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Liquid Biopsy: A Next Generation Diagnostic And Prognostic Tool In Solid Malignancies

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LIST OF ABREVIATTIONS

AC	Colon Adenocarcinoma
APC	Adenomatous Polyposis Coli
AR-V7	Androgen Receptor Splice Variant 7
ARS	Androgen Receptor Signaling
AUP	Animal Use Protocol
Cc-RCC	Clear Cell RCC
CCAC	Canadian Council Of Animal Care
Cf-DNA	Cell-Free DNA
Cf-RNA	Cell-Free RNA
CLL	Chronic Lymphocytic Leukemia
CNV	Copy Number Variations
CRC	Colorectal Cancer
CRPC	Castration-Resistant Prostate Cancer
Ct-DNA	Circulating Tumor DNA
CTC	Circulating Tumor Cells
DdPCR	Digital Droplet PCR
ELISA	Enzyme-Linked Immunosorbent Assay
EV	Extracellular Vesicles
FPR	False Positive Rate
HRM	High Resolution Melting
INDEL	Insertions/Deletions
ISP	Ion Sphere Particles
IVC	Inferior Vena Cava
MCRPC	Metastatic Castration-Resistant Prostate Cancer
MiRNA	Micro-RNA
NGS	Next Generation Sequencing
OS	Overall Survival
PCR	Polymerase Chain Reaction

PD	Disease Progression
Pre-MiRNA	Precursor-MiRNA
Pri-MiRNA	Primary MiRNA
PSA	Prostate-Specific Antigen
Pv	P-Value
QPCR	Quantitative PCR
QRT-PCR	Quantitative Real Time PCR
RBC	Red Blood Cells
RCC	Renal Cell Carcinoma
REST	Relative Expression Software Tool
RISC	RNA Induced Silencing Complex
ROC	Receiver Operating Characteristic
SD	Standard Deviation
SiRNA	Short Interfering RNA
Tam-Seq	Tagged-Amplicon Deep Sequencing
TEP	Tumor Educated Platelets
TPR	True Positive Rate
VAF	Variant Allele Frequency
WBC	White Blood Cells

CHAPTER 1

Summary, Aims & Outline Of The Thesis

SUMMARY, AIMS AND OUTLINE OF THE STUDY

The main goal of this thesis is to contribute to elevate the knowledge about liquid biopsy and its potentials regarding cancer. Specially, we aimed to improve liquid biopsy compartments' role as prognostic and predictive biomarkers in patients with solid tumors.
Every chapter of this thesis corresponds to a paper submitted, in process to be published, or an ongoing research to be submitted; at the moment of thesis delivery.
After the initial chapter which is summary, aims and outline of the thesis, in the second chapter which is divided into two sections; we provide an overview of the importance of liquid biopsy in the field of cancer prognosis and diagnosis as well as an introduction to various fractions that can be studied through liquid biopsy in the last section of this chapter.
In the third chapter, we described predictive roles of cell-free DNA in Colorectal Cancer patients. We also compared prognostic and diagnostic potential of exosomes with cell-free DNA in this malignancy. Furthermore our results demonstrated the genomic background of DNA from exosomes derived from Plasma of colorectal cancer patients with regard to the mutation in the tissue and cell-free DNA for the first time.
Chapter four however is focused on a brief review about the roles that miRNAs might play in the development of cancer. Subsequently; chapter five and six describes two of our projects designed to study some selected miRNAs in the tissue as well as the blood stream of new groups of colorectal cancer patients. We proposed that differences in miRNA expression profiles in tissue, tissue margin and blood of patients with solid tumors makes them beneficial factors in monitoring the characteristic features of malignancies.
In chapter seven we discus about possibilities of using different compartments of liquid biopsy such as extracellular vesicles and white blood cells; for detection of specific mutations regarding Renal Cell Carcinoma through a study containing in vitro and in vivo sections as well as a research on patients. It is this last step where we elaborate that extracellular vesicles including exosomes might be capable of mirroring information about mutations related to the disease. To conclude, the last chapter is a short discussion and some concluding remarks on how liquid biopsy and the repertoire of its fractions might affect the future of cancer research in patients with solid tumors.

CHAPTER 2
Liquid Biopsy

2.1. Liquid Biopsy Through Cell-Free Nucleic Acids

2.2. Encapsulated Circulating Tumor Materials; With Special Focus On Exosomes

CHAPTER 2.1.

Liquid Biopsy Through Cell-Free Nucleic Acids

Partly Adapted From

From Tumor To Circulation In Packed Form: When Liquid Biopsy Becomes The Pandora's Box

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Circulating Nucleic Acids – Next-generation Sequencing Strategies and Trends in Clinical Utility

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Introduction

Cancer related deaths have not been significantly reduced for decades even though there have been enormous efforts in treatment approaches [1]. Targeted and immune-based therapies have extended life of a fraction of patients with advanced solid tumors; however, treatment outcomes would be enhanced if applied earlier. As such, an early diagnosis of cancer can positively affect the survival by five to ten times compared to late stage detection [2]. Tumors at early stages of progression are relatively small. This presents a technical challenge for tumor detection by imaging-based methods that are currently used in the clinic. In addition, current screening modalities are usually unable to distinguish between small early-stage tumors and benign lesions, leading to over-diagnosis, and eventually over-treatment in many cases. For example, false positive detection of prostate cancer by prostate-specific antigen (PSA), lung cancer by low-dose helical computed tomography, and breast cancer by digital mammography have imposed unnecessary burdens by effecting both the precious time of clinicians and increasing medical expenses associated with patient follow-up and therapy designs [3-5]. Hence, there is an unmet need for robust and new generation screening strategies that are sensitive and specific for cancer diagnosis.

Liquid biopsy, a promising era in cancer research

Traditional techniques, which are used routinely in the clinic, play vital roles in tumor diagnosis; however, for therapeutic guidance, the specificity of traditional serum biomarkers is unsatisfactory. Imaging techniques on the other hand cannot be used for 'real-time' detection due to economic concerns and exposure to the radiation [6-9]. During the early stages of cancer formation and progression, tumor-specific information is released into biofluids, including blood or urine by various vehicles such as DNA, RNA, proteins, extracellular vesicles or tumor cells [10]. These data can be specifically captured, enriched and analyzed for early diagnosis of cancer. As such, collecting biofluids provides a platform for the development of novel diagnostic strategies called as liquid biopsies. The concept of liquid biopsy was introduced a few years ago to study CTCs in the blood stream of cancer patients [11]. However, presently liquid biopsy also includes the analysis of circulating cell-free DNA, extracellular vesicles and even blood platelets that all

can harbor tumor-specific data [12, 13]. Liquid biopsies can exploit the tumor-associated molecules such as aberrantly-expressed proteins and mutated nucleic acids released into the blood stream by cancer cells to detect cancer. Therefore, analysis of liquid biopsies offers alternative diagnostic strategies to monitor the presence or progression of cancer by making use of the wide variety of circulating tumor biomarkers. Accordingly, liquid biopsies have supported the development of novel diagnostic strategies for cancer detection, monitoring response to drug treatment, predicting outcome and cohort stratification based on drug sensitivity and resistance [14]. These possibilities together with non- or minimally-invasive nature of liquid biopsy have highlighted the advantages of this approach in comparison to conventional diagnostic methods. However, a number of challenges persist to exploit liquid biopsies in the clinical setting. The clinical utility, sensitivity and specificity are still major challenges to be addressed to pave the way toward clinical applications of liquid biopsy. Below, we review different forms of circulating tumor materials in biofluids and their biological and technical characteristics.

Next generation sequencing (NGS)

NGS has emerged as a powerful tool for liquid biopsy analysis, which allows the detection of cancer-related genetic and epigenetic alterations such as mutations, copy number variations (CNVs), and DNA methylation changes across wider genomic regions in many cancer types [15, 16]. However, detection of cancer with high specificity and sensitivity in liquid biopsies is still challenging, especially in early-stage cancers, as there exist many barriers to the utilization of circulating nucleic acids in clinical applications, including lack of well-accepted sample collection protocol and sensitive detection approaches. Furthermore, analysis of sequencing data requires specialized bioinformatics tools to identify robust biomarkers for clinical practice. In this part, we focus on major applicative areas for circulating nucleic acids in cancer after briefly addressing the importance of different next generation sequencing technologies in bypassing the limitations of liquid biopsies.

Cell free Nucleic acids

Cell free DNA (cfDNA)

During apoptosis or necrosis, cell DNA is released into blood stream as a part of cellular destruction [17]. In healthy individuals, phagocytes normally infiltrate and clear the debris of apoptotic and necrotic cells, and hence the levels of cfDNA are maintained in low levels. However, in cancer patients, cellular debris accumulates because of poor clearance by immune cells [18]. The DNA from the debris is hence released into blood stream in these patients which can be captured and analyzed [19]. In some *in vivo* studies with cultured cell lines, direct release of DNA into blood stream has been confirmed [20-22]. Several hypotheses have been proposed suggesting that the released DNA could be oncogenic and could transform the normal healthy cells at distant sites into malignant [23, 24]. Likewise, multiple reports emphasize that cfDNA levels in blood increases significantly in cancer patients and correlates with tumor progression [25, 26]. Although the yield of cfDNA is higher in serum, plasma is considered a better source to analyze cfDNA originated from tumor cells because of the lack of/ less contamination by DNA released by lysed immune cells [27]. Accordingly the limited background concentration of wild-type DNA from healthy cells is a technical advantage when using plasma. Even though cfDNA could be informative for monitoring tumor status and progression in cancer patients, there are currently many challenges associated with deploying cfDNA for diagnostic purposes. The low copy number of tumor-specific DNA molecules and short half-life of cfDNA make it difficult to work with cfDNA samples [28]. Additionally, the range within which cfDNA presents the tumor data is considerably large between early and late stage cancer patients, respectively with levels from undetectable to few hundred thousand copies in a unit volume of plasma [17, 29]. Hence, enriching the cfDNA for tumor information to effectively capture and specifically analyze the tumor data remains a challenge.

Cell free RNA

Circulating gene transcripts are also shed into the blood stream and in spite of high levels of RNases, they are interestingly stable and well protected by extra cellular packaging [30, 31]. Using

novel strategies for the capture and isolation of these extracellular vesicles, the levels of gene transcripts that they contain can be quantified by platforms such as microarrays, quantitative real time PCR, digital PCR, etc [32]. For example, cell-free transcripts from TERT and EGFR genes were quantified in blood stream of lung cancer patients in earlier studies [33]. The levels of TERT in blood was associated with tumor size, presence of metastasis and recurrence, whereas increased level of EGFR in blood correlated with advanced stage carcinomas. Also, in breast cancer patients, CCND1 transcript levels in blood were often associated with poor overall survival [34].

Circulating miRNAs also have many diagnostic and prognostic values. Because of the strong connection between miRNA levels and cancer maintenance, circulating miRNAs have a great potential as effective biomarkers for future targeted therapy. Circulating miRNA expression signatures are also associated with tumor classification, diagnosis and cancer progression [35-39]. For example, presence of miR-21, miR-210 and miR-155 in plasma were correlated with diffuse large B cell lymphoma [40]. In blood stream of breast cancer patients, miR-21 associate with early stage tumor progression, dysregulation of miR-10b and miR-155 levels correlate with metastases, and, high levels of miR-34a indicate advanced stage tumors. In patients with non-small-cell lung cancer, significantly higher levels of circulating miRNA-486, miR-30d, miR-1 and miR-499 were observed in patients with shorter survival [41-43]. Another study has shown that the abundance of miR-92 in plasma can distinguish between patients affected by colorectal or gastric cancers. Similarly, oncomiRs such as miR-500 is reported to be useful in the diagnosis of hepatocellular carcinoma [44].These findings highlight the clinical relevance of circulating nucleic acids for cancer diagnosis.

Applications of Next Generation Sequencing to Liquid Biopsies

Monitoring Tumor Data Using Targeted Sequencing

Metastases evolve many years after primary tumor resection and can harbor unique genomic alterations. As mentioned in the previous sections of this review, the peripheral blood contains cells and/or ctDNA derived from different metastatic sites and may therefore provide a more comprehensive picture than the analysis of a single metastatic lesion [17]. Murtaza *et al*. have now established proof of principle that exome-wide analysis of ctDNA can identify mutations

associated with acquired drug resistance in advanced cancers [45]. The authors serially analyzed ctDNA over 1–2 years in six patients with advanced breast, ovarian and lung cancer with very high ctDNA concentrations in their blood plasma. Even though exome sequencing in their report revealed numerous mutations in ctDNA, the authors were able to identify specific mutations like an activating mutation in *PIK3CA,* a truncating mutation in *RB1* and *MED1,* splicing mutation in *GAS6* and a resistance conferring mutation in *EGFR* in the post-therapy samples. Several groups have now reported that mutations present in ctDNA are highly concordant with those present in the matched tumor. In a study from Diehl *et al.,* mutations in ctDNA were quantified in patients with advanced colorectal cancers. These patients were reported to consistently present mutant adenomatous polyposis coli (*APC*) DNA molecules in their plasma [46]. The median number of *APC* DNA fragments in such patients was 47,800 per ml of plasma, of which 8% were mutant. Mutant *APC* molecules were also detected in >60% of patients with early, presumably curable colorectal cancers, at levels ranging from 0.01% to 1.7% of the total *APC* molecules. In another study, Dawson *et al.* used targeted deep sequencing to screen for point mutations in *PIK3CA* and *TP53* from ctDNA of plasma from metastatic breast cancer patients [47]. They found that around 50% of the patients participated in their study harbored these mutations providing a proof of concept analysis that ctDNA is an informative, inherently specific, and highly sensitive biomarker of metastatic breast cancer. Using targeted sequencing of ctDNA, Girotti *et al.* have also reported various mechanisms of resistance to therapy in melanoma patients [48]. They identified *NRASQ61K* mutation in ctDNA that was not present at the baseline of a melanoma patient under treatment, emerged at the onset of resistance. Targeted sequencing of the ctDNA also revealed a *de novo* $PIK3CA^{E545K}$ mutation that emerged coincident with resistance to chemotherapy when treatment was withdrawn. Takai E and Yachida S have reported targeted deep sequencing analysis of cfDNA using a modified Illumina platform and a targeted gene panel for pancreatic cancer [49]. The gene panel consisted of 60 genes, including 17 potentially actionable targets. In their study targeted deep sequencing of cfDNA in 48 pancreatic patients was carried out, including 43 cases that had ≥ 1% tumor DNA in total cfDNA and 5 patients with obvious distant organ metastasis. Their study reported somatic mutations in potentially targetable genes in almost 30% of the participated patients. In addition, analysis of somatic CNV using targeted sequencing data for cfDNA, revealed potentially targetable gene amplifications such as in *CCND1* and *ERBB2.* Although there are many promising studies, current ctDNA analysis is usually restricted to one or

a few mutation sites due to technical limitations. In the case of massively parallel DNA sequencers, the number of false positives caused by a high read error rate is a major problem. In addition, the final sequence reads do not represent the original DNA population due to the global amplification step during the template preparation. To address these problems, Kukita *et al.* established a high-fidelity target sequencing system of individual molecules identified in plasma cfDNA using barcode sequences [50]. This system consists of two steps. In first step, a novel target sequencing method adds barcode sequences by adaptor ligation. This method uses linear amplification to eliminate the errors introduced during the early cycles of polymerase chain reaction. In the second step, the erroneous barcode tags are monitored and removed. This process involves the identification of individual molecules that have been sequenced and for which the number of mutations has been absolute quantitated. Using plasma cell-free DNA from patients with gastric or lung cancer, the authors demonstrated that the system achieved near complete elimination of false positives and enabled *de novo* detection and absolute quantitation of mutations in plasma cell-free DNA. The authors have analyzed plasma cfDNA from gastric cancer patients. The ctDNA level represented by the TP53 mutation was zero or low during the early period of the disease and increased during the later period when the disease had progressed. In their study, an interesting model screening experiment using cfDNA from lung cancer patients was also reported. *KRAS* and *CTNNB1* were used as the target genes in this study. In lung cancer, the *KRAS* and *EGFR* mutations are exclusive and rarely co-exist in the same patients [50]. Whereas *CTNNB1* mutations are infrequent in lung cancer [51]. So, it can be speculated that *KRAS* hot spot mutations should appear only in *EGFR* mutation-negative lung cancer, and no mutations should be found in samples from normal individuals. However, the authors reported that *KRAS* variant-positive lung cancer cases could also harbor *EGFR* mutation and *CTNNB1* variant-positive cases could be a result of false-positive detection. Because redundancy in sequenced molecules could be removed by their novel method, the authors could discriminate experimental errors and true mutations from distribution patterns of base changes. Their results agreed well with previous knowledge of the mutations. Because KRAS mutations have been found in 25% of lung cancers, these promising studies suggest that most of the KRAS mutation-positive lung cancers were likely to be detected using plasma cfDNA [52]. In another approach Forshew *et al.* developed a method for tagged-amplicon deep sequencing (TAm-Seq) and screened 5995 genomic bases for low-frequency mutations [53]. Using this method, the authors identified cancer mutations present in circulating DNA at allele

frequencies as low as 2%, with sensitivity and specificity of >97%. Mutations throughout the tumor suppressor gene TP53 in circulating DNA from 46 plasma samples of advanced ovarian cancer patients were identified. In another case, an EGFR mutation in plasma was identified that was originally not found in an initial ovarian biopsy. Additionally, TAm-Seq was used to monitor tumor dynamics, and the authors tracked 10 concomitant mutations in plasma of a metastatic breast cancer patient over 16 months period. This low-cost, high-throughput method could facilitate analysis of circulating DNA as a noninvasive "liquid biopsy" for personalized cancer genomics. Hence, even though exome-wide sequencing is an interesting discovery tool, in clinical practice, it can be speculated that targeted sequencing for a panel of known drug resistance genes is a less expensive and more sensitive method.

