Analysis of cancer-related mutations in extracellular vesicles RNA by Droplet Digital™ PCR

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ABSTRACT

Extracellular vesicles (EVs) are taking their place as potential biomarkers in the field of liquid biopsy. In this study, EVs were isolated from plasma samples of 31 patients with colorectal cancer and melanoma via differential centrifugation and Droplet Digital™ PCR (Bio-Rad, CA, USA) was used to profile *BRAF* V600E/K, *KRAS* G12A/C/D/V and *KRAS* G13D mutations from EV-derived cDNA. The concordance rates with corresponding tissue were 54% and 44% in the colorectal cancer and melanoma cohort, respectively. Two patients displayed mutations in EVs not previously detected in tissue as evidence for emerging molecular resistance to anti-EGFR and BRAF/MEK inhibitor therapy prior to radiological evidence of tumor progression. We concluded that EV-derived nucleic acids may provide clinically relevant diagnostic information and mirror evolution of the disease.

METHOD SUMMARY

Extracellular vesicles (EVs) derived from patient plasma were isolated via serial centrifugation, followed by EV-RNA isolation and EV-cDNA amplification. Amplified EV-cDNAs were analyzed with the Bio-Rad QX200™ ddPCR system (CA, USA). EVs were also morphologically visualized with transmission electron microscopy, while their proteomic content was verified by western blotting.

KEYWORDS:

colorectal cancer • ddPCR • exosomes • extracellular vesicles • liquid biopsy • melanoma

Liquid biopsy was introduced as a noninvasive approach to assist the gold standard tissue biopsy in predicting treatment response, disease progression, prognosis and early cancer detection [1,2]. The analysis of solid tissue is spatially limited and consequently it only describes the section of the tumor that was probed. In contrast, liquid biopsy is increasingly helping us to understand the heterogeneous landscape of the tumor [3]. Consisting of circulating tumor cells, circulating cell-free DNA (cfDNA) and extracellular vesicles (EVs), liquid biopsy has been thoroughly studied to provide molecular features of various tumor types [4].

EVs are nanosized, cup-shaped vesicles that play a role in intercellular communication in both the physiological and the pathological environment. The vesicles are capable of preparing premetastatic niches by orchestrating the ideal environment before guiding metastatic cells to the destination [5]. EVs are formed by inward budding of the plasma membrane and are released into the bloodstream at the end of the endosomal recycling pathway, while carrying cargo from the cell of origin [6]. EVs are considered to be a suitable cancer biomarker due to their ability to protect their biological cargo (protein, lipid, nucleic acid) in the circulation [7]. Nucleic acid mutations in patient-derived EVs have been detected in studies of glioblastoma [8], pancreatic cancer [9,10], colorectal cancer [11], advanced pancreatic and biliary cancer [12].

In our study, the oncogenes *KRAS* and *BRAF* were analyzed with Droplet Digital™ PCR (ddPCR; Bio-Rad, CA, USA) to compare gene status in EV-derived cDNA (EV-cDNA) isolated from patients' plasma with that in corresponding tissues to understand concordance rate in 31 colorectal cancer (CRC) and melanoma (MM) patients.

Materials & methods

Patients

A total of 13 patients with advanced CRC and 18 with MM were enrolled into our study from the Department of Medical Oncology and Hematology and the Skin Tumor Center of the Charité Medical University. Among the 31 samples, three patients were previously recruited for OncoTrack from the Charité and the Medical University Graz [13]. All individuals provided informed consent. The study was approved by ethics committees from both Charité University Medicine (EA1/069/11, EA4/090/08, EA4/063/13) and Medical University Graz (23-015 ex 10/11). Patient demographics are summarized in Table 1.

Table 1. Demographics, clinical characterization and therapy information of participants from (A) the colorectal cancer and (B) the melanoma cohort.

