNA could provide clues for acquired cetuximab resistance and provide evidence for subsequent treatment.

The predictive function of TP53 mutations in mCRC patients treated with targeted therapies has not been so far established [27]. TP53 was the most frequently mutated gene in our study, we analyzed the value of acquired TP53/KRAS co-mutation in the group of patients treated with cetuximab. Among the 28 mCRC patients treated with cetuximab, 5 patients were detected with acquired TP53/KRAS co-mutation. We found TP53/KRAS co-mutation was related with shorter OS in patients with mCRC. Our study suggests that patients

with secondary KRAS mutations after treatment with cetuximab have a worse prognosis if TP53 mutations are present at the same time. We speculate that TP53 genotyping could have an additional prognostic value in mCRC patients treated with targeted therapies, which should be confirmed on larger mCRC series.

6. Conclusion

In conclusion, monitoring of cetuximab-resistant gene mutations in EV DNA could be attractive diagnostic methods and provide a comprehensive overview of the acquired resistance mutational landscape. Major limitation of this study is that the findings were established in a relatively small cohort. Future investigation should be designed to provide strong evidence supporting the validity and feasibility of large-scale analysis of EV DNA.

7. Supplementary Method

Exosomes isolation. The ultracentrifugation (UC) method was optimized according to the method previously described [13]. At 37°C, after thawing, the plasma samples were centrifuged to remove the cell debris at 3,000 g for 15 min. Next, the supernatant was diluted with seven-fold volume of PBS, centrifuged at 13,000 g for 30 min, and treated with a 0.22 μm filter to remove the large particles. The supernatant was ultracentrifuged using a P50AT2-986 -rotor (CP100NX; Hitachi, Brea, CA, USA) at 100,000 g for 2 h at 4°C to pellet the EVs. The particles were resuspended in PBS and centrifuged at 100,000 g for 2 h at 4°C. After washing with PBS, the EVs pellets were resuspended in PBS.

Nanoparticle tracking analysis (NTA). ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) was used with a 405 nm laser to detect the vacuole enrichment suspension with concentration between 1×107/ml and 1×109/ml, and to determine the size and number of isolated particles. A video with a duration of 60 s was shot at a frame rate of 30 frames per second, and NTA software was used to analyze particle movement (ZetaView 8.02.28).

Transmission electron microscopy (TEM). 20 μl EVs enriched solution was placed on a copper mesh and incubated at room temperature for 10min. After washing with sterile distilled water, EVs enriched fraction was contrasted by uranyl oxalate solution for 1 min. Dry samples under incandescent lamp 2 min. The copper mesh was observed and photographed under a transmission electron microscope (JEOL-JEM1400, Tokyo, Japan).

Extraction of EV DNA. Before DNA separation, the samples were treated with 2000 U/ml DNase I (New England BioLabs, Frankfurt, Germany) at 37°C for 2 h to remove possible nucleic acid contaminants. After treatment, the enzyme was heated to an inactivated state at 75°C for 10 min (this step was only used to verify the serum DNA distribution). Firstly, total DNA was extracted from EVs using DNA lysis buffer [0.5% SDS, 0.05M EDTA, 0.01M Tris-HCl, pH 8.0, 0.1M NaCl, 200 μg/ml protease K (Amresco, Solon, Ohio, USA)]. 400 μl DNA lysates were added to each tube of EVs. After mixing, the mixture was incubated at 55°C for 24 h. Secondly, the balance of phenol and chloroform was used for deproteinization. Thirdly, 3 M CH3COONa, glycogen and anhydrous ethanol were used to precipitate DNA for 24 h at -20°C, and TE (0.001 M EDTA, 0.01 M Tris-HCl, pH 8.0) was used to suspense DNA at 37°C for at least 16 h. EV DNA was quantified on a nanometer drop ND-2000 spectrophotometer (Thermo Fisher Scientific) [12].

PCR analysis and sequencing. PCR analysis was performed using specially designed primers for preamplified DNA products from EVs (Supplementary Table 4). The PCR product was sent to Invitrogen Biotechnology for sequencing analysis. 18 DNA fragments of 8 mCRC development and resistance related genes were covered, including TP53 (R175-R179, R213, G245-R248, R273-R282), KRAS (G12-G13, Q61), PTEN (R233), NRAS (Q61), PIK3CA (E542-E545, H1047, Exon 19), EGFR (E746-P753, L833-H835, L858, G465), ERBB2 (V777), PDGFRA (R981) and BRAF (V600), with sequencing depth over 10000×.

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9. Ethics Approval

This study was approved by the Ethics Committee of the Fifth Medical Center of PLA General Hospital (No. KY-2011-8-3), and was conducted in accordance with International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). Written informed consent was obtained from each patient.

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