Future Directions and Conclusion

Analysis of circulating nucleic acids has emerged as promising biomarker for early cancer detection and monitoring disease progression due to the relatively easy access to liquid biopsies. The advent of novel NGS strategies provides a unique advantage to systematically examine the circulating nucleic acids for tumor-specific aberrations. This review attempted to highlight one of the many applications of cf-nucleic acids in cancer research. It focused on studies identifying point mutations, copy number abnormalities and nucleosome positioning patterns. Using sophisticated bioinformatics analysis, advances have been made to better understand the property of cf-nucleic acids through fragmentation and nucleosome spacing patterns. There is a great potential of using circulating nucleic acids to analyze methylation biomarkers for identification of cancer cell origin. Moreover, patterns of CNV through the WGS analysis can further reveal the extent of tumor heterogeneity. As most of the cfDNA interrogations to date are proof-of-principle studies, large-scale, multi-site cohort studies that systematically investigate all these aspects of molecular profiles are needed to evaluate the complementary nature of their screening power so that liquid biopsy signatures can be refined, validated, and utilized in clinical practice. Nevertheless, to move cell-free nucleic acids into routine clinical practice for better patient management, future studies will need to address several issues related to efficient capture and analysis of tumor data from liquid biopsies. We conclude by emphasizing the need for more studies and concerted international

efforts in realizing the promise of precision medicine which will eventually lead to the identification of new oncological biomarkers for early disease detection and outcome prediction.

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CHAPTER 2.2

Encapsulated Circulating Tumor Materials; With Special Focus On Exosomes

Extracted From

From Tumor To Circulation In Packed Form: When Liquid Biopsy Becomes The Pandora's Box

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Introduction

Cancer has a significant impact on public health worldwide. One strategy to lower its burden is through cancer screening and early diagnosis. Tissue biopsy is the most widely-used tool for cancer detection, staging, and prognosis, but sometimes tumor tissue can be difficult to obtain, especially in metastatic diseases. Liquid biopsy is used in the detection of next-generation analytes, such as tumor cells, cell-free nucleic acids and exosomes in peripheral blood and other body fluids from cancer patients. It is considered one of the most advanced minimally-invasive to completely non-invasive diagnostic system in order to enable clinically relevant actions for precision medicine. Medical actions include, but are not limited to, early diagnosis, staging, prognosis, anticipation (lead time) and the prediction of therapy responses, as well as follow-up. Blood contains many types of tumor associated biologic materials like circulating tumor cells (CTCs), tumor educated platelets, extracellular vesicles, cell-free circulating nucleic acids such as cfDNA, cfRNA including miRNA [1]. Conventionally, the applications of liquid biopsies in cancer have mostly focused on CTCs. More recently, this analysis has been extended to circulating free nucleic acids associated with cancer providing novel approaches for potential applications in development of multi-marker diagnostic, prognostic and therapeutic signatures.

Compared to traditional cancer diagnosis using tissue biopsy, liquid biopsy is more feasible and less invasive and is more comprehensive than tissue biopsy to evaluate tumor heterogeneity [2]. Facilitated by the rapid development of next-generation sequencing (NGS) technologies, nowadays, liquid biopsy can achieve much higher sensitivity than tissue biopsy and can be designed for different purposes [3]. Further, liquid biopsies avoid some key limitations of conventional tumor tissue biopsies, including invasive tumor sampling, under-representation of tumor heterogeneity and poor description of clonal evolution during metastatic dissemination, strongly reducing the need for multiple sampling. On the other hand, liquid biopsies also suffer from important drawbacks such as the fragmentation and/or degradation of cell-free nucleic acids, the instability of RNA, the low concentrations of certain analytes in body fluids and the confounding background-presence of normal, as well as aberrant DNAs and RNAs. In this review, we discuss potentials of liquid biopsy analysis as a tool for early diagnosis and prognosis for solid tumors. We will concentrate this review on encapsulated forms of tumor-associated nucleic acids

in biofluids, and will describe the source, characteristics, methodological progress of capture/detection for various circulating vehicles of tumor materials, including circulating tumor cells (CTCs), extracellular vesicles (EV) and tumor educated platelets (TEP).

Repertoire of encapsulated circulating tumor materials

Circulating Tumor Cells (CTCs)

CTCs are cancer cells that may have been shed from primary or metastatic tumor sites into the blood stream. Although Thomas Ashworth identified CTCs for the first time in 1869, the association of CTCs with the treatment outcomes including overall survival had not been studied intensively until the late 1990s [4]. It should be noted that even though CTCs are in very low levels they play a central role in tumor progression and metastasis. For instance, independent studies have reported a positive correlation between CTC levels in peripheral blood and clinical stages including metastasis in lung cancer patients [5-9]. Moreover, a significant correlation between CTC levels and poor survival has been reported in SCLC patients [10, 11]. Additionally, a reduction in CTC levels after chemotherapy in these patients was significantly associated with better clinical outcomes [12, 13]. Several studies have shown that a high abundance of CTCs may serve as a prognostic marker of poor survival in breast cancer [14-17]. There is also growing evidence on clinical relevance of using CTCs, both qualitatively and quantitatively, as prognostic markers in non-metastatic breast cancer [15, 18, 19]. Earlier studies also were able to stratify patients into early and late stage breast cancer groups based on the absolute cut-off levels of CTCs in the blood [15, 20, 21]. These studies suggest that enumerating CTCs could potentially be used as prognostic and biomarkers for clinical diagnostic screenings in the future [22, 23].
CTCs were reported to be effective prognostic markers for predicting response to and relapse after therapies [15, 22, 24, 25].In case of NSCLC patients, a recent study has reported on the successful detection of activating EGFR mutations in 11 out of 12 CTCs captured from patients with such mutations in their tumors [26]. Particularly, a drug resistance-conferring T790M mutation in EGFR was identified in CTCs isolated from these patients following treatment with tyrosine kinase inhibitors.

Likewise, circulating tumor cell-based AR-V7 detection can serve as a treatment selection biomarker in castration-resistant prostate cancer (CRPC) [27]. The detection of androgen receptor splice variant 7 (AR-V7) in CTCs from men with CRPC was shown to be associated with primary resistance to enzalutamide and abiraterone therapy but not to taxane chemotherapy [28]. In men with AR-V7-positive CTCs, taxanes appear to be more efficacious than enzalutamide or abiraterone therapy, whereas in men with AR-V7-negative CTCs, taxanes and enzalutamide or abiraterone may have comparable efficacy.

Additionally, Scher HI et al. have reported that the measurement of the "nuclear" AR-V7 in CTCs was shown to be a treatment-specific biomarker in metastatic castration-resistant prostate cancer (mCRPC) at therapeutic decision points in the clinical setting [29]. A critical decision in the management of mCRPC is when to administer an androgen receptor signaling (ARS) inhibitor or a taxane. This finding by Scher HI et al. has validated CTC nuclear expression of AR-V7 in men with mCRPC as a treatment-specific biomarker that is associated with superior survival on taxane therapy over ARS-directed therapy in a clinical practice. Continued examination of this biomarker in prospective studies will further aid clinical utility. Hence, CTCs play an important role in cancer diagnostics, and their isolation, enumeration and characterization may improve therapy selection, drug response monitoring. Nevertheless, in order to harness the full potential of cancer-associated information of CTCs major technical limitations need to be addressed. For example, the number of CTCs that can be isolated from blood is variable between tumor types; however, the absolute number of CTCs that can be captured for analysis usually remains very low, even in patients with metastatic cancers [30, 31]. This significantly low number of CTCs is a major hurdle in using CTCs for investigating molecular signatures of tumors. For example, approximately 6 picograms of DNA is available from a human single cell and reports suggest that less than 10 CTCs can be harvested from 7.5mL of blood providing minute amounts of DNA for genomic analysis [32-34]. The development of more sensitive genomic methodologies will be of great help in this context.

Exosomes

Circulating carriers of tumor-cell materials span beyond CTCs and include non-cellular vehicles, including various extracellular vesicles, released from tumor cells into blood circulation [35]. There are two broadly-classified types of extracellular vesicles: micro-vesicles, which are released from cell membrane by budding; and exosomes, which are released into bloodstream by exocytosis

of multi-vesicular bodies fusing with plasma membrane [36]. Exosomes are tiny vesicles around 40-100 nm in size and were first reported by Pan and Johnstone in early 1980s [37]. These tiny vesicles are released by immune cells, endothelial cells and blood cells including platelets, and play significant roles in the exchange of cellular information [38, 39, 40, 41]. It has been shown that exosomes released by a cell can be involved in regulation of diverse cellular processes in other cells by encapsulating and transferring effector molecules like proteins and different types of nucleic acids, including DNA, RNAs, and miRNAs from the host cell to target cells [38, 39, 42]. These functional properties of exosomes are hijacked by cancer cells to impact target cells for a specific phenotype change at a distant site. For example, exosomes released by cancer cells can reprogram tumor microenvironment to facilitate tumor invasion and metastasis [43, 44]. Exosomal miRNAs such as miR-105 and miR-214 in particular have been shown to play important roles in tumor progression by regulating angiogenesis, and thereby stimulating metastasis [43, 45, 46]. It has also been reported that the drug-resistance phenotype can be transmitted to other cancer cells via exosomes, similar to antibiotic-resistance transmission by plasmids [47]. The transmission of these functional properties is believed to be mediated by tumor-specific molecules that travel between cells as cargo of exosomes. Indeed, tumor-specific mutations and transcriptome signatures can be effectively identified in exosomes [48]. Accordingly, exosomal cargo can serve as cancer biomarkers. Exosomes have already been elucidated as diagnostic biomarkers using surface proteins or miRNAs [49, 50]. For example, Nilsson et al. conducted a pilot study to validate the concept of urine exosomes as a potential source of cancer biomarkers in prostate cancer patients. They noted that we potentially could consider mRNAs and miRNAs as not only as cancer detection biomarkers but also as a biomarker for classifying severity of the tumor phenotype and following the tumor response to treatment [51]. In a study by ogata-Kawata et al., it has been shown that the serum exosomal levels of seven miRNA (including let-7a, miR-1229, miR-1246, miR-150, miR-21, miR- 223, miR-23a) were significantly higher in patients with colorectal cancer compared to healthy controls, which after surgical resection of tumors, their levels down-regulated significantly [52]. In a recent study, Liu et al. found that plasma exosomal miR-23b-3p, miR-10b-5p and miR-21-5p could serve as prognostic biomarkers for non-small-cell lung cancer patients [53]. Immense research is carried out to identify the exosomal cargo to define both the parent cancer cells and the actual target cells. To date few proteins and other factors had been identified that might be involved in miRNAs packaging and sorting into exosomes. As a result of some

limitations in isolation techniques, it is difficult to determine which miRNAs may be secreted as exosome cargo and also which miRNAs are packaged into exosomes [54].

The packaging of RNA transcripts into exosomes seems to be selective and non-random, at least for some transcripts. For example, Shurtleff et al. developed biochemical assays in which they identified YBX1 as an RNA-binding protein that is critical for the efficient packaging and secretion of miR-223 in vitro and in cultured human cells, respectively [54]. The exact mechanisms regulating this selective packaging is not completely known; however, it has been reported that the packaging of miRNA in the exosomes is regulated mainly by sequence specific motifs, present in RNAs that are recognized by packaging proteins during exosome formation in parent cells [55, 56, 57].

Tumor Educated Blood Platelets (TEP)

Several studies have previously focused on circulating cell free nucleic acids, CTCs, and exosomes to approach liquid biopsy concept [58, 59]. Recently, Best et al. has reported significant findings about tumor-educated blood platelets [60]. The same group and others had reported earlier that blood platelets and tumor cells interact with each other to promote tumor growth and invasion [61, 62]. It has now been shown that gene expression profiles in blood platelets change along with the RNA profiles of cancer cells when they interact with each other [60]. In these studies, transcriptome profiles of blood platelets from cancer patients was able to detect the tissue origin of tumors with 71% accuracy across six different cancer types. Notably, Best et al. report no significant changes in the RNA profiles of blood platelets between patients with metastatic and non-metastatic tumors [60]. This suggests that the regulation of RNA profile in blood platelets is majorly influenced by the tumor cells rather than by the tumor microenvironment. Remarkably, tumors presenting MET or ERBB2-positive gene signatures or KRAS, EGFR, or PIK3CA mutations were distinguished using mRNA profiles of blood platelets obtained from cancer patients. The findings that blood platelet transcript profiles can distinguish between tumors mutated for KRAS, EGFR, or PIK3CA is very promising for cancer patients stratification to receive personalized therapies based on these profiles [60]. This potential application is of particular interest for patients suffering from metastatic cancers. Over the past decade, several drugs have been developed to specifically target the cellular signaling pathways in metastatic cancer cells [63]. However, the patients are stratified and targeted therapies are majorly designed

by analyzing the primary tumors after resection. In many patients, metastases occur after several years of initial tumor diagnosis and the metastatic sites present with unique genomic and transcriptomic signatures different from the tumor tissue of origin [64]. Hence, novel strategies to detect and capture tumor information directly from metastatic cells with non-invasive approaches like analyzing tumor educated blood platelets, would strengthen personalized cancer therapy in metastatic diseases.

Given this diversity in the spectrum of circulating carriers of tumor material in blood, it is interesting to perform prospective comparative studies between blood platelets, CTCs, cell-free DNA or exosomes for tumor content, and analyze which form of these liquid biopsy compartments provide the most informative molecular signatures of carcinogenesis and metastasis. Such studies will help to improve the screening and diagnosis of cancer, metastasis risk estimation, prognostic approaches, patients-cohort stratification and monitoring of therapy-response and emergence of resistance. Studies on large patient cohorts can significantly help to understand the full potential of tumor specific information that liquid biopsy offers.

Methods for capturing and isolation of EVs for next generation sequencing

Extracellular vesicles

Multiple approaches are available today for the EVs enrichment and profiling of EVs but the approaches are not standardized on the method. The most widely used method for EV isolation is differential centrifugation that can be applied to either body fluids and cell conditioned media [65, 66, 67]. Differential ultracentrifugation is the "gold standard" and the most economical protocol for EV isolation especially when large numbers of exosomes are required. In this method, cellular debris and larger EVs (>150 nm) are eliminated before pelleting remaining EVs at ≥100,000 g which requires more than 10 hours of centrifugation. Other methods such as ultracentrifugation and centrifugation isolation methods results in lower yields of exosomes due to the formation of aggregates [68-71].

Ultrafiltration is another method for capturing exosomes using filters with specific pore sizes . For example, the Amicon ® Ultra-15000 kDA tube approach retain vesicles with a maximum diameter of 0.22 um One way of improving the purity of the exosome population obtained along

with minimizing the time required for isolation is to pair ultracentrifugation with ultrafiltration [72, 73]. Urbanelli et al. comprehensively reviewed the commercial kits and patented approaches of exosome isolation that developed to improve and simplify the exosome isolation process [74]. New emerging isolation devices developed to decrease the time period of exosome isolation [75]. ExoChip is an example of these new approaches that works relatively cost-effectively in exosome separation. ExoChip is a microfluidic device which is suitable for capturing pure concentrations of exosomes as confirmed by Western blots and immuno-electron microscopy [76].

Exosomes also carry surface markers from the cell of origin, which can be used for enrichment strategies, similar to CTCs [77]. For example, characterization and analysis of exosome surface proteins hold great promise for the ability to identify, separate, sort and enrich exosomes originating from diverse cell sources. While the development of methods that allow for the routine analysis of exosome surface proteins has been a challenge, a number of recent advances have demonstrated the potential. Immunoaffinity-bead based capture methods, microfluidic chip methods and antibody-based exosome arrays using both label and label-free detection platforms have all successfully exploited specific exosome surface proteins. This has enabled the capture, enrichment and characterization of unique populations of exosomes in the blood of healthy donors and of patients with pancreatic cancer, ovarian cancer and lung cancer [76, 78, 79]. Surface protein-based exosome isolation methods combined with exosomal RNA extraction and qPCR detection assays have proven to be rapid and sensitive enough to monitor therapeutic response and resistance using exosomes from the blood of patients with glioblastoma [80, 81, 82].

Additionally, the rapid advancement of a novel method of nanoscale fluorescence activated cell sorting called nanoFACS has further advanced methods of exosome isolation and sorting and allowed for the study of discrete, free, individual exosomes from body fluids [83]. This technique of holds great promise for future diagnostic applications where isolation and examination of individual exosomes is paramount. Finally, in addition to proteins, analysis of exosome protein-to-lipid ratios can be used to further isolate and characterize subpopulations of exosomes in body fluids [84].

NanoSight system (NanoSight, Amesbury, UK) has been employed to measure the number and size distribution of EVs [85]. It is clearly evident that ideal purification of exosomes facilitates the proteins, DNA and miRNA analysis in exosomes [86]. Although the potentially significant role of exosomes in both physiological and pathological conditions is known, but their relevance as either

biomarkers or in disease intervention has doubted. One possible limitation regarding this method is the contamination from other EVs. Another reason for these discrepancies is the lack of standardization that leads to not implementing of this method as a routine clinical practice. Therefore, establishing a standardized technique for exosomes isolation is one of the most important challenges [87].

After isolation, Exosome-derived nucleic acids and proteins can be studies through sequencing, qPCR, Western blot, ELISA and flow cytometry. Such analyses provide new insights about tumor data but the broad spectrum of isolation methods makes it challenging to compare results between studies. At the DNA level, the mutational analysis of exosomal DNA is comparable to data from cell-free DNA and CTCs. Nevertheless, before we can choose exosomal DNA over other liquid biopsy compartments for routine mutation analysis; an in-depth comparison study is required [88]. Exosomes are also rich in RNA transcripts. The RNA contents of exosomes likewise other extracellular vesicles can be analyzed using NGS and qPCR 6 [86]. In addition to RNA, it has been reported that several exosomal protein also play important roles in cell-cell communication [59]. Studies have demonstrated that exosomal proteins and RNA transcripts are able to serve as prognostic and even predictive biomarkers in cancer patients [88]. Hopefully, a standardized method for isolation of the exosome and capturing the tumor data from them will be developed in the near future. This can maximize the relevance of laboratory-based researches of exosomes in the clinical setting. Conclusively, integrated systems for exosome detection and isolation serve to facilitate exosome research [86, 89].

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CHAPTER 3

Prognostic & Predictive Roles Of Liquid Biopsy In Colorectal Carcinoma

Potential prognostic and predictive roles of liquid biopsy in colorectal carcinoma; With A Special Focus on Circulating DNA and Exosomes

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Abstract

Background: In order to have the chance to evaluate both the response to therapy and possible causes of resistance, the repeatability of blood draw is a notable advantage of Liquid Biopsy. Consequently, liquid biopsy is considered a repeatable, non-invasive and dynamic tool. It could be able to recover cancer-specific information (miRNAs, circulating-free DNA (cfDNA), circulating tumor DNA (ctDNA) and exosomes) from liquid samples (especially blood). Liquid Biopsy therefore is there to overcome the limitations associated with traditional tissue biopsy.