(A) Colorectal cancer		(B) Melanoma	
Total patients (n = 13)	Number	Total patients (n = 18)	Number
Gender:		Gender	
– Male	11	– Male	12
– Female	2	– Female	6
Age (years)	59 (median)	Age (years)	54 (median)
Localization:		Stage:	
– Right-sided colon cancer	5	– 1	2
– Left-sided colon cancer	5	– 2	0
– Rectum	3	– 3	3
AJCC:		– 4	13
– 1	0	<i>BRAF</i> MUT	13
– 2	0	<i>BRAF</i> WT	5
– 3	0	Therapy:	
– 4	13	– Nivolumab	2
<i>KRAS</i> MUT	7	– Nivolumab/ipilimumab	7
<i>KRAS</i> WT	6	– MEK inhibitor	2
Therapy:		– <i>BRAF</i> + MEK inhibitor	7
– Cetuximab	3		
– FOLFOX + cetuximab	3		
– FOLFOX + bevacizumab	6		
– FOLFOX + bevacizumab + panitumumab	1		

AJCC: American Joint Committee on Cancer staging system; CRC: Colorectal cancer; MM: Melanoma; MUT: Mutation; WT: Wild-type.

Isolation of plasma-derived EVs

Peripheral blood was collected in BD Vacutainer® EDTA tubes (BD, NJ, USA) and centrifuged at $1811\times g$ for 7 min and $3061\times g$ for 10 min at room temperature. Plasma was stored at -80°C until further processing. Plasma (1 ml) was thawed at room temperature to isolate EVs via ultracentrifugation, as shown in Supplementary Figure 1. This was conducted with the Sorvall Discovery 90 SE ultracentrifuge and T-1270 fixed angle titanium rotor (Thermo Scientific, MA, USA).

Isolation of cell lines-derived EVs

For assay optimization and as positive controls, EVs were isolated via ultracentrifugation from cell lines (SK-MEL-28, IGR-1, RPMI 8226, MIA PaCa-2, LS174T, SW480 and HCT 116) that express mutations of *BRAF* V600E/K, *KRAS* G12A/C/D/V and *KRAS* G13D. Initially, cell lines were validated by single nucleotide polymorphism profiling with Multiplexion GmbH (Friedrichshafen, Germany) and tested negative for mycoplasma using Promokine PCR Mycoplasma Test KIT I/C based on the manufacturer's instructions (PromoCell GmbH, Heidelberg, Germany). At 80% confluency, primary fetal bovine serum was removed by rinsing adherent cells twice with phosphate-buffered saline (PBS) and reculturing them with advanced DMEM or advanced RPMI (Thermo Fisher Scientific, MA, USA) without fetal bovine serum. The EV-rich supernatant was harvested after 48 h of incubation at 37°C and 5% CO_2 . The remaining cells were pelleted at $2000\times g$ for 20 min at 4°C and the supernatant was filtered with a $0.22\text{-}\mu\text{m}$ cellulose acetate filter (Merck, Darmstadt, Germany) to remove larger particles. The supernatant was concentrated with a Centricon Plus-70 Filter (Merck) following the supplier's instructions and was then filtered again, followed by two cycles of ultracentrifugation as depicted in Supplementary Figure 1. Cell lines, their corresponding mutation status and culture conditions are listed in Supplementary Table 1.

RNA isolation & whole transcriptome amplification of extracellular vesicles

After isolation of EVs, RNA was extracted using the miRCURY RNA Isolation Kit – Cell & Plants (Exiqon, Denmark) for cell line-derived EVs and the miRCURY RNA Isolation Kit – Biofluids (Exiqon, Denmark) for EVs from plasma. Due to the discontinuation of both Exiqon kits, the miRNEASY micro kit (Qiagen, Hilden, Germany) was used as a replacement for 50% of the samples. EV-RNA was isolated according to manufacturer's instructions. DNase inhibitor (Thermo Fisher Scientific) was added to RNA samples to avoid possible DNA contamination. The samples were purified, concentrated with the RNA Clean & Concentrator™-5 kit (Zymo Research, CA, USA) and eluted in $10\text{ }\mu\text{l}$ nuclease-free, diethyl pyrocarbonate-treated water (Carl Roth, Karlsruhe, Germany) based on the manufacturer's instructions.