Aim: Our main aim was to investigate the potential prognostic and predictive role of blood cancer-related biomarkers such as cfDNA or ctDNA and Exosomal DNA in colorectal cancer (CRC) patients.

Results: cfDNA/ctDNA and Exosomal DNA appeared to have a potential applicability in CRC management. New generation technologies are able to identify clinically relevant genomic alterations proving comparable performance to the ones in tissue.

Introduction

Circulating cell-free DNA (cfDNA) is derived from apoptotic or necrotic cells (1, 2). Being isolated from the plasma of cancer patients, cfDNA reflects the genomic mutation profile of the tumor. cfDNA levels are measured to be used for various clinical and research purposes, most importantly diagnosis of cancer (3).

Genomic mutations have always been one of the hallmarks of cancer (4). Molecular characterization of cancer needs the analysis of tissue specimen but using a single tissue specimen will provide only limited information of the disease (5, 6). However, serial analyses of cell-free DNA (cfDNA) describes the heterogeneity of a given cancer and supports more suitable decisions for treatment (7, 8).

Whether cfDNA must complement routine analyses of tissue in all types of cancer remains to be studied (9, 10), but cfDNA has the potential to assess cancer evolution superior to tumor tissue DNA (11, 12).

Colorectal cancer (CRC) is one of the most common cancers worldwide (13, 14). CRC is a dynamical disease and is recognized that most CRC develops slowly over years (15, 16). Genetic and epigenetic events during the CRC progression differs from tumor to tumor considerably (15, 17, 18). Thus, CRC is not a unique disease; it includes different genetic pathways' encompassing which causes various clinical behaviors (15, 17-19).

Understanding the underlying mechanisms of cancer diversity and biomarker identification to predict responsiveness to treatments seems essential to improve treatment options. With regard to the molecular level, CRC illustrates a complex framework of altered genes (20).

Recent progress allows the study of biomarkers relevant to colorectal cancer. Besides, collection of blood specimens ("liquid biopsies") is a less invasive and therefore an attractive approach. Moreover, tumor DNA is released into the blood with regard to cell turn-over (21). Therefore, cfDNA mutation analysis captures a "real-time" mutational profile of the tumor(s) (22). This

however gives access to tracking of dynamic changes in tumor biology throughout individual treatment. It has been reported that cfDNA genomic landscape provide insight to track CRC status (23) and recurrence (24-26).

Exosomes are small vesicles of 30-100 nm found in the circulating blood, in which microRNA (miRNA/miR), mRNA and DNA fragments can be detected (27-30).

Studies demonstrated that the level of exosomes released from cancer cells are increased in comparison to normal cells (31, 32). Exosomal DNA, may provide information about the origin of cancer cells (33, 34). Exosomes may be a sensitive and specific diagnostic tool to monitor cancer patients (35).

The reported advantages of liquid biopsy, are mainly related to its easy repeatability and the possibility of avoiding the selection bias related to tumor heterogeneity. Moreover, gene studies require neoplastic tissue for further genetic analysis, but in some conditions, the impossibility of a re-biopsy (because it is technically not feasible or because the patient is deemed unfit for invasive procedure) could lead to situations of "undertreatment" that could be avoided with the introduction of liquid biopsy in clinical practice. This new tool could potentially be able to improve CRC management during diagnosis, real-time monitoring or acquired resistance phases.

To the best of our knowledge, clinical utility and practicality of obtained data from liquid biopsy compartments (cfDNA & Exosomal DNA) in accordance with CRC are still under debate. On the other hand, Exosomal DNA genotyping assays in patients with colorectal cancer (CRC) have not been fully described. Therefore, we designed a research regarding the mutational landscape of Exosomal DNA in accordance with diagnosis, recurrence of the diseases and emerging changes.

The steps we followed were: the analysis of the concordance between specific mutations (KRAS, NRAS and BRAF) found in the tissue as well as in the blood. Following with the search of the prognostic value of "mutation load". Last but not least we did the comparison between the genetic information carried by cfDNA and Exosomal DNA, regarding their use in clinical practice.

Materials and Methods

Patient selection

A diagnosis of colorectal cancer was the required criteria to be met prior to inclusion in the study. Patients with diagnosed colon adenocarcinoma (colon AC) had been recruited for blood collection.

Written informed consent have been obtained from all participants. A sample of 40 CRC patients were enrolled during chemotherapy with or without targeted agent at the U.O.C. of Medical Oncology of the University Hospital Policlinico Paolo Giaccone of Palermo, recruited on a voluntary basis.

Peripheral venous blood samples have been collected in EDTA containing plasma preparation tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). One or more blood samples were collected primarily before the beginning of chemotherapy infusion. Blood samples were collected initially at the time of the first instrumental evaluation and every 2-3 months. (Table 1)

Blood sample preparation

Plasma was separated from the cell fraction within one hour from the collection using two successive centrifugations: the first 1200 gx 10 minutes at 4 ° C to avoid cell lysis leading to the separation of the plasma from the corpuscular part of the blood and the second to 3000 gx 10 minutes at 4 ° C to eliminate cellular debris and red blood cells from plasma. Thereafter, Plasma samples were transferred into new 2 ml tubes and stored at -80 °C until use.

Circulating Cell-Free (cfDNA) DNA Extraction

cfDNA was extracted from 1 mL plasma using the QIAampÒ circulating nucleic acid kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. This kit uses a vacuum pump to increase the extraction speed and efficiency. The procedure is easy and suitable for the simultaneous extraction of multiple samples. The extraction involves: a first phase of lysis, a purification phase through various washes, during which the DNA is retained by a membrane present in the column provided by the kit, and a final phase of elution in which the purified DNA is removed from the membrane by centrifugation after imbibition with the elution solution.

Circulating DNA quantification

3µl of the extracted cfDNA were used to measure the concentration obtained. Quantization of free circulating DNA was performed using the Qubit TM dsDNA HS Assay Kit (Invitrogen, Life Technologies, CA, USA) in a Qubit 3.0 fluorometer (Invitrogen, Life Technologies, CA, USA). This is a very sensitive and specific test that can quantize even small concentrations of DNA (between 0.01-100 ng/µl). This quantization system allows to "measure" the emission of

fluorescence through the use of a fluorophore, which specifically binds the double-stranded DNA (dsDNA).

Exosomal DNA Extraction

Following the manufacturer's instruction, we extracted the exosomes with Exoeasy kit (Qiagen). This kit uses a column inside, a membrane able to specifically bind the entire spectrum of extracellular vesicles present in the sample, including also the exosomes. Exoeasy Maxi kit allows a rapid isolation of the exosomes using a quantity of 0.2-4 ml of plasma. It consists of a series of washes and centrifugations, which aim to remove from the membrane all that is not represented by extracellular vesicles. The last important elution step serves to detach the isolated vesicles from the membrane of the column and to keep them inside the tube.

The DNA inside the exosomes have been extracted with the help of XCF Exosomal DNA Isolation kit (System Bioscience, XCF200A-1). Obtained DNA then passed through the NGS step same as cfDNA.

Next Generation Sequencing Analysis

The NGS analysis was conducted both on cfDNA and on isolated exosomes. In particular, the sequenced cfDNA samples, expressed with the CRC code, were 17 (2 for each patient except CRC-7). Among these 9 patients, 3 were chosen, of which the exosomes were isolated and the Exosomal tumor DNA sequenced (CRC-3, CRC-13 and CRC-20). Also in this case, for each of the 3 patients, 2 plasma samples were used, taken at two different time points.

Approximately 10 ng of DNA was used for next-generation DNA sequencing. Massively parallel sequencing was performed using the Ion AmpliSeqTM Cancer Hotspot Panel v2 (CHPv2) according to the manufacturers' instructions. CHPv2 targets 207 amplicons for 2885 mutations in 50 cancer-associated genes.

The Ion AmpliSeq Library kit 2.0 (ThermoFisher) kit was used to prepare the libraries following the instructions given on the protocol.

The resulting libraries were then subjected to further amplification by PCR emulsion on the Ion OneTouch2 TM (ThermoFisher) instrument. During this phase the library fragments are linked to the Ion Sphere Particles (ISP) thus forming the template which will then be used during the

sequencing phase. At the end of the emulsion PCR, we proceed with the enrichment of the ISP using the Ion OneTouch TM ES instrument. The enriched ISPs are then loaded onto the Ion 530 TM chip which is then subjected to sequencing within the IonS5 instrument. During the run planning through the Torrent Suite TM Software, all the information that will be used in the following sequencing phase is provided (number of flows, barcodes and reference files). The Ion 520 TM & Ion 530 TM Kit-OT2 (ThermoFisher) kit was used to complete the above steps. The analysis of the data and the annotation of the variants was finally conducted through Ion Reporter Software TM by applying the AmpliSeq CHPv2 peripheral / CTC / CF DNA single sample workflow.

Statistical Analysis

Statistical analyzes were conducted using the MedCalc ver 14 software and SPSS software version 19.0. To compare the PFS and OS of patients with values of cfDNA less than the median and those with values higher than the median, a LogRank test was performed. Linear regression and the LogRank test were used to study the correlation between DSS, PFS and "tumor mutational load". Numerous statistical analyses evaluated the distribution of factors such as the concentration of cfDNA and the "tumor mutational load" of patients grouped on dichotomous variables (eg patients with right-sided and left-sided colon cancer). The Student and Levene t tests were used provide our results.

Results

Patients

NGS succeeded in 8 out of 10 patients. Mutations were detected in the blood of three of the patients with noticeable coverage with the results in tissue.

Later on, we continued with the exosome part in that patients each in two time points and later we continued with the NGS for the DNA from exosomes. In one of those three patients, however we succeeded to find relevant mutations with very high frequencies inside the exosomes. The two time-points of the same patient had a significant coverage with regard to Exosomal DNA.

Mutational Analysis of Colorectal Tumors Identified by CHPv2

The median output per sample by Ion Xpress sequencing was 110 bases for ctDNA and 66 bases for Exosomal DNA. The sequence reads however were 12,015,041 for ctDNA and 416,187 for Exosomal DNA. The coverage depth for the samples which were a median close to 0,0 were considered insignificant.

cfDNA and prognostic role

Overall survival (OS) was evaluated. However, given the short duration of follow-up for some patients, the median was not reached after 5 patients out of 40 died (mOS 28,3 months) (Figure 1) The same analysis for PFS was performed. The PFS was calculated between the first and second lines. Unlike the OS, in this case the median was reached and was equal to 14,5 months. Furthermore, it has been showed that patients with a concentration of cfDNA higher than the median value (0.47 ng/µl) had worse, statistically significant, PFS (p=0,048), according literature data. (Figure 2)

As regards cfDNA value and primitive neoplastic site, although patients with right-sided colon cancer reported a higher mean (0.704 ng/µl) than those with left-sided colon cancer (0.643 ng/µl), the difference was not statistically significant (p>0,05). (Figure 3)

A statistically significant correlation has been shown between cfDNA value and histological mucinous adenocarcinoma subtype. The mean concentrations of cfDNA in the mucinous adenocarcinoma and adenocarcinoma group was respectively 0,79 ng/µl and 0.57 ng/µl, statistically significant. (Figure 4)

cfDNA and predictive role

A possible cfDNA level difference has been showed in patients with disease progression (PD) if compared to patients who did not (PD was defined RECIST 1.1 criteria). The result obtained shows that the mean cfDNA concentrations of progressing patients (0.77 ng/µl) were statistically lower than the average of non-progressing patients (0.63 ng/µl) (p=0,07) (Figure 5), although a non-statistically significant difference was observed between the mean concentrations of cfDNA of patients receiving anti-EGFR (0.81 ng/µl) and the mean concentration patients treated with anti-VEGF (0.73 ng/µl).

Tumor mutational load and prognostic role

Among the 17 samples analyzed in NGS, only in 2 samples a mutation in the KRAS gene was found respect to the 5 mutations identified on the tissue. The correlation between KRAS mutations in the tissue and in the cfDNA was therefore 40%. NGS sequencing of the NRAS gene produced a different result. Two mutations were detected in the CRC-3D and CRC-10A samples. However, no mutation of NRAS was detected on the tumor tissue of both patients. Mutation of the CRC-4 was not confirmed by liquid biopsy. The BRAF V600E gene of the CRC-3 sample was not detected in the cfDNA and overall the NGS analysis did not allow to detect mutations in the BRAF gene. Despite the small number of samples studied, it could be suggested a correlation between disease-specific survival (DSS) and the number of non-synonymous mutations, statistically significant at the second time-point. As shown in Figure 8, the number of non-synonymous mutations showed an inverse and significant correlation with the DSS ($p = 0.034$). Using the median value of non-synonymous mutations (3.5), it was found that patients with a number of non-synonymous mutations below the median value had a trend for better DSS, (LogRank test $p= 0.08$) (Figure 9). A similar result was obtained for PFS. The cumulative progression time reported a statistically significant increase ($p = 0.037$) as the number of total mutations decreases (Figure 10). However, also in this case, by subdividing this parameter according to the median value (17.5 mutations), the result was not statistically significant (LogRank test $p= 0.0896$) (Figure 11), probably due to the small number samples.

Next generation sequencing results of cfDNA

In the second part of the study, we selected nine patients with particular medical records (17 samples) to perform Next Generation Sequencing cfDNA. As shown in Tables 2 and 3, 2 samples of cfDNA collected from the blood of 9 patients were analyzed, at two different time points, with the exception of the CRC-7 code patient of which only one sample was available, for a total of 17 samples sequenced.

As shown in Table 3, a total of 309 mutations were discovered. Among these, 65 are non-synonymous somatic mutations, (61 have a single nucleotide substitution (SNV) and 4 are insertions/deletions (INDEL)). The remaining 244 are synonymous and intronic mutations.

The mean allelic frequency (variant allele frequency, VAF), expressed as a percentage, of non-synonymous somatic mutations found in the 17 samples was 38.3, with a VAF range of 1.62-100.

Figures 6 and 7 showed, for each gene involved, the number and frequency of non-synonymous mutations. The highest number of mutations was detected in the TP53, where the most frequent SNV was c.215 C>G. Mutations in the APC, NRAS, PIK3CA and KRAS genes, notoriously involved in the Fearon and Vogelstein "adenoma-carcinoma sequence", showed a lower number of mutations. The KDR gene, which codes for VEGFR2, was found to be mutated in 3 metastatic patients treated with antiangiogenetic. The other genes, such as EGFR, PTEN and ERBB2 implicated in the CRC carcinogenic process by blocking the EGFR cascade, demonstrated a low frequency of mutations. Among the 17 samples analyzed in NGS, only in 2 was found the mutation in the KRAS gene, against the 5 mutations identified on the tissue. The correlation between KRAS mutations in the tissue and in the cfDNA is therefore 40%. NGS sequencing of the NRAS gene produced a different result since 2 mutations were detected in the CRC-3D and CRC-10A samples. However, no mutation of NRAS was detected on tumor tissue of both patients. At the same time, the NRAS Q61K mutation of the CRC-4 sample was not confirmed by liquid biopsy. Tissue mutation of the BRAF V600E gene of the CRC-3 sample was not detected in the cfDNA and overall the NGS analysis did not allow to detect mutations in the BRAF gene in any of the samples analyzed.

Next generation sequencing results of Exosomal DNA

A further step of the study was the Exosomal DNA NGS analysis in comparison with the sequencing of cfDNA. Plasma exosomes samples were collected from 3 CRC patients (CRC-3; CRC-13; CRC-20), at 2 different time points (T1 and T2). Exosomal DNA and cfDNA were quantized and sequenced from the same blood sample. An interesting result emerged from the sequencing of the Exosomal DNA of the CRC-20 sample. Mutations identified in these samples are sumarised in table 4. Table 5 however shows the most mutated genes in all samples for ctDNA and Exosoemal DNA as well.

As shown in Figure 12, 2 blood samples were collected from CRC-20 (the first at the third cycle and the second at the seventh CAPOX q-21 adjuvant cycle). Over a period of 6 months from the start of the treatment, an extensive lymph node and metastatic spread recurrence was CT evaluated and PET confirmed. Both the CEA and cfDNA values showed a trend that is consistent with the patient's clinical history, in fact a CEA 100-fold increase and a cfDNA 2-fold were observed.

The sequencing of the cfDNA extracted from the patient's blood to the first and second time points revealed a number of 5 and 8 non-synonymous somatic mutations respectively. The KRAS gene mutation was detected in the T2 sample, but not T1. In addition to mutation in the KRAS gene, functionally significant mutations were found in other genes such as: PIK3CA and ERBB2 (linked to KRAS and the EGFR pathway) and NOTCH1 (whose activity is able to suppress the Wnt expression even when the degradation of the ß catenin by the APC complex is disabled by the mutation in the latter gene). The same analysis was performed on the Exosomal DNA: at T1 17 missense mutations were identified, with a high average allelic frequency (VAF = 81). At T2 the result was not significant, probably for technique reasons. However, T1 Exosomal DNA result could explain this case-report. I reported the KRAS mutation appearance in the T1 Exosomal DNA according to neoplastic tissue mutational status. Interestingly, KRAS gene mutation was detected in cfDNA only at T2. Among the other genes, other mutations have been identified, in PIK3CA, ERBB4, SMARCB1 and SMAD4. SMAD4 gene mutation could assume a considerable role because of its prognostic role CRC. Finally, From the comparison with cfDNA, it would seem that the exosomes should be object of more in-depth studies as they could be able to highlight RAS mutations earlier than cfDNA.

Discussion

In recent years, numerous scientific reports have evaluated the diagnostic, prognostic, predictive and monitoring role of cfDNA and ctDNA. Both are promising non-invasive biomarkers that have contributed to the recent rise of liquid biopsy (36, 37).

One of the best important application of cfDNA concentration as a biomarker for monitoring the response to therapy was the evaluation of the variations during chemotherapy infusion. CfDNA concentrations on the basis of radiological progression showed a statistically significant trend, although the type of targeted therapy did not. However, its essential to reach a number of samples for each patient that allows a more accurate dynamic evaluation of the tumor and mutation load of CRC.

About the site of the primary tumor, our result suggests higher cfDNA value in patients with right colon CRC patients, although the is not statistically significant. Of note, a statistically significant result was obtained for histological type. In fact, the mucinous forms of CRC showed significantly

higher levels of cfDNA than those obtained from patients with classic adenocarcinoma suggesting greater tumor activity linked to mucinous type to explain its already known prognostic role.
The limited number of available blood samples did not allow a precise dynamic monitoring of the genetic profile. However, analyzing the results obtained, interesting cases have emerged. First, some tissue mutations have not been confirmed by the cfDNA. Secondly, the cfDNA allowed to highlight mutations not found in the tumor tissue (NRAS patient). In the first case, the non-concordance could be due to a limitation of the technique used (DNA extraction and sequencing). On the other hand, however, we must consider that most of the patients have undergone the resection of the primary tumor, as well as extreme variability of the metastases number and size. So, the different tumor burden could influence gene mutations found in cfDNA. In the second case, we can instead assume that the number of mutated copies of the NRAS gene at the time of tissue biopsy was not sufficient to detect it. In contrast, over time, the increase in the number of mutated copies of the NRAS gene was seen in the cfDNA.
Regarding "mutational load" predictive and prognostic role, different trends were observed between the number of mutations, the VAF and the survival estimates. This encouraging result surely pushes us to expand the sample number and to seek further confirmations on the reliability of the mutational tumor load as a promising biomarker in CRC.
Moreover, there is increasing evidence that the intercellular communication mediated by exosomes plays an important role in the process of carcinogenesis. In fact, they intervene in angiogenesis, premetastatic niche development and immune system tumor escape (38, 39). The case under report has shown that the Exosomal DNA is able to intercept the KRAS gene mutation before cfDNA gene. Clinically, it could allow to define disease recurrence and the secondary resistance phases. From the analysis of the Exosomal DNA cargo, other mutations have also emerged among which the SMAD4 gene, which has a prognostic role CRC. Biologically, the above mentioned diagnostic anticipation could be explained by the ability of the exosomes, equipped with a membrane, to transport and protect the load of proteins and nucleic acids within them. On the contrary, since the cfDNA in the blood is subjected to continuous degradation and denaturing processes, a longer time is required for mutated genes to exceed the sensitivity limit of the NGS. In addition to sensitivity, a further important difference between the cfDNA and the Exosomal DNA is related to specificity. In fact, ctDNA, which derives from the processes of necrosis and apoptosis of neoplastic cells, constitutes only a small fraction of the cfDNA. Vice versa, the exosomes are actively released

from the cancer cells and their content seems to be fundamental in the tumor economy, so the sequencing of their DNA allows a specific assessment of the genetic picture of the neoplasm. Consequently, these reasons justify the need to know the diagnostic accuracy of the technology available today to remove the limits of genomic analysis and to improve the cost/effectiveness ratio.

Although our study compared the two sources of DNA in only one patient, we hypothesized that the sequencing of Exosomal DNA can guarantee a true "real time" evaluation of tumor dynamics, thus constituting a highly reliable non-invasive biomarker in the disease course.

In light of this, the most fascinating perspective is undoubtedly that of using a Next Generation Sequencing panel that allows to study the mutational status of single genes related to the pathogenesis of CRC and "tumor mutational load". New generation technologies allow the analysis of multigenic panels and several patients at the same time, guaranteeing high performance standards and economic savings. Several articles have discussed the potential role of ctDNA in CRC by highlighting the detection of higher levels in more advanced stages of disease. It has also been hypothesized that elevated levels of ctDNA in CRC are correlated with poor outcome, as its primary post-surgery finding may suggest the presence of minimal residual disease and its potential role for the definition of actionable targets. Although, CRC are highly heterogeneous, their intra-tumoral heterogeneity features are less studied than other cancer types. Recent findings show that intra-tumoral heterogeneity can be tested by single-cell sequencing; the single-cell genome sequencing permit quantitative characterization of both single nucleotide variations and somatic copy number variations in individual tumor cells (40). Recently, a Nature Opinion article suggests that the main operative unit of a cancer is the genetically and epigenetically modified single cell. The single-cell analysis can allow to clarify the intra-tumoral genetic heterogeneity and cancer genome evolution in order to develop new tools able to provide robust interpretation of the mechanisms related to diagnosis, tumor recurrence and the functioning of new generation molecular target agents and the development of secondary resistances modifying cancer patient management (41, 42). Despite these potentialities, the use of liquid biopsy in routine clinical practice has historically been burdened by still substantial costs, heterogeneity in methodology and results from studies or in different threshold values that could be influenced by the noise caused by normal tissue cfDNA. For these reasons, the level of evidence to date is not yet considered as high to recommend the use of ctDNA RAS testing in routine clinical practice. In addition, our

expectation is that by ceaselessly escalating experience of findings and varios analyzing methods regarding ctDNA and Exosomal DNA; one could overcome the aforementioned barriers place ctDNA and Exosomal DNA in regular clinical practice for most of the CRC cases.

Conclusions

Our results demonstrated the genomic background of DNA from exosomes derived from Plasma of colorectal cancer patients with regard to the mutation in the tissue and ctDNA for the first time. In comparison with our previous studies on tissue, we firstly aimed ctDNA and get relevant results in both time points of three patients. Later we revealed the possibility that there is a very exact genomic profile picture inside the exosomes. Interestingly our results confirmed the same mutations in Exosomal DNA most importantly with high frequencies. This suggests the fact that exosomes selectively mirror the Genetic landscape of the disease. They may also be able to specifically reveal the stage, process and dynamics of the disease. Last but not least, they can be used as sources of information regarding metastasis, resistance and response to therapy. Considering these, they can be considered novel agents for diagnosis and prognosis of colorectal cancer and other types of cancer as well.

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Figures & Tables

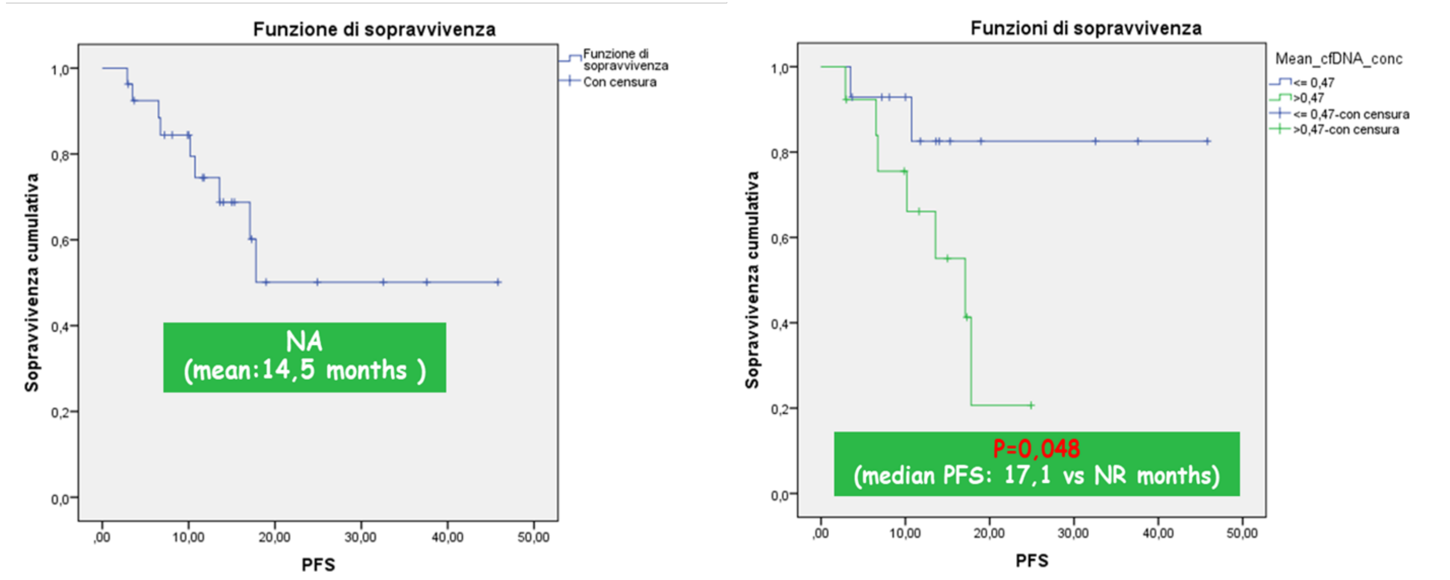

Figure 1: Median PFS (A) and PFS according to median [cfDNA] (NA: Not applicable)

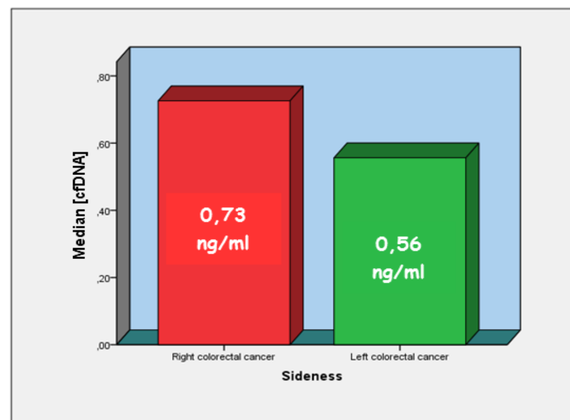

Figure 2: [cfDNA] and primary mCRC sidedness

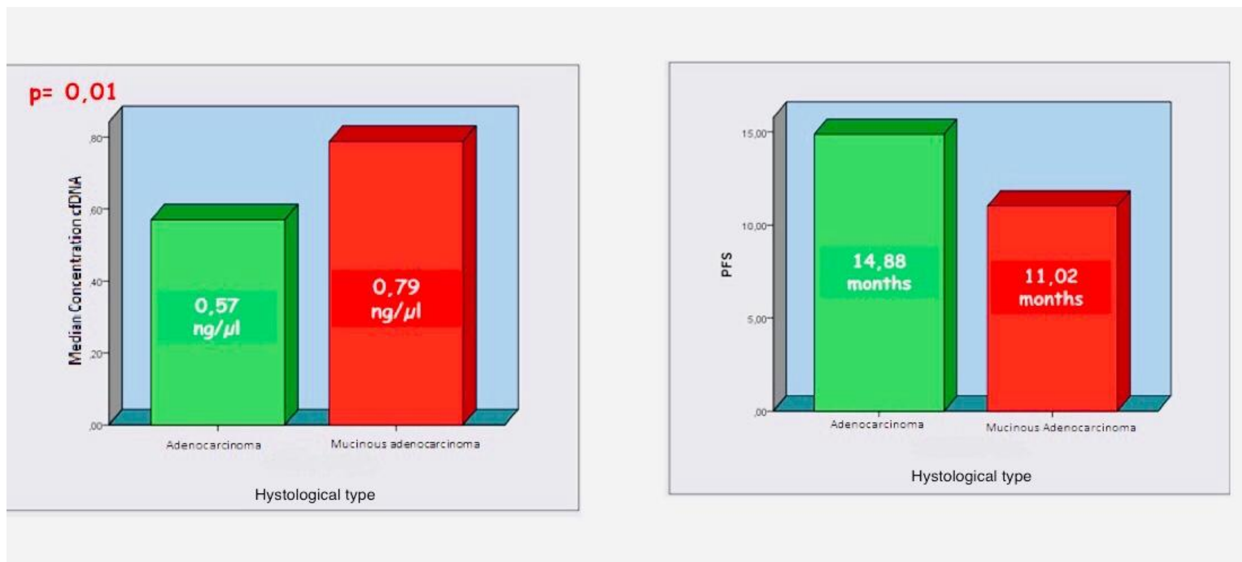

Figure 3: [cfDNA] and PFS and histological type

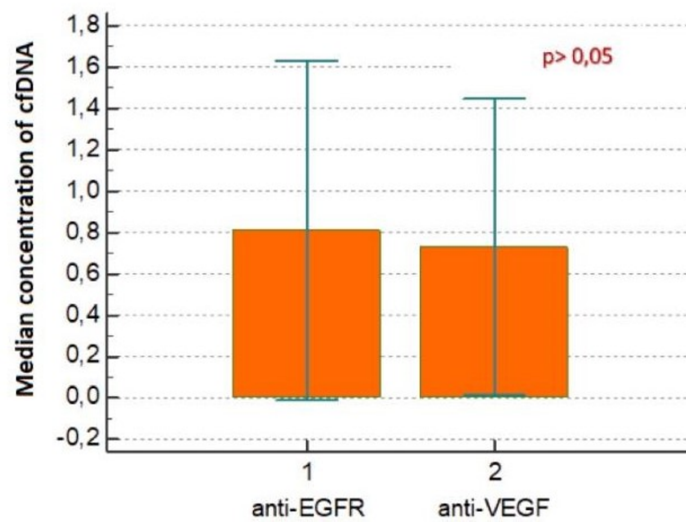

Figure 4: [cfDNA] and targeted agents

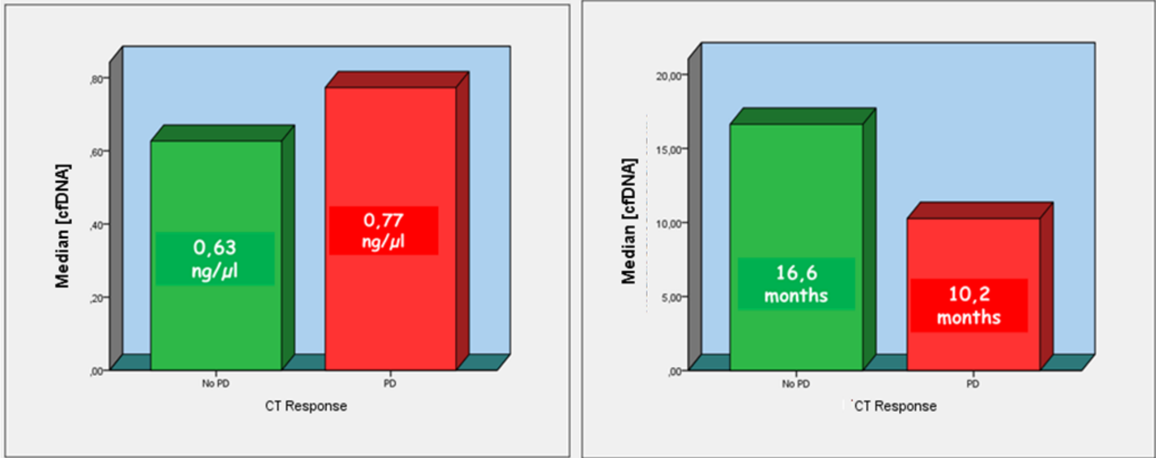

Figure 5: [cfDNA] and PFS and TC response

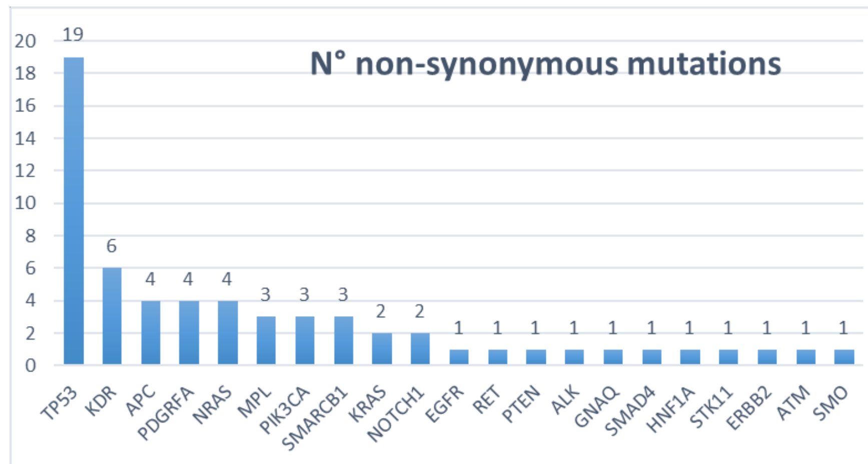

Figure 6: Non-synonymous mutations (%)

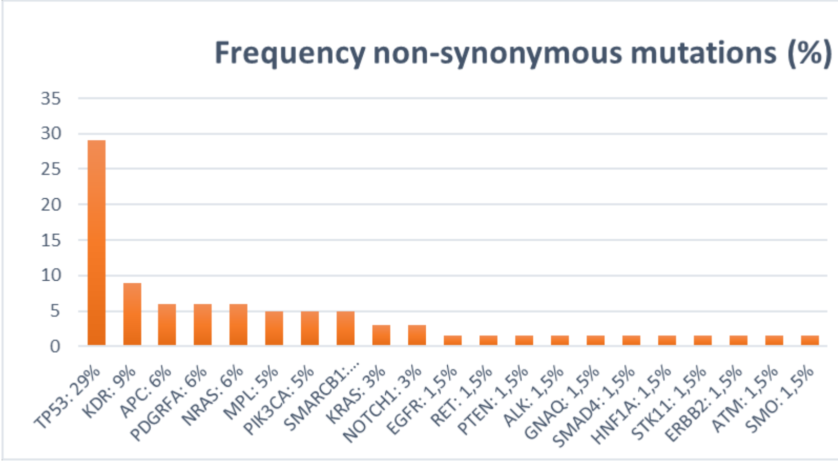

Figure 7: Non-synonymous mutations (%)

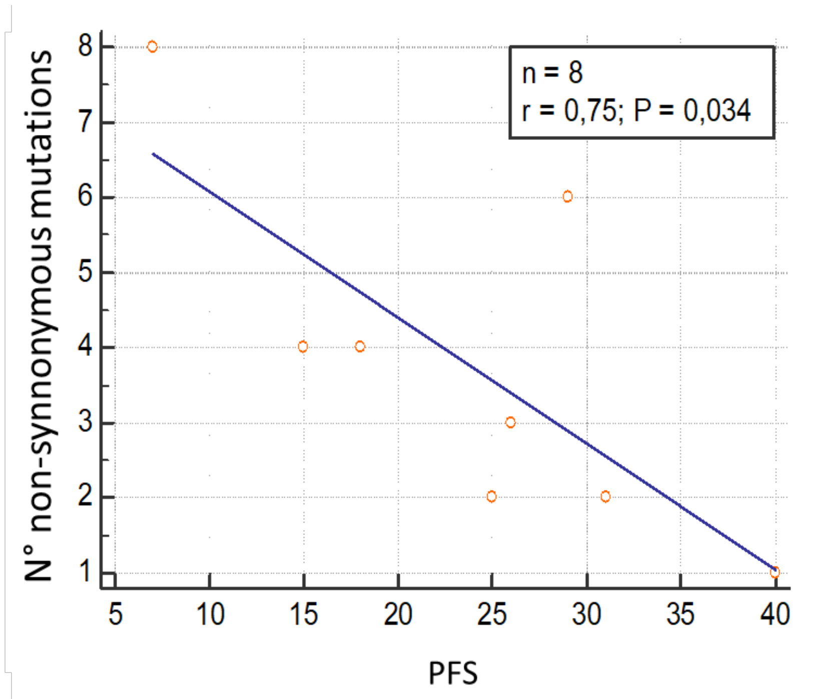

Figure 8: Correlation between sample collection 2 (P2) and disease-specific survival (DSS)