Whole transcriptome amplification was performed with the REPLI-g WTA kit (Qiagen) according to the supplier's instructions. The EV-derived RNA was converted into cDNA and amplified, resulting in a final volume of $19\text{ }\mu\text{l}$ amplified cDNA. Samples were purified,

concentrated with the RNA Clean & Concentrator-5 kit (Zymo Research) and eluted in 30 µl nuclease-free, diethyl pyrocarbonate-treated water (Carl Roth) based on the manufacturer's instructions.

Droplet Digital polymerase chain reaction

Primers and probes for ddPCR assays were designed using Primer blast [14]; 2 µl cDNA and 18 µl of total reaction volume – consisting of primers, wild-type and mutation probes and ddPCR Supermix for Probes (no dUTP) (Bio-Rad, CA, USA) – were converted into droplets with the QX200™ droplet generator (Bio-Rad). Droplet-partitioned samples were transferred to a 96-well plate and PCR was performed on the T-100 thermal cycler (Bio-Rad) with the following protocol: 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 58–61°C for 1 min, with a final step of 98°C for 10 min (annealing temperatures varied with the different mutation assays). Primer and probe sequences and PCR conditions are listed in Supplementary Table 2. Samples were analyzed as duplicates in the FAM (MUT) and HEX (WT) channels using the QX200™ Droplet Reader (Bio-Rad). Droplet fluorescence data were analyzed with QuantaSoft™ version 1.7.4 (Bio-Rad) and detected events were expressed as copies/µl (CPM). The results were then calculated to [15]:

$$\text{CPM} = \frac{\text{copies of target} \times \text{template volume}}{\text{volume analyzed of the duplicates}}$$

For ddPCR optimization, cDNAs from cell lines with wild-type or mutation status in *KRAS* and *BRAF* were utilized. The finalized mutation assays were repeated in triplicate. Optimization steps for the ddPCR are described in Supplementary Figures 2 and 3.

The false-positive rate was evaluated using the number of unspecific events detected in the mutation channel when analyzing non-template controls and high and low concentrations of cell line-derived wild-type cDNA (500 and 100 CPM, respectively). The limit of detection was identified by analyzing mutant cDNA diluted in a background of constant wild-type cDNA.

Assay specificity was verified, demonstrating that probes did not bind to other mutations at the same nucleotide position. For all assays, the false-positive rate ranged from zero to one event and the limits of detection were 0.1%. Prior to analysis of patient samples, EVs derived from cell lines were utilized to validate detection of mutations.

Whole-mount immunoelectron microscopy

EV-rich fractions were fixed and deposited on Formvar carbon-coated (Plano, Wetzlar, Germany), glow-discharged (MED 020, Leica) nickel grids. The grids were washed in PBS and incubated in a blocking buffer containing 1% bovine serum albumin (Sigma-Aldrich, Darmstadt, Germany) in PBS. This was followed by an exposure to primary antibody CD63 produced in mouse (Bio-Rad) for 1 h and secondary antibody goat antimouse IgG conjugated to 5-nm gold particles (Plano) for 30 min. Subsequently, grids were stained with 2% phosphotungstic acid (Sigma-Aldrich), pH 7.0, for 10 min and incubated in a mixture of 2% methylcellulose and 4% uranyl acetate (Merck) for 10 min on ice. Stainless steel loops were used to remove grids, excess fluid was gently blotted on Whatman Grade 1 qualitative cellulose filter paper (Merck) and the grids were left to dry. Analysis was performed using a Zeiss transmission electron microscope 912 equipped with a digital camera (Proscan 2K, Slow-Scan CCD-Camera, Zeiss, Oberkochen, Germany) and image analysis was performed with ITEM software (Olympus). For the negative controls, primary antibodies were omitted.

Western blot

EV pellets were homogenized in RIPA lysis buffer (50 mM Tris/HCL, pH 7.2–7.4, 150 mM NaCl, 0.1% SDS, 1% Na-Deoxycholate, 1% Triton X100), supplemented with Protease Inhibitor Cocktail (Thermo Scientific) and boiled at 95°C; 20 µg protein was loaded in 4–15% gradient Mini Protean® TGX™ Precast Gels (Bio-Rad). Proteins were electrophoretically transferred to polyvinylidene difluoride membranes and blocked in 5% bovine serum albumin in TBST (0.1% Tween-20). Membranes were incubated with antibodies against tetraspanins CD9 (1:1000 dilution), CD81 (1:500 dilution) and *cis*-matrix Golgi marker GM 130 (1:1000 dilution) (System Bioscience, CA, USA). Protein bands were visualized via Li-Cor Odyssey Imaging System (Li-Cor, NE, USA). Western blot analysis was performed in duplicate.

Results & discussion

Characterization of extracellular vesicles

The features of EVs were examined using whole-mount immunoelectron microscopy and were determined based on their size, morphology and immunoreactivity for proteins enriched in EVs. Using these criteria, CD63-positive EVs ranging from 10–100 nm, with morphologies varying from cup-shaped to round with an intact membrane and a central depression, were identified in EVs derived from both cell lines and patient samples, consistent with reports by Trajkovic, Raposo and Colombo [16–18]. Based on the methods mentioned by György [19] and Théry, Ostrowski and Segura [20], the typical morphology of EVs was confirmed by including 2% phosphotungstic acid and 4% uranyl acetate as controls. To confirm the endocytic origin of the EVs and to distinguish vesicles of interest, immune-labeling of anti-CD63 conjugated to 5-nm gold nanoparticles was applied based on a refined method previously described by Théry *et al.* [21]. The expression level of tetraspanins fluctuated between different types of EVs and the density of CD63 labeling between EV fractions or between areas within a given fraction was not homogeneous. Some EVs were labeled, but there also appeared to be a population of larger unlabeled EVs (>100 nm) (Figure 1).

Figure 1. Immunoelectron microscopy images of whole-mount extracellular vesicles from colorectal cancer cell line and patient. (A & B) Electron microscopy images of whole-mount extracellular vesicles from colorectal cancer cell line (SW-620) labeled for CD63. Arrowheads indicate CD63-positive extracellular vesicles (5-nm immunogold particles) and arrows demonstrate unlabeled microvesicles. **(C)** Insets illustrate magnified images of extracellular vesicles. **(D–F)** Electron micrographs of whole-mount extracellular vesicles from patient 146 labeled for CD63.

For western blot analysis, tetraspanins CD9 and CD81 were detected in EVs isolated from cell lines and plasma samples. Due to the lack of a specific marker found ubiquitously in EVs, GM130 (a *cis*-matrix Golgi marker) was included as a negative control to label cell line-derived proteins which are not present in EVs (Figure 2).

Mutation detection

A total of 18 MM and 13 advanced CRC patients with corresponding tissue samples were recruited in this study. Pretreatment tissue profiling revealed the presence of *KRAS* mutations in seven of 13 CRC patients (54%). *BRAF* was mutated in 13 of 18 MM patients (72%). *KRAS/BRAF* wild-type was present in six CRC patients (46%) and *BRAF* wild-type in 5 MM patients (28%). Thus EVs from MM patients were screened for *BRAF* V600E and *BRAF* V600K mutations, whereas EVs from CRC patients were screened for five *KRAS* mutations (*KRAS* G12A/C/D/V and *KRAS* G13D). In the MM cohort, *BRAF* wild-type was detected in ten EV samples, mutations were detected in six patients (V600E and V600K in two and four patients, respectively), and in two samples neither the wild-type nor any mutation was detected. In the CRC cohort, *KRAS* wild-type was detected in seven EV samples, mutations were present in three patients (G12D and G12V in two and one patient, respectively), and in three patients neither wild-type nor any mutation was detected.

EV-based ddPCR analysis was compared with tissues profiled during routine analysis (Tables 2 & 3). Among the 18 MM patients, 44% (8/18) EV-derived results were in concordance with the gene status in tissue. *BRAF* wild-type instead of expected mutations was detected in 28% (5/18) of the patients and 17% (3/18) of the samples had mismatched mutations (V600K was detected twice instead of the expected V600E and V600E was detected once instead of wild-type). In CRC, 54% (7/13) of EV-based profiles were in concordance with the respective tissue, whereas in 15% (2/13) of the samples wild-type was detected instead of the expected mutations (and vice versa for one patient).