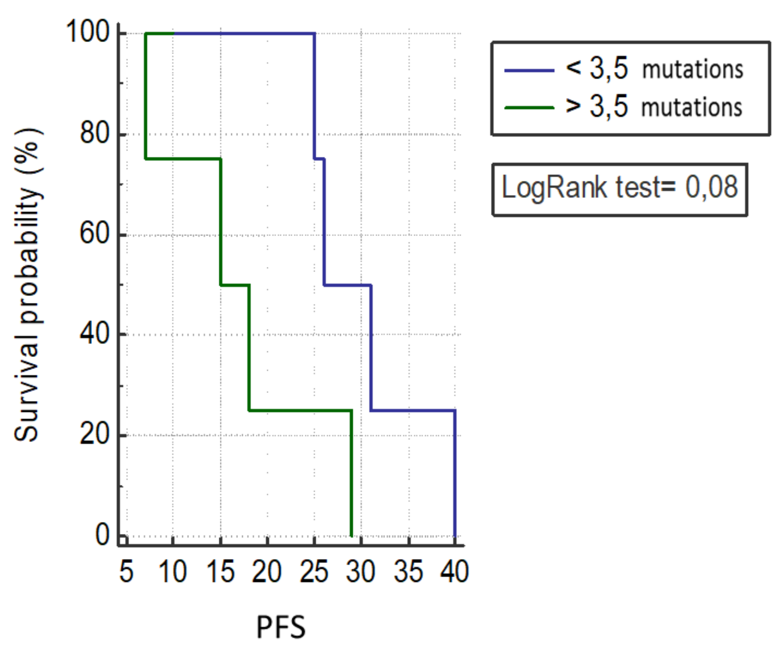

Figure 9: Disease-specific survival (DSS) and non-synonymous mutations

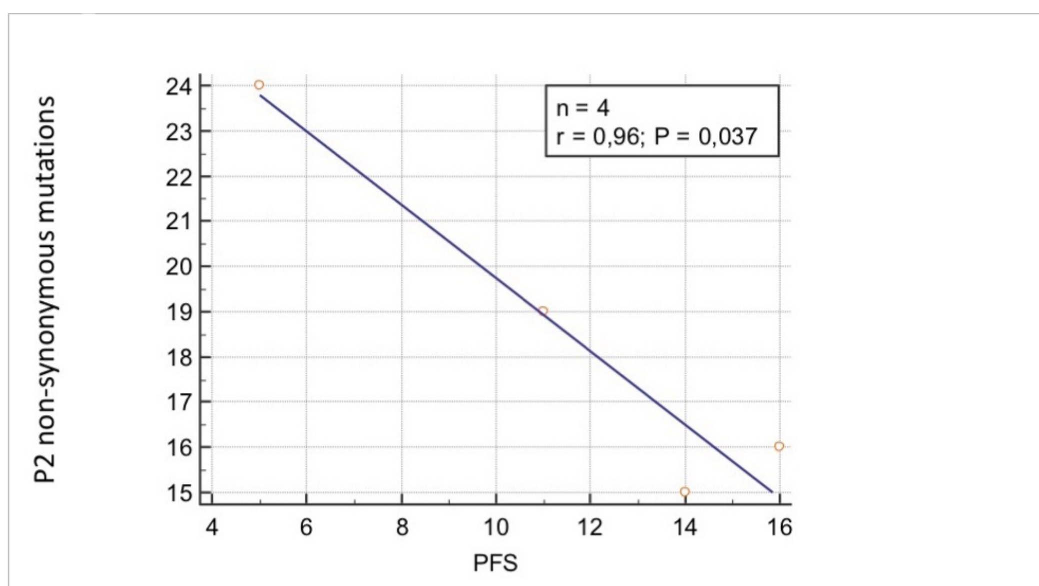

Figure 10: Correlation between sample collection 2 (P2) and progression-free survival (PFS)

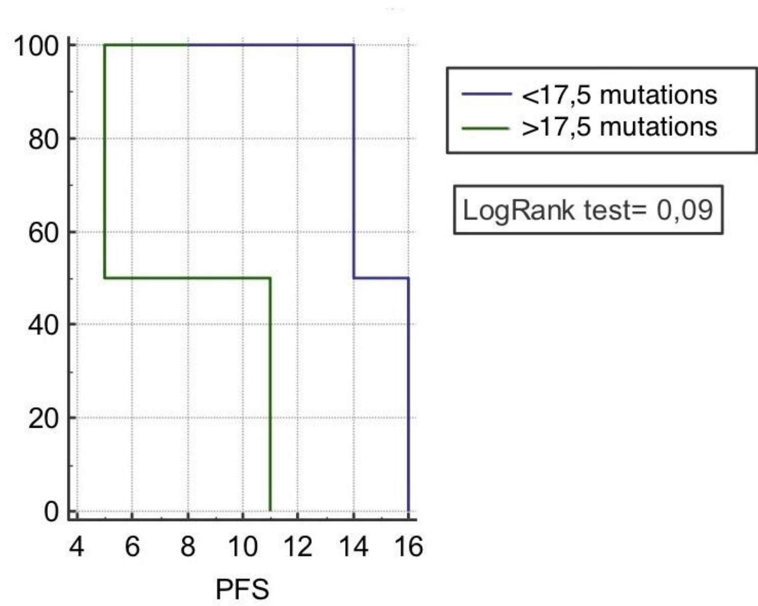

Figure 11: Progression-free survival (PFS) and non-synonymous mutations

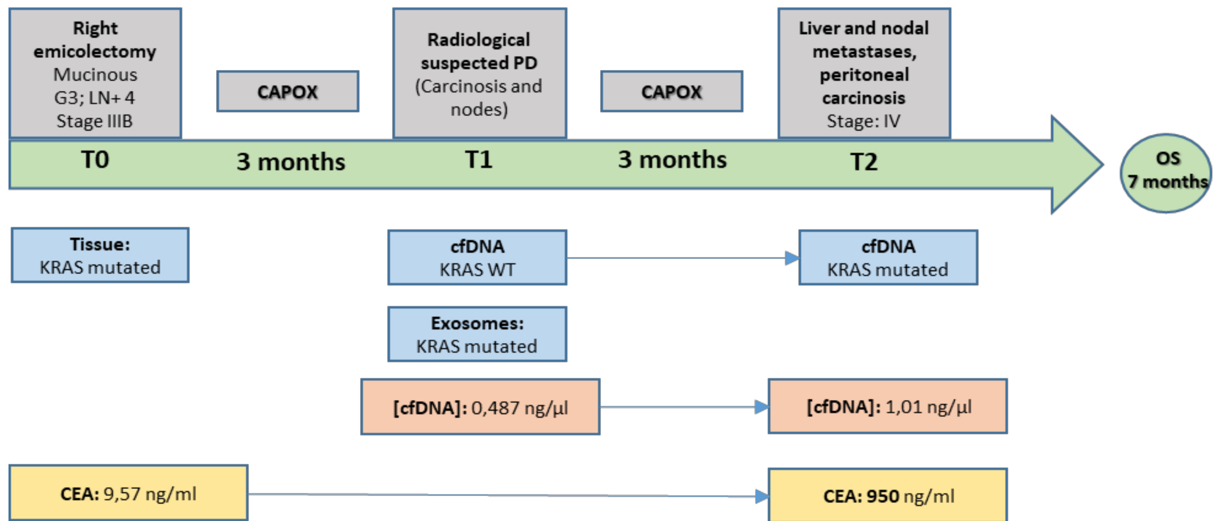

Figure 12: CRC-20 case report

Table 1: Clinical features of patients included in the research project

N° Patients	9
Mean age	66 (52-75)
Sex	
Female	6 (67%)
Male	3 (33%)
Primary tumor sideness	
Right colon	5 (56%)
Left colon	4 (44%)
Neoplastic nodes	
< 10	1 (11%)
≥ 10	7 (78%)
NA	1 (11%)
Grading	
G1	0
G2	3 (33%)
G3	5 (56%)
NA	1 (11%)
Hystological type	
Adenocarcinoma	5 (56%)
Mucinous Adenok	4 (44%)
Diagnosis TNM stage	
IV	8 (89%)
III	1 (11%)
Linea di terapia	
Adjuvant	1 (11%)
I line	3 (33%)
II line	4 (44%)
III line	1 (11%)
Survival status	
Alive	8 (89%)
Dead	1 (11%)

Table 2: Clinical features of patients included in NGS

N° Patients	40
Mean age	62 (48-81)
Sex	
Female	17 (42.5%)
Male	23 (57.5%)
Primary tumor sideness	
Right colon	16 (40%)
Left colon	24 (60%)
Neoplastic nodes	
< 10	32 (80%)
≥ 10	5 (12.5%)
NA	3 (7.5%)
Grading	
G1	2 (5%)
G2	23 (57.5%)
G3	10 (25%)
NA	5 (12.5%)
Hystological type	
Adenocarcinoma	29 (72.5%)
Mucinous Adenok	11 (27.5%)
Diagnosis TNM stage	
IV	22 (55%)
III	12 (25%)
II	4 (20%)
I	2 (5%)
CT Lines	
Adjuvant	2 (5%)
I line	20 (50%)
II line	8 (20%)
III line	10 (25%)
CT Type	
CT	7 (17.5%)
CT+Anti-VEGF	29 (72.5%)
CT+Anti-EGFR	4 (10%)
Survival status	
Alive	35 (87.5%)

Table 3: NGS results. CT: Computed tomography response; SD: Stable Disease; PR: Partial Response; PD: Progression o
specific survival; PFS: Progression-free survival, OS: Overall survival; P1: Blood sample collection; P2: Blood

Code	[cfDNA]	number of non-synonymous mutations	Total number of mutations	Mean VAF% [non-synonymous]	Number of cycles P1-P2	CT	P S
CRC-1	0.423	5	21	22.604%	18	SD	
	0.130	1	15	96.25%			1
CRC-3	2.79	2	16	46.085%	21	PD	
	0.428	6	22	17.8%			
CRC-4	0.867	2	14	50.19%	20	PD	1
	1.11	3	16	34.4%			
CRC-7	0.523	6	17	48.31%	NA	NA	1
CRC-10	0.362	4	19	15.4%	5	SD	N
	0.813	2	15	71.62%			
CRC-13	0.374	5	18	31.55%	18	PD	N
	0.225	4	16	39.55%			
CRC-16	0.253	3	17	57.43%	6	PR	1
	0.747	4	19	50.37%			
CRC-20	0.457	5	24	48.61%	4	PD	.
	1.01	8	24	15.7%			
CRC-26	0.293	3	20	49.46%	13	SD	N
	0.624	2	16	72.95%			
Total		**65**	**309**	**38.3%**			

Table 4: Identified mutations for samples whom went through NGS for Exosomal DNA as well as cfD

BRAF				KRAS				KRAS	
CRC3A	**3A_exo**	**CRC3D**	**3D_exo**	**CRC13A**	**13A_exo**	**CRC13C**	**13C_exo**	**CRC20A**	**20A_exo**
APC	FLT3	APC		APC	KDR	APC	SMAD4	APC	APC
CSF1R	SMAD4	ATM		ATM		CSF1R		ATM	ATM
EGFR		CSDE1		CSF1R		EGFR		CSF1R	ERBB4
EGFR-AS1		CSF1R		EGFR		ERBB4		DERL3	FGFR2
ERBB4		EGFR		ERBB4		FGFR3		EGFR	FGFR3
FGFR3		EGFR-AS1		FGFR2		FLT3		EGFR-AS1	HRAS
FLT3		ERBB4		FGFR3		HMGXB3		ERBB4	IDH2
HMGXB3		FGFR3		FLT3		KDR		FGFR3	KDR
KDR		FLT3		HMGXB3		MET		HMGXB3	KIT
PDGFRA		HMGXB3		KDR		PDGFRA		HNF1A	KRAS
RET		KDR		KRAS		PIK3CA		HRAS	NOTCH1
STK11		NOTCH1		PDGFRA		RET		KDR	NPM1
TP53		NRAS		PIK3CA		SMARCB1		MET	PDGFRA
		PDGFRA		RET		TP53		PDGFRA	PIK3CA
		RET		SMAD4				PIK3CA	RB1
		STK11		TP53				RET	SMAD4
		TP53						SMARCB1	SMARCB1
								TP53	
								VHL	

Table 5: Most mutated genes in all patients

Most Mutated	
CtDNA	**ExoDNA**
KDR 10	SMAD4 8
APC 9	PIK3CA 6
PDGFRA 9	ATM 3
RET 9	ERBB4 3
TP53 8	RB1 3
ERBB2 7	APC 2
FGFR3 7	FGFR3 2
PIK3CA 7	FLT3 2
CSF1R 6	IDH2 2
EGFR 6	KDR 2
HMGXB3 6	KRAS 2
ATM 5	NOTCH1 2
EGFR-AS1 4	NPM1 2
FLT3 4	
MET 3	
SMARCB1 3	
STK11 3	
DERL3 2	
HRAS 2	
KRAS 2	
NOTCH1 2	
NRAS 2	

CHAPTER 4
MicroRNA and Cancer

The role of microRNAs in cancer regulation

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Submitted To Gene

Abstract

MicroRNAs (miRNAs) are a large family of evolutionary conserved small non-coding RNA molecules that firstly discovered in 1993. They regulate gene expression of about 50% of protein-coding genes at the post-transcriptional level. MiRNAs can target numerous messenger RNAs and subsequent misexpression of them can affect many different signaling pathways. They are playing a pivotal role in cancer development by regulation of the genes expression which involved in the proliferation, survival, differentiation, apoptosis or metastasis of the cancer cells. Several treatment approaches such as inhibition of oncomiRs and restoration of tumor suppressor miRNAs have been established in certain types of cancers and some other miRNA-based strategies are in development for cancer prevention and treatment. Nowadays, cancer is the most important target of miRNA therapeutics and the specific mechanisms by which miRNA mediates cancer pathways needs more research and study.

Keywords: MicroRNA; Cancer; Proliferation; Metastasis; Therapeutic molecule

Introduction

MicroRNAs (miRNAs) are a large family of evolutionary conserved single-stranded small RNA molecules which occur as non-coding RNAs of 19-24 nucleotides in length [1, 2]. MiRNAs regulate gene expression of about 50% of any protein-coding gene at the post-transcriptional level [3]. In the post-transcriptional level, miRNAs act through degradation of their target mRNA or translational inhibition of the entitled mRNA [4, 5]. MiRNA coding genes are located either in intergenic regions or in the introns and are transcribed by RNA polymerase II into long primary transcripts called primary miRNAs (pri-miRNAs) [6]. In the nucleus, these are processed by RNAase III endonuclease Drosha and double-stranded RNA-binding protein Pasha, into a structure called precursor-miRNA (pre-miRNA) [7]. Pre-miRNAs are transported to the cytoplasm. There, they are cleaved and a short RNA duplex molecule is generated [8]. Later a helicase forms the mature miRNA. Mature miRNA is then assembled into the RNA induced silencing complex (RISC) [9, 10]. RISC regulates target mRNA's function by binding to it and silencing its expression [11–13]. In addition, by acting on regulatory sequences of their target gene, miRNAs can promote the expression [14, 15]. MiRNAs mostly can target numerous mRNAs, thus in case of misexpression in a single miRNA, expression of several hundreds of proteins can be disrupted and many different signaling pathways may be affected. This also can cause cancerous transformation [16, 17]. The processing of miRNAs is demonstrated in figure 1.

MicroRNA discovery

MicroRNA was first discovered by Victor Ambros' laboratory in 1993 during research on Caenorhabditis elegans. Simultaneously, Gary Ravkun reported the first miRNA target gene which resulted in the identification of a novel mechanism of post-transcriptional gene regulation [4]. Later, Ravukon and Horvitz found let-7 in the same model nematode species. Also, a class of short interfering RNA (siRNA) involved in the process of RNA interference was discovered. Following these findings, the various number of miRNAs have been discovered and reported in mammals and more than 700 miRNAs which were identified in humans, have been fully sequenced [5].

MicroRNA and cancer

Proliferation, Differentiation, development, and metabolism are examples of multiple biological functions of this class RNAs. In addition, miRNAs are playing an important role in cancer, diabetes, autism, fragile X syndrome, Alzheimer's and heart diseases [18–21]. Cancer has been known as a common complex disease worldwide. Series of genetic and epigenetic factors alter certain balances which cause uncontrolled cellular proliferation. Due to the complexity of cancer, a single therapeutic strategy will not be able to produce a lasting cure. MiRNAs, however, got the capacity of targeting several protein-coding genes at the same time. As a result, a small change in miRNA expression can lead to meaningful alterations in the expression profile of several protein-coding genes and therefor cause changes in cellular phenotype [22–24]. MiRNAs can be classified as oncomiR and tumor suppressor miRNAs from which a large number of can be used as diagnostic and prognostic biomarkers of the cancers [25, 26].

Tumor formation is the result of alterations in miRNA expression by decreasing the expression of necessary genes that are needed in the proliferation or survival of the cells. However, in another study, it has been indicated that cancer progression or tumorigenesis does not contribute directly to miRNA [27]. It is not completely found out that the changes in miRNA expression are either because of the pathological state of cancer or the cancer is the direct reason for it. Nevertheless, miRNA expression is affected directly or indirectly by several alterations that happen in cancer cells. Some changes such as gene mutations, changes in epigenetic regulation of miRNA, abnormalities in miRNA genes or proteins which are involved in their construction and genomic rearrangements are some of the examples for alterations that might affect miRNA expression.

One of the main factors of changing miRNA expression in tumor cells in the presence of miRNAs in tumor-related genomic regions or genomic fragile areas. This specifically causes influences miRNA and mRNA connectivity features which can be named the direct effect of the mutations. Incomplete translational processes are the result of altered miRNA interactions [28]. MiRNAs are capable of regulating a broad set of genes efficiently and silence target genes simultaneously. Since cancer is a heterogenic disease, miRNA's characteristic features are beneficial for treatment. MiRNAs target cancer cells in spite of targeting endothelial cells and fibroblasts. This helps the inhibiting of angiogenesis and tumor fibrosis. Therefore, the required process during metastasis

and tumor formation is blocked [23, 29–31]. Moreover, dysregulated miRNAs are implicated in the pathogenesis of Cancer due to having an effect on oncogenes and/or tumor suppressor genes [32].

MicroRNAs function

The role of miRNA in cancer has already been intensively evaluated and either clinical studies or in vitro and in vivo experiments demonstrated their importance on this occasion [24]. MiRNA's role was first studied in association with chronic lymphocytic leukemia (CLL) [33]. Later multiple miRNAs were reported in accordance with plenty of other cancers [34–42]. Dysregulation of miRNAs is also linked to cancer in various studies [43–46]. It has been proved that they play important roles in metastasis, initiation, and progression as well as therapeutic resistance [46]. There also exist researches describing miRNAs playing two separate acts in carcinogenesis (to be both as "oncomirs" and as "tumor suppressors") [47–50]. In support of this thought, scientists demonstrated the fact that miRNA expression can be up- or down-regulated in cancer cells in comparison with normal cells. They also seem to be deregulated in hematological malignancies as well as many solid tumors [44, 51, 52]. When it comes to location however, about 50% of miRNA genes are embedded in genomic instability regions [53]. This strengthens the evidence of cancer being related to miRNAs. Besides, miRNAs regulate 20-30% of all protein-coding genes [54, 55], which supports the probability of miRNA's signature providing efficient information about tumors [51].

Notably, expression patterns of miRNAs are tumor- and tissue-specific [34, 51, 56–58]. For example, miR-155 is a multifunctional miRNA and is involved in inflammation, immune response and cancer development [59], over-expressed in leukemia and lymphoma [34, 60–64] and down-regulated in melanoma, gastric cancer, ovarian cancer and endocrine tumors [65–70]. These capabilities make miRNAs valuable agents for diagnosis and therapy of certain kinds of cancer. Another example of cancer-specific miRNAs is miR-21 which has been investigated by several groups. Subsequently scientists found out that miR-21 is over-expressed in malignancies like breast [52], colon [71], lung [34], liver [72], thyroid [73] and leukemia [34]. These findings suggested that this oncomir is a good example of a cancer-specific miRNA [74].

Dysfunction or misexpression of miRNA can affect a broad range of processes involved in tumor progression such as metastasis, apoptosis, angiogenesis and cell cycle regulation [75–80]. There are reports of five families of miRNA who target cell cycle regulators. These miRNAs are the let-7, the miR-15a/16 cluster, miR-34 families, the miR-17/20 cluster and the miR-221/222 cluster. The entitled miRNAs are capable of controlling cell cycle checkpoints. Malfunction of these miRNAs may cause a rise in cell proliferation, which is necessary for tumor growth [81]. However, some studies demonstrated miRNAs as anti-apoptotic regulators of key pathways in cancer. These miRNAs are highlighted to maintain cancer cell survival and drug resistance contribution [82–84]. Besides, pro-apoptotic miRNAs serving as anti-cancer agents [85]. MiRNAs also play a key role in the metabolism of cancer cells [86]. They regulate nutrient uptake, targeting transporters, and metabolic enzymes and modulating cancer cell metabolism. They increase the accumulation of materials to control metabolic flux and support proliferation [87–90]. We summarized some important onco-miRNAs and their functions in table 1.

Metastasis-mediating miRNAs have also been discovered. They regulate distinct steps of the metastasis, affecting both signaling pathways in the cancer cell and interactions of cells with one another and with tumor stroma. According to studies, they can activate or suppress metastasis [91, 92]. Some miRNAs are implicated in suppressing apoptosis and stimulating tumorigenesis [93]. Other miRNA families contribute to both tumor growth and metastasis [40]. They are able to silence multiple oncogenes and are down-regulated in several tumors [94–96]. Pro-metastatic miRNAs, however, are another example of metastasis-promoting miRNAs [97, 98]. Subsequently, due to the important role of miRNAs in cancer, there exists a wide range of strategies based on miRNA in oncology. They can be used for cancer classification [51, 99] or tissue origin identification of cancers with the unknown primary origin [100, 101]. Their expression can serve as a useful prognostic or diagnostic marker [93, 102–107]. Interestingly, miRNA signatures have been established as predictive factors of response to therapy [108–112] and drugs [113–116].

MicroRNA in cancer therapy

Since miRNAs discovery, a debate has risen that miRNAs could be regarded as a promising biomarker to improve response to cancer treatment [27]. The advent of miRNA-based therapy,

however, has opened new avenues to use targeted therapy for clinical applications, since there are some limitations for current cancer therapies [22, 117, 118]. All of the applications in the previous section are possible when dealing with primary tumors. The fact that miRNAs are more stable than mRNAs is the key point since this stability enables them to be detected in the circulation and serve as biomarkers. Circulating miRNAs can be measured with regard to a wide variety of cancers [119]. Therefore, studies are currently highlighting their employment in cancer therapeutics [120]. Based on several studies in recent years, miRNAs can be used as highly potential molecules in CRC therapy [32].

Several strategies associated with miRNAs can prevent cancer progression (Figure 2). Some examples include cutting oncogenic miRNAs by artificial miRNAs which are capable of pairing with mRNAs, inhibition of the entitled oncogenic miRNAs, inducing the tumor suppressor miRNAs or decreasing miRNA expression using various epigenetic factors like promoter methylation. To reduce the miRNA expression, antisense oligonucleotides can also be used. They are particularly paired with miRNA [27]. One type that is artificially made is Antagomir [121]. In comparison with other cancer treatment methods, these molecules are less toxic and create stable inhibition [28].

MiRNAs can also be agents or targets of cancer therapy according to their function, stage of cancer and type of cancer [122]. In order to use combined therapies targeting multiple miRNAs, tumor-secreted miRNA who are messengers and/or effectors must be characterized as the first step. Due to the correlation between their levels and metastasis, circulating or exosomal miRNAs can be quantified to select patients with high risks of metastasis in a certain type of cancer. As a result, these patients will benefit from a preventive strategy which targets the miRNA effectors [114]. There also have been studies indicating that treatment interventions such as inhibition of oncomiRs and restoration of tumor suppressor miRNAs might be beneficial for certain types of cancers [26].

In addition to the fact that cell-free miRNAs are functionally effective in metastatic progression, they are also nominated for potential novel therapeutic targets [114, 123]. An interesting advantage of cell-free miRNAs is that their expression levels can be monitored when treatment is started [124]. In this regard circulating miRNAs are discovered to be potential diagnostic and therapeutic agents in association with cancers [14, 43, 44, 76, 77, 91, 125–127]. Another aspect of targeting miRNAs is that they are observed to be beneficial for improved response to drugs. Hence,

circulating miRNA's expression level in blood is useful for prognosis determination [116, 128]. Moreover, compared to other gene-therapy methods and drug molecules, miRNA showed low toxicity [113]. Accordingly, in case of safe delivery to cancer cells, miRNA-based therapeutics seems to be promising anti-cancer guardians.

Conclusion

Once miRNAs discovered, significant progress in the identification of these novel family has confirmed that these small and non-coding RNAs are a numerous class of regulatory RNAs. Also, the skeleton of a biochemical mechanism for their functions in gene regulation has specified. The most attractive part of miRNA therapeutics is their capability to target any genes, which is not possible or difficult by protein-based drugs or small molecules. Nowadays, cancer is the most important target of miRNA therapeutics among the numerous diseases being studied. We briefly clarified the particular roles and the importance of miRNAs in the regulation of gene expression. Additionally, the specific mechanisms by which miRNA mediated repression needs more research and study.

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Figures & Tables

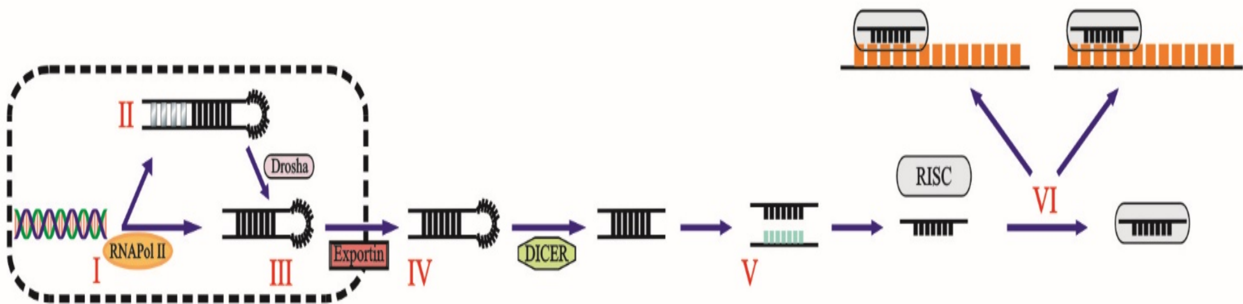

Figure legend

Figure 1. MicroRNA processing. RNA polymerase II and proper transcription factors excite the transcription of the microRNA gene (I) in the production of a pri-miRNA. The primary transcript (II) is then processed by an RNAase III enzyme called Drosha, to produce a ~ 65 nucleotide (nt) pre-miRNA. The pre-miRNA, which has a short stem of 2–3 nt 3′ overhangs (III) is then exported by exportin 5 (EXP5) to the cytoplasm for additional processing. In the cytoplasm, the precursor microRNA subsequently processed into a mature 19–24 nucleotide duplex (IV) by an enzyme called Dicer. Afterward, the duplex is separated to a primary and secondary strand (V); then the primary strand is embedded into the RISK (RNA-induced silencing complex). In the next step, the microRNA with RISC targets complementary mRNA transcripts (VI) at the seed region to induce either block translation (right) or mRNA degradation (left).

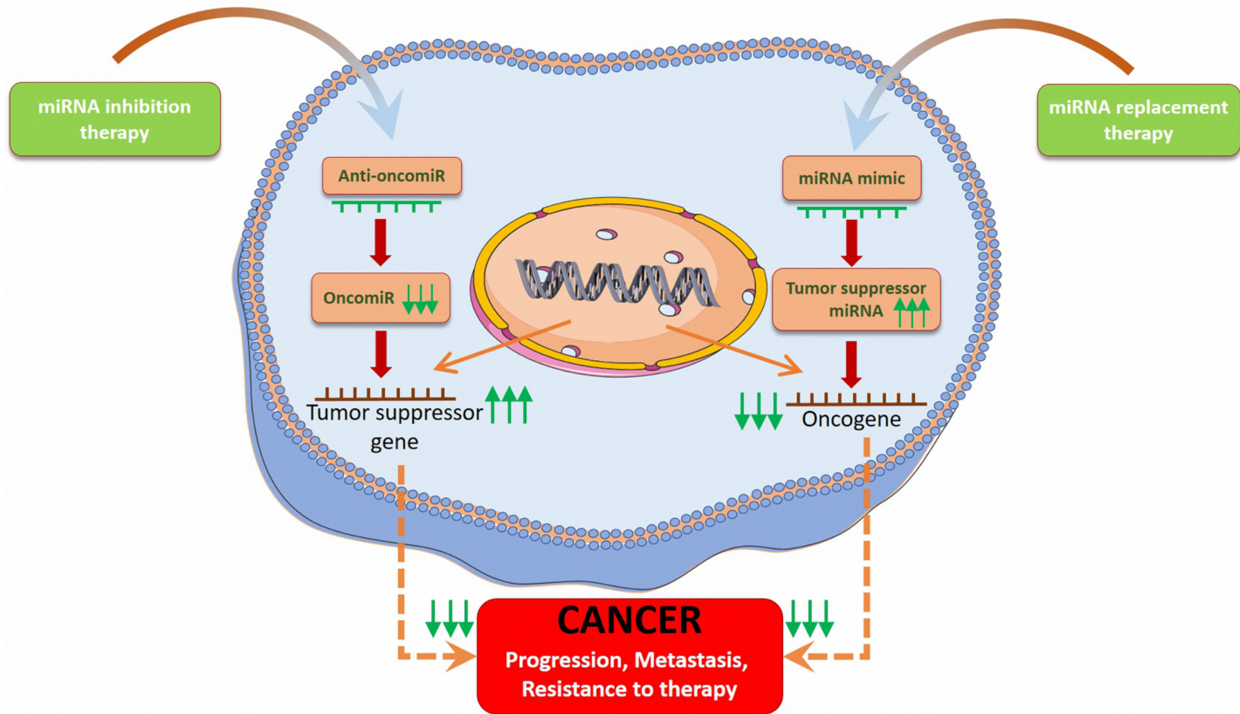

Figure legend

Figure 2. Different miRNA-based therapeutic strategies against cancer progression, metastasis, and resistance to therapy.

Table 1: Some important onco-miRNAs and their function.

MICRORNAS	TARGET GENE(S) OR PROTEIN	FUNCTION
miR 15 and miR 16	NA	B-cell lymphocytic chronic Leukemia
miR-17 ~ 92 cluster	NA Myc	Lung and other malignancies Tumorigenesis and angiogenesis
miR-21	Pdcd4, BMPRII & LRRFIP1	Promote apoptosis through activation of caspases
miR-155	TP53INP1	Overexpression in pancreatic cancer and breast cancer progression
miR-371 ~ 3	LATS2	Cell proliferation and tumor development

NA: Not applicable

CHAPTER 5

Clinical Significance Of MicroRNA Expression In Colorectal Carcinoma

Expression profiles of miR-196, miR-132, miR-146a and miR-134 in human colorectal cancer tissues in accordance with their clinical significance: comparison regarding KRAS mutation

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// Abstract

Purpose: Colorectal Cancer (CRC) is among the most widespread malignancies in the world. MicroRNA (miRNA) has been identified as an important modulator of biological processes of the cells. This group of noncoding RNA also has a pivotal role in the growth and development of human malignancies, including CRC. Among these miRNAs, miR-196, miR-132, miR-146a, and miR-134 have fundamental impacts on the regulation of different cancers. The current study aimed to determine the involvement of these miRNAs in CRC patients.

Methods: In this study, 50 pairs of tumor and tumor marginal samples of CRC patients were investigated to assess the expression levels of miR-196, miR-132, miR-146a and miR-134 in this cancer. For this purpose, firstly, the quantitative real-time PCR (qRT-PCR) was performed. Also, KRAS mutation and clinicopathological characteristics of the CRC patients were analyzed in the study groups.

Results: The findings demonstrated the overexpression of miR-196 (P-value= 0.0045) and miR-146 (P-value= 0.0033) in tumor tissues compared to controls. Conversely, the expression levels of miR-132 (P-value= 0.00032) and miR-134 (P-value<0.0001) were down-regulated in tumor tissues. Also, miR-RNA146a was the only miRNA with significant expression change in case of the KRAS gene mutation. Interestingly, the expression ratio of these miRNAs was significantly correlated with some of the clinicopathological features of the patients such as lymph node and distant metastasis.

Conclusion: Our data demonstrated that these miRNAs appear to be potential novel biomarkers for early diagnosis of CRC and may pave the way for the future establishment of novel therapeutic options for CRC.

Keywords: Colorectal cancer; miRNA; Quantitative real-time PCR, KRAS mutation, Clinicopathological characteristics

Introduction

Colorectal Cancer (CRC) is the third most widespread malignancy which its rate is increasing especially in developing countries (1, 2). CRC is heterogeneous cancer, which happens following the increase of tumor cells in the colon, rectum, and appendix (3). The two main factors that are affecting the occurrence of this cancer are environmental and genetic (4). Prevalence and mortality rates reported for CRC are about 1,400,000 and 700,000 cases per year (1). Overall, about 40-50% of patients die because of distant metastasis, thus disregarding of diagnoses and standard therapy of this type of cancer, patients' life is in tie with tumor stage at the diagnosing time (5,6). MicroRNAs (miRNAs) are a group of noncoding RNA which contains about 18-25 nucleotides that influence post-translational gene expression (7–9). It is approximated that the miRNAs control about 30% of the genome encoding various proteins (10).

Although some therapeutic options such as surgery and chemotherapy are available for CRC patients, the prophecy of the disease is inauspicious and in many cases who undergone surgery the cancer recurrence and metastasis occurs. Late diagnosis of this malignancy is one of the great challenges, and clinically wise. Therefore, molecular studies that result in the recognition of biological biomarkers should have a clinical emphasis. To date, expansive research shows that miRNAs are unique and helpful biomarkers for early determination and treatment of CRC (9). The direct function of miRNAs in cancer is confirmed through the interaction of miRNAs with targeted genes. This has effects on various cancer properties including apoptosis, differentiation, and cell proliferation. However, for tumors classification in various groups with distinct features such as etiology and cancer cell types, miRNAs are even more accurate than messenger RNA (mRNA) (9). In cancer samples, many miRNAs are abnormally expressed that is indicated by miRNAs structure and their functions. The stages of cancer are in association with miRNAs expression patterns (9).

Some variants of miRNAs have been introduced as potential biomarkers recently. Our target in this study is to come up with a more precise identification of expression patterns of four selected miRNAs (miR-196, miR-132, miR-146a, and miR-134). MiR-196 is reportedly overexpressed in various human malignancies, including CRC (11). It has been suggested that miR-196a and miR-196b have participated in tumor progression and tumorigenesis of CRC (12). Studies revealed that miR-132 is down-regulated in CRC tissues with distant metastases and that the expression of miR-132 particularly inhibits cell invasion in CRC cell lines (13). MiR-132 also has a key function in

both prostate and pancreatic cancers (14, 15). Moreover, miR-132 represents a promising biomarker for the targeted therapy of CRC (16). MiR-146a has been particularly studied due to its role in tumor progression. MiR-146a has been indicated to be overexpressed in tumor tissues as well as cancer cell lines (17). Dysregulated miR-146a however, is involved in tumorigenesis. Reports demonstrated the down-regulation of miR-146a in various malignancies including breast, lung, pancreatic and gastric carcinomas (18–20). MiR-134 was found to be able to suppress the migration of cancer cells. It has been investigated that miR-134 induces apoptosis in CRC cells as well (21). Subsequently, our main goal was to clarify the entitles miRNAs expression profiles in tumor tissue and marginal tissue (i.e. control samples). We also studied the expression changes concerning the mutations detected in KRAS. Moreover, we aimed to do a wide comparison of clinical relevance with these expression patterns in all these miRNAs.