Figure 2. Characterization of extracellular vesicles by western immunoblotting. EVs derived from cell lines and plasma from three patients were analyzed by immunoblotting with antibodies against exosomal proteins CD9 and CD81 and nonexosomal protein, GM130. Whole cell lysates were included as positive control for GM130.

EV: Extracellular vesicle; K: Kelly, L: LS174T, S: SK-Mel-28.

Table 2. Concordance between gene status of extracellular vesicles and tumor tissue for (A) melanoma and (B) colorectal cancer cohort.

(A) Melanoma	
ddPCR results	Number of patients (%)
Total number of MM patients	18
Concordance:	8 (44.4%)
– <i>BRAF</i> WT	5 (27.7%)
– <i>BRAF</i> V600E	1 (5.5%)
– <i>BRAF</i> V600K	2 (11.2%)
Discordance:	
– <i>BRAF</i> WT instead of expected <i>BRAF</i> MUT	5 (27.7%)
– <i>BRAF</i> V600K instead of expected <i>BRAF</i> V600E	2 (11.2%)
– <i>BRAF</i> V600E instead of expected <i>BRAF</i> WT	1 (5.5%)
Undetectable	2 (11.2%)
(B) Colorectal cancer	
ddPCR results	Number of patients (%)
Total number of CRC patients	13
Concordance:	7 (53.9%)
– <i>KRAS</i> WT	5 (38.5%)
– <i>KRAS</i> G12D	1 (7.7%)
– <i>KRAS</i> G12V	1 (7.7%)
Discordance:	
– <i>KRAS</i> WT instead of expected <i>KRAS</i> MUT	2 (15.4%)
– <i>KRAS</i> MUT instead of expected <i>KRAS</i> WT	1 (7.7%)
Undetectable	3 (23%)

CRC: Colorectal cancer; ddPCR: Droplet Digital™ PCR; MM: Melanoma; MUT: Mutation; WT: Wild-type.

Discordant cases of *KRAS* & *BRAF* mutation status

Two cases stood out with discordant mutation status between tissues and EVs. Patient 16 was diagnosed with melanoma and brain metastasis. Based on the *BRAF* V600E mutation status in cervical lymphatic nodules, the patient received dabrafenib, followed by dabrafenib and trametinib (*BRAF*/MEK inhibitor) combination therapy. The patient was recruited prior to receiving combination im-

Table 3. Cancer stages, tissue profiling and extracellular vesicles Droplet Digital™ PCR results for (A) colorectal cancer and (B) melanoma cohort.

(A) Colorectal cancer				
Patient	Cancer stage (AJCC)	Tissue sequencing	ddPCR	CPM
6	4	KRAS G12D	KRAS WT	4.18
10	4	KRAS G12D	No results	No results
19	4	KRAS G13D	No results	No results
21	4	KRAS G12D	KRAS WT	33.3
22	4	KRAS WT	KRAS WT	1.87
23	4	KRAS WT	KRAS WT	3.63
25	4	KRAS WT	KRAS WT	8.58
26	4	KRAS WT	KRAS WT	39.6
27	4	KRAS G13D	No results	No results
30	4	KRAS WT	KRAS WT	2508
302	4	KRAS G12D	KRAS G12D	5.2
338	4	KRAS WT	KRAS G12D	13.86
356	4	KRAS G12V	KRAS G12V	3.5
(B) Melanoma				
Patient	Cancer stage	Tissue sequencing	ddPCR	CPM
1	4	BRAF WT	BRAF V600E	61.6
6	3c	BRAF WT	BRAF WT	0.88
10	4	BRAF V600E	BRAF WT	3091
11	4	BRAF V600E	BRAF WT	1.6
15	4	BRAF V600E	BRAF WT	1.76
16	4	BRAF V600K	BRAF V600K	3.08
19	4	BRAF WT	BRAF WT	0.9
20	4	BRAF V600E	BRAF WT	2251
22	3a	BRAF V600K	BRAF V600K	16.5
25	4	BRAF V600E	BRAF V600K	6.26
28	4	BRAF V600E	No results	No results
29	3c	BRAF V600K	BRAF WT	298.1
30	4	BRAF V600E	BRAF V600K	36.6
33	1a	BRAF V600E	No results	No results
37	4	BRAF V600E	BRAF V600E	16.5
38	1	BRAF WT	BRAF WT	0.44
39	4	BRAF WT	BRAF WT	7.48
40	4	BRAF WT	BRAF WT	0.55