Material and Methods

Study population

The study included 50 patients with primary CRC diagnosed at Imam Reza Hospital in Tabriz, Iran during 2017-2018. To achieve a genetically pure sample population, all the patients were selected from those native to East Azerbaijan, North West of Iran. Patients who received chemotherapy and radiation therapy were excluded during sample gathering. The clinicopathological parameters of the patients are presented in table 1. For each case, samples from the primary tumor and the matched normal marginal tissues were gathered. All collected tissues were histologically confirmed to be an adenocarcinoma of the colon. The samples were immediately transferred to an RNase inhibitor solution (Qiagen) and stored until subsequent implementations.

DNA extraction

DNA from tissues was isolated using Tripure isolation kit (Roche) considering the manufacturer's manual. The DNA quality and purity were assessed using NanoDrop spectrophotometer (Thermo Fisher Scientific).

KRAS mutation detection

High Resolution Melting (HRM) analysis was done by Step One plus Real-time PCR system (Applied Biosystems, Foster City, CA). Mutation detection for exon2 of the KRAS gene in tumor samples was carried out using gene-specific primers and SYBR green master mix (TAKARA). All susceptible samples were sent to sequencing in order to confirm the mutation. The primer sequences used in the PCR are presented in table 2.

RNA isolation and transcriptase reactions

As DNA, total RNA content of tumor and the matched normal tissues was isolated using Tripure isolation kit (Roche) considering the manufacturer's manual. A NanoDrop Spectrophotometer was used to check the RNA quality and purity. Besides, to generate complementary DNA (cDNA) of extracted RNAs, TAKARA cDNA synthesis kit was used because of using the stem-loop method instead of using OligoT primers which was mentioned in the kit instruction guide specific primers were used (Table 2).

Quantitative real-time PCR

Quantitative analysis was done by the real-time PCR system. Relative quantification of the expression level of genes from tumor and normal marginal tissue samples was carried out using gene-specific primers and SYBR green master mix. Expression of the U6 gene was also measured in each sample as the housekeeping gene. Primers were designed using oligo 7 software. The average score of duplicated C_t values was measured for each sample and comparative C_t ($2^{\Delta\Delta Ct}$) method was used to determine the relative expression level of target genes.

Description analysis of miR-196, miR-132, miR-146a, and miR-134 as tumor markers

The relative expression analysis of miR-196, miR-132, miR-146a, and miR-134 was done by a randomization test applying the Relative Expression Software Tool (REST) (22). Receiver operating characteristic (ROC) curve was plotted to assess the specificity and sensitivity of predicting CRC from normal tissues by these miRNAs expression levels. For this purpose, the sensitivity in function of the specificity at the different cut-off grades was analyzed using Sigma Plot 12.5 software. This plot is a graph of the true positive rate (TPR) and false positive rate (FPR)

for different descriptors threshold (22). Hence, the vertical axis in the ROC curve indicates TPR and horizontal axis indicates FPR. The bigger vertical axis values for a hypothetical horizontal axis value shows the better performance of a descriptor.

Statistical analysis

GraphPad Prism 6 (Graph Pad Software Inc. San Diego, CA) was used for statistical analysis. The data were shown as mean ± standard deviation (SD) based on 3 independent experiments. Independent sample *t*-test was carried out to determine difference genes expression level between primary tumor tissues and corresponding normal tissues. For evaluation of the association between the clinical feature of the patients with relative expression of genes, Cross tab (Eta) analysis was performed. $P < 0.05$ was considered as statistically significant level.

Results

In this study, the HRM technique was used to identify KRAS gene mutation. 16 subjects with mutations in the KRAS gene were detected. Later the expression levels of our target miRNAs were evaluated both in tumor and marginal tissues and their expression in relation with KRAS mutation were analyzed. Moreover, the expression patterns were compared in association with clinical features such as age, sex, lymph node metastasis, distant metastasis, differentiation, and tumor stages.

MiR-196 expression in CRC

We determined the relative expression of miR-196a in 50 CRC cancer tissues compared with adjacent tumor-free normal tissues. U6 gene expression was used as an internal control for normalizing of these data. Our data revealed the up-regulation of miR-196 in tumor tissues (P-value (Pv) = 0.0045). Also, the relationship analysis of miR-196 expression with clinicopathological traits of patients showed a statistically significant association between the expression of miR-196a with lymph node (Pv= 0.042) and distant metastasis (Pv <0.0001). Due to the KRAS mutation, there is no significant change in KRAS positive samples in comparison to KRAS negative in our study (Figure 1).

MiR-132 expression in CRC

According to our data, the expression ratio of miR-132 is down-regulated in tumor tissues in comparison to tumor-free marginal normal tissues (Pv= 0.00032). In relationship analysis, we found that there is a significant association between the expression level of miR-132 and tumor stages (Pv= 0.0067). As miR-196a expression level of miR-132 was not related to the KRAS mutation too (Figure 2).

MiR-146a expression in CRC

Our data revealed the up-regulation of miR-146 in tumor tissues compared to matched tumor-free normal tissues (Pv= 0.0033). In relationship analysis, we discovered a significant correlation between miR-146a expression level and distant metastasis (Pv= 0.0016). In our study, miR-RNA146a was the only microRNA with significant expression change in case of the KRAS gene mutation. This miRNA expression was significantly up-regulated in KRAS positive samples in compression to KRAS negative (Pv= 0.0102) (Figure 3).

MiR-134 expression in CRC

We ascertained the relative expression of miR-134 in CRC participants tissues compared with marginal normal tissues of the subjects and the results demonstrated significant down-regulated expression of miR-134 in tumor tissues (Pv<0.0001). Also, relationship analysis showed the correlation among the expression of this gene and tumor stages (Pv= 0.0026), lymph node (Pv= 0.021) and distant metastasis (Pv= 0.035) of samples. On the other hand in the case of KRAS mutation, this micro RNA was not significantly changed between two studied groups of our study (Figure 4). All the relevant clinical data regarding the entitled miRNAs expression profiles are summarized in table 3 respectively.

The capability of miRNAs for the diagnosis of CRC

ROC curve was applied to estimate the sensitivity and specificity of the miR-196, miR-132, miR-146a and miR-134 as candidate novel biomarkers for CRC. ROC curve data are shown in figure

5. The results of the statistical analysis of ROC curves for diagnostic evaluation are presented in table 4.

Discussion

MiRNAs are a class of non-coding RNAs that post-transcriptionally modulate the gene expression. Subsequently, activation of the immune system, regulation of cholesterol homeostasis, ontogenesis, and other numerous functions are some of the biological processes that are controlled by microRNAs (23–25). The patterns of expression of miRNAs are distinct in tumor tissues and body fluids like saliva, serum, urine, and plasma (23). Differences between the origin of cancer cell and its surrounding stromal tissue could be results of differences in miRNAs expression profiles in different cancers (9). These expression patterns can shed light on the application of miRNAs as informative agents for diagnosis and prognosis. For this reason, we focused on the expression patterns of 4 hotspot miRNAs (miR-196, miR-132, miR-146a, and miR-134) in the tissue of CRC patients regarding their KRAS mutational background. It is designated that 12 miRNAs (including miR-196a and miR-134) are up-regulated and 8 miRNAs (including miR-146a) are down-regulated in the stool of CRC patients (26). Also, Iannone et al. could differentiate CRC incidences from healthy controls by analyzing these 20 miRNAs (27). They also managed to clarify different TNM stages with high specificity and sensitivity (27).

MiR-196 over-expression has been observed in several malignancies including CRC (28). Additionally, it has been demonstrated that miR-196 is up-regulated in colon cancer tissues (12). Our data also designated the up-regulation of miR-196 in tumor samples compared with adjacent tumor-free normal tissues. In research by Ge et al., it has been indicated that miR-196 is significantly high expressed in CRC compared to corresponding normal colorectal tissue. The results were also aligned with prognosis in patients with CRC. These results revealed that mir-196 may be correlated with a more aggressive clinical outcome in patients with CRC (29). There are also articles demonstrating that miR-196a may have pro-oncogenic effects in CRC (12, 28). Despite all the studies, miR-196's mechanisms of action in different cancers are unclear. It largely depends on the molecules that miR-196 targets. If miR-196 shows a dominant effect on the blockade of oncogenic molecules, this means that miR-196 is playing a tumor suppressor role; but if miR-196 targets the tumor suppressors, it mainly will perform oncogenic effects (30).

One study claimed the possible participation of miR-196 in the occurrence of CRC because of its overexpression. Besides, the expression levels of miR-196 were meaningfully linked with staging, as well as lymph node and distant metastasis. The same study indicated that the expression patterns were not associated with depth of invasion, size, location, differentiation, gender, and age (31). We observed that there is a statistically significant correlation among the expression level of miR-196a with lymph node metastasis and distant metastasis. One study provided evidence that miR-132 acts as a tumor suppressor through enhancing apoptosis as well as inhibiting cancer cell proliferation and migration (32). In pancreatic cancer progression, however, expression of miR-132 suppressed the proliferation and colony formation of cancer cells (33). Taken together, these findings illustrated that miR-132 behaves as a tumor suppressor (15). Our results revealed that miR-132 is down-regulated in tumor tissues compared to tumor-free marginal normal tissues. Increased expression of miR-132 reduces the incidence of colitis-associated tumors (34). MiR-132 can modulate cell proliferation, differentiation, apoptosis, metabolism, and growth (35, 36). Confirming our results, Zheng et al. also reported that miR-132 was down-regulated in CRC patients with distant metastases. This Down-regulation was correlated with adverse prognosis in CRC patients and more aggressive tumor phenotypes (13). Moreover, we found that there is a significant association among the expression level of miR-132 and tumor stages.

MiR-146a expression can reduce the proliferation of many types of cancer cells (37, 38) except melanoma, cervix and thyroid carcinoma in which miR-146a is up-regulated (18–20). Our data revealed the up-regulation of miR-146 in tumor tissues compared to matched tumor-free normal tissues. Several studies suggested that miR-146a is potentially involved in the initiation and progression of CRC. Pizzini et al. indicated that miR-146a is down-regulated in the metastasis tumor tissue in comparison to primary CRC tissue. On the contrary, as mentioned before, Ahmed et al. reported that decreased miR-146 expression in the stool of CRC patients compared with normal subjects (26, 39). Taken together it has been indicated that miR-146a may be involved in colorectal carcinogenesis.

Studies have shown that rs2910164 polymorphism in miR-146a may contribute to CRC risk (26,39,40). Wang et al. observed that miR-146a was significantly reduced in cancer tissue and that it was interestingly related to TNM stage, which suggested the potential connection between miR-146a and the disease pathogenesis (41). We discovered a significant association between miR-146a expression level and distant metastasis. Besides miR-RNA146a was the only microRNA

among the entitled four that had a significant expression change regarding KRAS gene mutation. The expression was significantly up-regulated in KRAS positive samples in compression to KRAS negative. The expression level of miR-134 is down-regulated in glioma tissues (42) as well as breast cancer tissues (43). A study also revealed that miR-134 can be validated as a marker for oral cell carcinoma diagnosis (44). As mentioned above miR-134 is up-regulated in the stool of CRC patients (26, 27). Our results have shown the significant down-regulated expression of miR-134 in tumor tissues compared with marginal normal tissues of the subjects. To the best of our knowledge, miR-134's association with CRC and its clinical relevance remains unclear. We observed a meaningful correlation among the expression level of this gene and tumor stages as well as lymph node metastasis.

Despite the elevating number of successful therapeutic options for CRC treatment, the clinical achievements concerning inhibition of recurrence and metastasis are still poor. This results in low long-term survival rates. Over the past two decades, a growing list of biomarkers associated with prognosis and progression of CRC have been attributed. However, the majority of these biomarkers happened to fail in clinical studies validation. Thus, there is an urgent demand for novel biomarkers to diagnosis early metastasis as well as to predict recurrence in CRC patients. Although the expression patterns of several miRNAs in CRC are partly studied, little is known about the association between these expression profiles and the clinical features of such an aberration. However, in its early research stage, miRNAs present great potential for detection and therapy. Therefore, further investigations regarding their expression profiles in CRC tissue will shed a light on their role as biomarkers for early diagnosis and as agents for monitoring the disease.

Conclusion

The diagnosis and therapeutic outcomes for cancer could be improved by the availability of miRNAs. Moreover, the type of tissue, differentiation, invasion, and response to therapeutic agents are the biological and clinical characteristics of tumors that are associated with the expression of miRNAs. Subsequently, these miRNAs appear to be novel biomarkers for early diagnosis of CRC. Our data serves as a basis for further research before these miRNA can be used as biomarkers for CRC. Detection of miRNA target molecules and demonstrating their expression patterns concerning specific mutations will help discover the mechanisms of cancer. Last but not least,

taking a vital step for early detection and proper monitoring of CRC, using miRNAs as diagnostic and prognostic markers is very beneficial.

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Figures & Tables

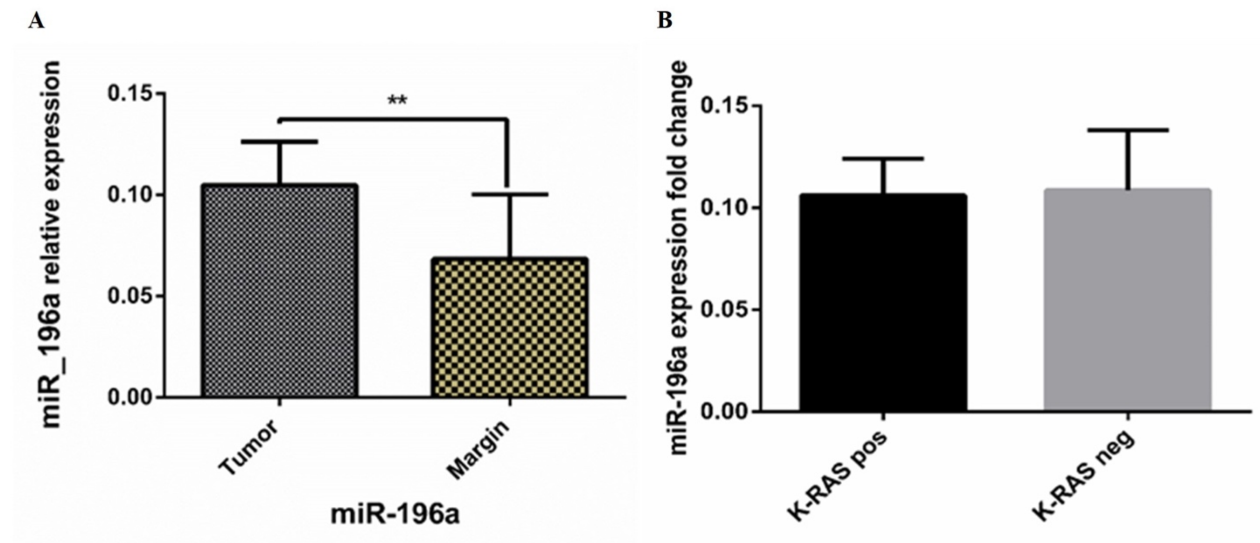

Figure 1. (A) Expression level of miR-196a in tumor and marginal tissues. (B) Expression level of miR-196a in K-RAS negative and K-RAS positive tissues. The expression of the U6 gene was measured in each sample as the housekeeping gene. **P <0.01 in comparison with margin group.

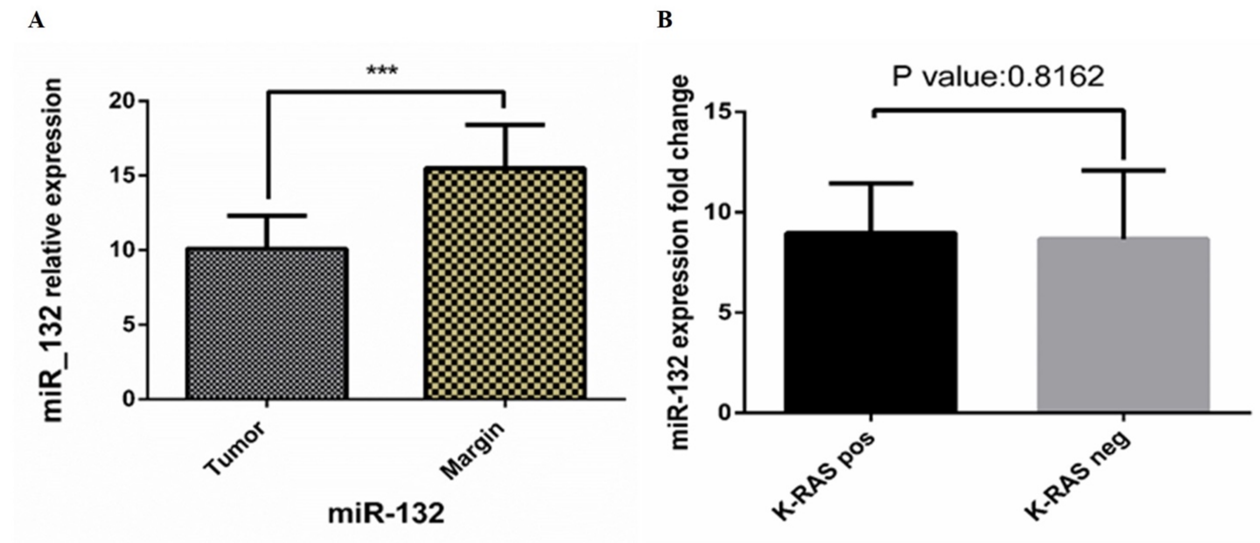

Figure 2. (A) Expression level of miR-132 in tumor and marginal tissues. (B) Expression level of miR-132 in K-RAS negative and K-RAS positive tissues. The expression of the U6 gene was measured in each sample as the housekeeping gene. ***P <0.001 in comparison with margin group.