• CPM = $\frac{\text{copies of target} \times \text{template volume}}{\text{volume analyzed of the duplicates}}$
 AJCC: American Joint Committee on Cancer staging system; ddPCR: Droplet Digital™ PCR; MUT: Mutation; WT: Wild-type.

munotherapy with nivolumab and ipilimumab. Our EVs analysis revealed a *BRAF* V600K mutation. One year after recruitment into our study, staging analysis showed subcutaneous, cerebral, intraabdominal, intradural and vertebral metastatic progress. Metastatic tissue from submammary skin was sequenced due to the advanced progress, verifying the mutation switch from *BRAF* V600E to *BRAF* V600K.

Patient 338 was diagnosed with sigmoid colon cancer and synchronous hepatic metastasis. Routine pathology analysis revealed *KRAS* wild-type in biopsied tissue from the rectum and sigmoid colon. The patient received FOLFOX/cetuximab combination therapy for 4 months, but presented with lung and hepatic metastasis 1 year after treatment. A blood sample was collected prior to resection of the liver metastasis. EV analysis detected subclones resistant to anti-*EGFR* therapy, harboring a *KRAS* G12D mutation.

The availability of EVs in various biological fluids like plasma and urine has prompted multiple studies on their use as potential biomarkers [22]. EVs are known as reservoirs of proteins, DNA and RNA, with a lipid bilayer membrane shielding intracellular cargo from extracellular conditions. Among their many roles, the vesicles are able to reflect the cellular condition at the time of release and pass on information from cell to cell [23]. To date, the mechanisms of sorting cellular components into EVs and for their specific uptake by designated cells are still unclear. In this study, the possibility of mutation profiling in EVs was explored in patients' plasma via Droplet Digital PCR. The MM and CRC patient cohorts consisted of 28 patients with metastatic disease and two patients in the early stage of the

disease, all of whom underwent treatment prior to plasma sampling. EV-based mutational analysis was compared with tissue samples, demonstrating a concordance rate of 54 and 44% in the CRC and MM cohorts, respectively.

In previously published data from our group, *KRAS* and *BRAF* were profiled in cfDNA and compared with tissue samples using ddPCR. In a cohort of 65 CRC patients (stages I–IV), cfDNA was detected in 100% of the patients, with 55% correlation with tissue samples. Mutations were mainly detected in stage IV patients (and in one patient in stage II); two of ten patients had *BRAF* mutations and nine of 25 patients had mutations of *KRAS* [24]. The overall concordance rates demonstrated comparable utility of cfDNA (55%) and EVs (54%) analysis for tissue mutation detection in plasma of CRC patients. Three patients (patients 302, 338 and 356) from the cohort were included in our EVs study to allow direct comparison of EVs and cfDNA. Interestingly, EVs may be more accurate in matching the gene status of the tumor when compared with cfDNA (while acknowledging that the comparison of cfDNA and EVs was limited with only three patients). The *KRAS* G12D tissue mutation of patient 302 was verified in both EVs and cfDNA. However, in patient 356, EVs analysis displayed a *KRAS* G12V mutation, whereas cfDNA analysis only detected the wild-type. Disease progression in patient 338 correlated with EV-based ddPCR results, demonstrating a mutation switch; in contrast, only wild-type was detected in cfDNA. Our results corresponded with a report by Möhrman *et al.* [25], who analyzed EVs for *BRAF* V600 and *KRAS* G12/G13 via next-generation sequencing; cfDNA was analyzed with ddPCR and BEAMing digital PCR [25]. When compared with sequenced formalin-fixed, paraffin-embedded archival tissues, 95% of exosome samples matched the mutation status of the tissue. The concordance rate for cfDNA was comparable with EVs, with 90% for ddPCR and 92% for BEAMing digital PCR [25]. In another study of both liquid biopsy components, *KRAS* mutation detection was undertaken on exosome-derived DNA and cfDNA isolated from pancreatic ductal adenocarcinoma patients; when compared with tissue, exosome-derived DNA surpassed cfDNA in *KRAS* detection in metastatic patients (85 and 57.9%, respectively) [9].

The discordant results can be explained by the heterogeneous landscape of the tumor and/or the different timepoints of plasma sample and tissue collection. In our study the blood samples were collected after patients received the allocated therapy; therefore, tissue sampling prior to treatment and blood collection post-treatment were separated by a median time difference of 6 months. Thus we explored whether EVs could provide information on how the treatment affects the disease. Möhrman *et al.* reported that the discrepancies in their analysis when comparing gene status in exo-nucleic acid and archival tissue samples could be due to time variation of plasma and tissue collection. They reported a much longer time difference (median 20 months) than in our study and a 5% discordance rate between exo-nucleic acid and archival tissues [25]. Nevertheless, our study is not without limitations. Compared with other studies [9,25,26], our cohort is relatively small, and the patients were treated with diverse therapies.

As it is difficult to obtain repeated tissue samples, it is beneficial to incorporate blood-based detection methods to assist in treatment decision making. Two cases in our patient cohort suggested that EV analysis could be used as part of treatment monitoring to detect resistance. The patients representing both MM and advanced CRC not only demonstrated a mutation switch, but also a significant disease progression.

Clinical trials demonstrated improved progression-free survival and overall survival in melanoma patients who received selective *BRAF* (dabrafenib, vemurafenib) and *MEK* (trametinib) inhibitors compared with those receiving systemic therapy (e.g., dacarbazine, interferon or IL-2) [27]. However, unavoidably, subclonal activation during *BRAF/MEK* inhibition occurred at a median rate of 4–7 months post treatment [28]. This included a mutation shift, proliferation of subclones and eventually their detection by mutation testing. Van Allen *et al.* sequenced tissue samples originating from *BRAF* V600 mutant advanced melanoma patients and reported genetic alterations in the *MAPK* (*ERK*) signal transduction pathway in 51% of the patient cohort [29,30]. This was also noticed in patient 16 who demonstrated a significant progress 10 months after treatment pause, correlating with the presence of a mutation switch (*BRAF* V600E to *BRAF* V600K). Unlike sequenced tissue taken prior to *BRAF/MEK* inhibitor treatment, EV-based analysis demonstrated the *BRAF* V600K mutation. Interestingly, the EV-ddPCR results were reconfirmed by sequencing newly biopsied submammary metastatic skin tissue.

The anti-epidermal growth factor receptor (anti-*EGFR*) inhibitor cetuximab was approved as first-line single-agent therapy (or in combination with standard chemotherapy) for metastatic CRC patients who are intolerant to chemotherapy, either *EGFR*-expressing or *RAS* wild-type [31]. Molecular alterations have been reported in colorectal cancers that are *RAS* wild-type at baseline, eventually shifting the tumors to a mutated status resulting in a secondary resistance to anti-*EGFR* therapy [32]. We noticed a similar acquired resistance in patient 338 when we compared the EV status 1 year after FOLFOX/cetuximab combination treatment with that of primary tumor tissue biopsied prior to therapy. This disease progression was not detectable in our cfDNA study [24]. In cases from both the MM and CRC patient cohorts, EV-ddPCR indicated the mutation switch before known disease progression could be detected by either staging, routine PCR or tissue sequencing analysis.

We proved that EVs are able to provide a snapshot of disease evolution. Furthermore, EV-cDNA is able to detect mutation switches prior to clinically verified disease progression. Liquid biopsy is therefore a versatile addition to tissue biopsy for monitoring responses to therapy, a process that requires the possibility of repetitive real-time monitoring to understand the dynamic changes within the disease.

Future perspective

As a liquid biopsy component, EVs provide valuable information for real-time monitoring of disease and could be utilized as a cancer screening tool due to minimal invasiveness. Cancer is a heterogeneous disease and liquid biopsy could adequately reflect this information, unlike mutation analysis of tissue biopsy, which only provides information about the specific location where tissue was acquired.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2020-0028

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that all patients provided informed consent prior to blood donation and the study was approved by ethics committee.

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