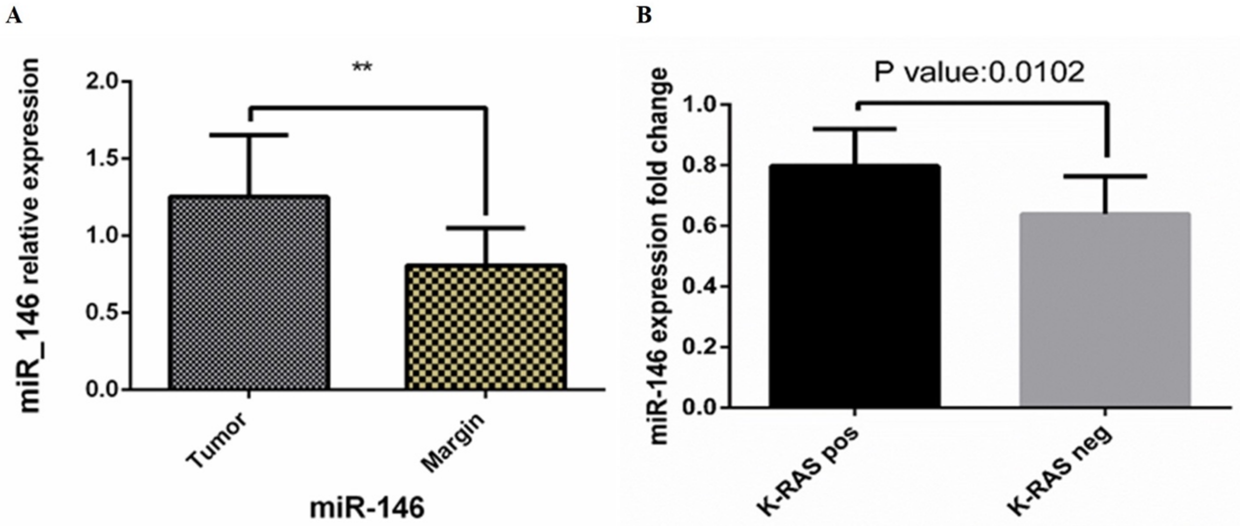

Figure 3. (A) Expression level of miR-146a in tumor and marginal tissues. (B) Expression level of miR-146a in K-RAS negative and K-RAS positive tissues. The expression of the U6 gene was measured in each sample as the housekeeping gene. **P <0.01 in comparison with margin group.

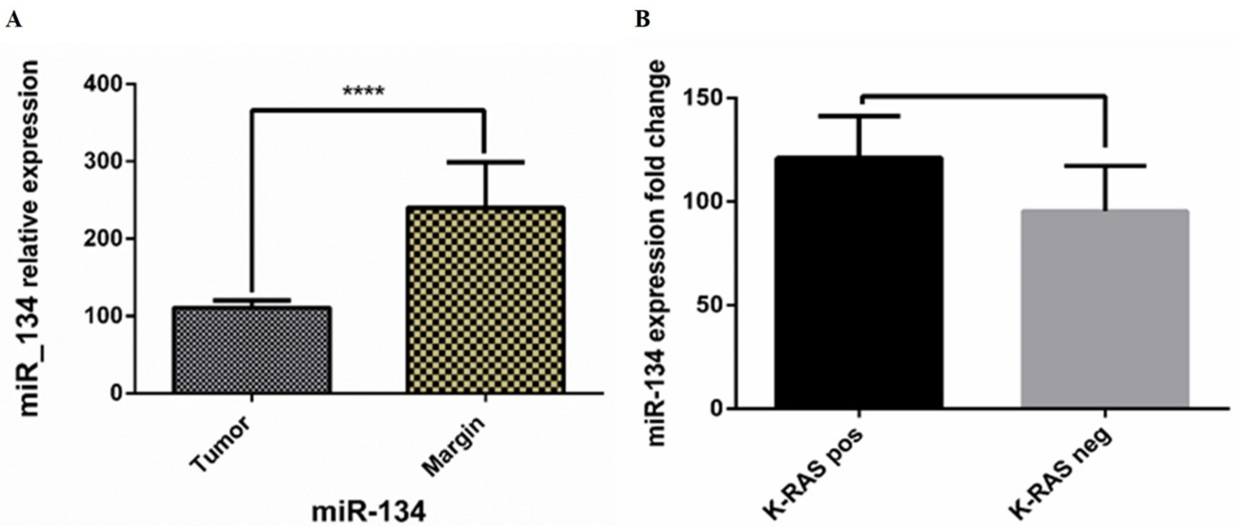

Figure 4. (A) Expression level of miR-134 in tumor and marginal tissues. (B) Expression level of miR-134 in K-RAS negative and K-RAS positive tissues. The expression of the U6 gene was measured in each sample as the housekeeping gene. ****P <0.0001 in comparison with margin group.

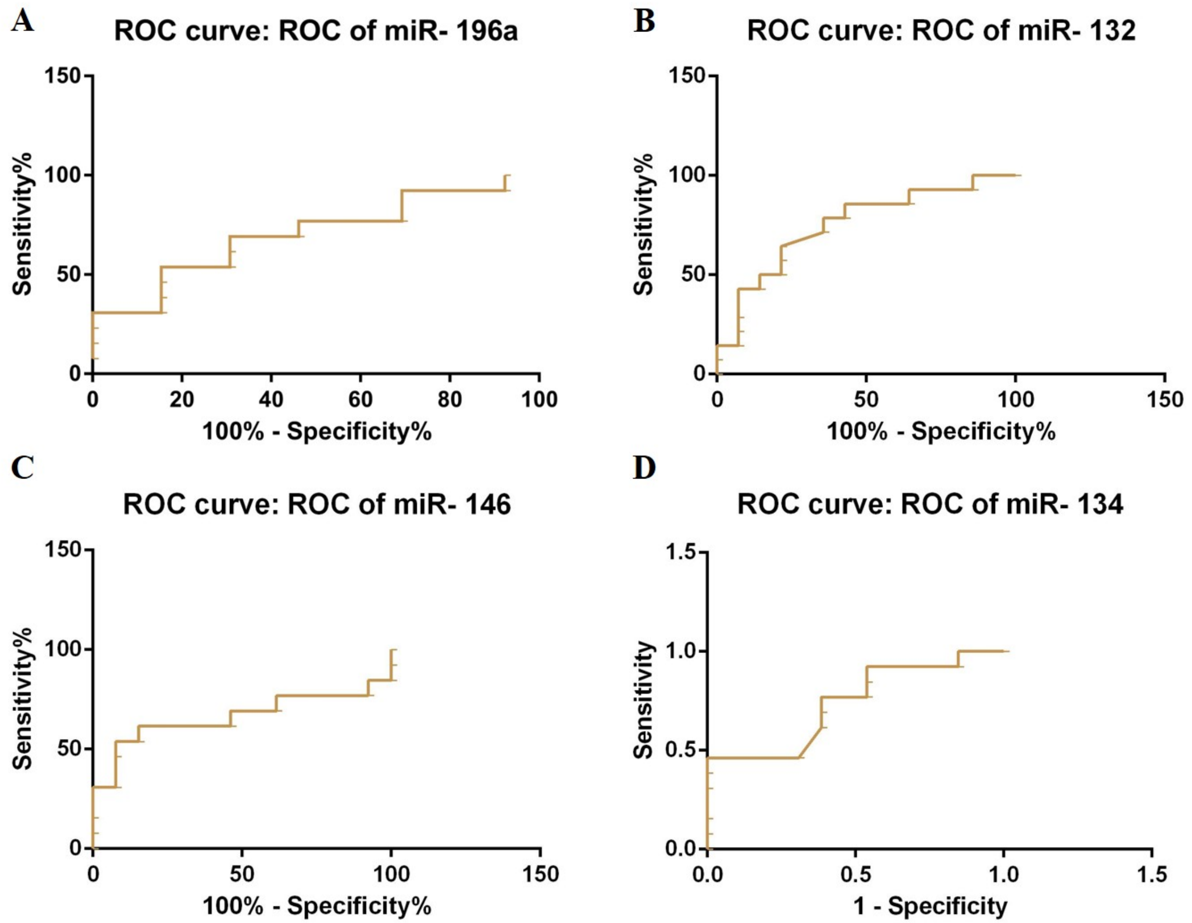

Figure 5. Receiver operating characteristic (ROC) curve for miR-196a (A), miR-132 (B), miR-146 (C) and miR-134 (D) which identifies CRC cases from normal controls.

Table 1. Clinicopathological characteristics of the CRC patients.

Characteristic	Value (n=50)
Age	
<60	26 (52%)
>60	24 (48%)
Sex	
Male	32 (64%)
Female	18 (36%)
Smoking	
Yes	31 (62%)
No	19 (38%)
Tumor metastasis	
pM0	42 (84%)
pM1	8 (16%)
Tumor location	
Rectum	13 (26%)
Right colon	21 (42%)
Left colon	16 (32%)
Differentiation pattern	
Poor	11 (22%)
Moderate	26 (52%)
Well	13 (26%)

Table 2. Primer sequences.

Primer	Sequence
u6 stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATAT
U6 Forward	GCTTCGGCAGCACATATACTAAAAT
U6 Reverse	CGCTTCACGAATTTGCGTGTCAT
hsa-miR-146a-5p stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCCT
hsa-miR-146a-5p Forward	CGTGCTGTGACCTATGCTG
hsa-miR-146a-5p Reverse	CCAGTGCAGGGTCCGAGGTA
hsa-miR-196a2-5p Stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCAAC
hsa-miR-196a2-5p Forward	CGAGCTGGTGACCTATGCTG
hsa-miR-196a2-5p Reverse	CCAGTGCAGGGTCCGAGGTA
hsa-miR-132-5p Stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTAAC
hsa-miR-132-5p Forward	GCTGGTGACCGTGGTCGTT
hsa-miR-132-5p Reverse	CCAGTGCAGGGTCCGAGGTA
hsa-miR-134-5p Stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCCTC
hsa-miR-134-5p Forward	AGTGGATTGTGACTGGTGG
hsa-miR-134-5p Reverse	CCAGTGCAGGGTCCGAGGTA

Table 3. MiRNAs expression profiles in association with clinicopathological parameters. (n.s: non-significant differences; Pv: P-value).

Gene	Change in tumor	Exact P-value	Age	sex	Lymph node metastasis	Distant metastasis	Differentiation	Tumor stages
miR-134	Down-regulated	< 0.0001	n.s	n.s	Significant (Pv=0.021)	Significant (Pv=0.035)	n.s	Significant (Pv=0.0026)
miR-132	Down-regulated	0.00032	n.s	n.s	n.s	n.s	n.s	Significant (Pv=0.0067)
miR-196a	Up-regulated	0.0045	n.s	n.s	Significant (Pv=0.042)	Significant (Pv <0.0001)	n.s	n.s
miR-146	Up-regulated	0.0033	n.s	n.s	n.s	Significant (Pv=0.0016)	n.s	n.s

Table 4: The statistical analysis of receiver operating characteristic (ROC) curve for diagnostic evaluation.

ROC curve data	Area under the curve	Std. Error	95% confidence interval	P-value	Controls (Margin)	Patients (Tumor)
miR-196	0.7551	0.09327	0.5723 to 0.9380	0.02164	46	46
miR-132	0.7551	0/09327	0.5723 to 0.9380	0.02164	49	49
miR-146a	0.6627	0.1164	0.4346 to 0.8909	0.1585	43	43
miR-134	0.7396	0.0987	0.5462 to 0.9331	0.03786	49	49

CHAPTER 6

Tissue & Liquid Biopsy MiRNA Profiles In Colorectal Carcinoma

The different profiling of microRNA-based tissue biopsy and liquid biopsy for colorectal cancer disease screening and detection

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Abstract

Background: Despite the variety of screening methods for colorectal cancer (CRC), there are some limitations to these screening tests. Thus, developing an efficient method to detect biomarkers for this disease is an urgent necessity. In this study, to identify potential biomarkers of CRC, we aimed to characterize the expression profiles of some microRNAs (miRNAs) in tumor tissues, marginal tissues, and plasma samples of CRC patients.

Methods: In this study, circulating miRNAs isolated from plasma of 50 patients and their tumor and tumor marginal samples were investigated to assess the expression levels of miR-146, miR-196a, miR-132, and miR-134 in these patients. For this purpose, the quantitative real-time PCR was applied to assess the expression levels of these selected miRNAs. Besides, the clinicopathological features of the patients were analyzed in the current study.

Results: The findings demonstrated the overexpression of miR-146 in tumor tissues when compared to margin tissue, but there was no significant change in the level of this miRNA in plasma samples. Also, the expression levels of miR-132 and miR-134 were down-regulated in tumor tissues. But, similar to miR-146 results, no significant changes were detected in the expression of the miR-132 and miR-134 in plasma samples. In addition, the expression of the miR-196a level is similar in both tissue and plasma samples. The expression ratio of these miRNAs correlated with the clinicopathological features of the patients.

Conclusion: Our data demonstrated the tissue expression levels, but not plasma levels of these selected miRNAs may be appropriate biomarkers for detection of CRC.

Keywords: Colorectal cancer, MicroRNA, Tissue biopsy, Liquid biopsy, Quantitative real-time PCR

Introduction

Colorectal cancer (CRC) is the third most frequent tumor worldwide that approximately results in more than 700,000 deaths per year and in Western countries, represents the second most common cause of cancer mortality (1,2). The incidence and mortality of CRC can be decreased by screening tests (3–6). Nonetheless, there are some limitations for these screening test and the invasive nature of some makes them a limited method of screening (6–8). Therefore, CRC screening compliance remains a problem and developing a sensitive, noninvasive, and cost-effective screening method to detect biomarkers for this disease is an urgent necessity.

Liquid biopsy is a novel noninvasive technique of cancer diagnosis which relies on the study of circulating cells or cell components in the blood (9). In recent years potential roles and diagnostic values of circulating microRNAs (miRNAs) as biomarkers for several cancers have been introduced (10). MiRNAs are small non-coding RNAs that their actions affect gene expression regulation by degrading and/or inhibiting the translation of mRNA which can specifically affect tumorigenesis genes (11–13). Tumors are a source of circulating miRNAs (14–17). Several characteristics such as being derived from cells, showing tissue-specific expression (18,19), and being present in peripheral blood in a remarkably stable form, making extracellular miRNAs potential cancer biomarker candidates (14,20,21).

Subsequently, we aimed to characterize the precise expression patterns of miR-196, miR-132, miR-146a, and miR-134. In CRC patients, along with various human malignancies, over-expression of miR-196 has been reported (22). Previously, the miR-196a and miR-196b contribution in tumor progression and tumorigenesis of CRC has been suggested (23). While miR-132 expression can inhibit cell invasion in CRC cell lines, observations appointed that its expression in CRC tissues with distant metastases is down-regulated (24). MiR-132 also is an encouraging biomarker for CRC therapy follow-up (25). Because of its role in tumor progression, miR-146a has been observed in different studies and over-expression of miR-146a in tumor tissues as well as in cancer cell lines has been indicated (26). Investigations revealed that miR-134 is able to prevent cancer cells migration and also to induce apoptosis in CRC cells (27). Therefore, the goal of this study was to examine the selected miRNAs expression profiles in plasma, tumor tissue and marginal tissue (i.e. control samples). In addition to examining the expression changes of these

miRNAs in tumor tissue and plasma samples, we studied the expression patterns of all these miRNAs in comparison to their clinical relevance.

Material and Methods

Study population

Fifteen subjects with primary CRC diagnosed at Imam Reza Hospital in Tabriz, Iran during 2017-2018 were enrolled at the study. Exclusion criteria included the use of chemotherapy and radiation therapy during sample gathering. The selection of all patients from native patients to East Azerbaijan province of Iran insured the genetic purity of the sample population. Samples of the primary tumor and the matched normal marginal tissues for every patient were collected and transferred to an RNase inhibitor solution (Qiagen) and kept until being used in assays. The clinicopathological parameters of the patients are summarized in table 1.

DNA extraction

DNA from tissues was isolated using a commercial isolation kit from Tripure (Roche) exactly as described by the manufacturer's manual. NanoDrop spectrophotometer (Thermo Fisher Scientific) was used to assess the quality and purity of the DNA.

RNA isolation and transcriptase reactions

Like DNA, total RNA content of tumor and the matched normal tissues was isolated using a commercial isolation kit from Tripure (Roche) exactly as described by the manufacturer's manual. NanoDrop spectrophotometer (Thermo Fisher Scientific) was used to assess the quality and purity of the RNA. TAKARA cDNA synthesis kit was used to generate complementary DNA (cDNA) of extracted RNAs. As mentioned in the kit instruction guide specific primers (Table 2) were used for stem-loop assay instead of using OligoT primers.

Circulating miRNA isolation from plasma

Total RNA was purified from 200 µl of serum using a commercial isolation kit from Tripure (Roche) exactly as described by the manufacturer's manual. NanoDrop spectrophotometer (Thermo Fisher Scientific) was used to assess the quality and purity of the RNA. TAKARA cDNA synthesis kit was used to generate complementary DNA (cDNA) of extracted RNAs. As mentioned

in the kit instruction guide specific primers (Table 2) were used for stem-loop assay instead of using OligoT primers.

Quantitative real-time PCR

The real-time PCR was done to quantitative analysis of samples. Gene-specific primers and SYBR green master mix were used to carry out the relative quantification of the expression level of genes from the tumor, normal marginal tissue, and plasma samples. The housekeeping gene was identified by measuring the expression of the U6 gene in each sample. Oligo 7 software was used to design primers. With respect to determining the relative expression level of target genes, we measured and used the average score of duplicated C_t values and comparative C_t ($2^{\Delta\Delta Ct}$) method for each sample. Table 2 shows the primer sequences used in the qRT-PCR.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 (Graph Pad Software Inc. San Diego, CA). The results are presented as mean ± standard deviation (SD) based on 3 independent experiments. An independent sample *t*-test was used to analyze the difference genes expression level between tumor and normal tissues and plasma samples. Cross tab (Eta) analysis was carried out to examine the association between the clinical features of the patients with relative expression of genes. The level of significance was set at $P < 0.05$.

Results

Our findings showed the overexpression of miR-146 (P-value= 0.0002) in tumor tissues when compared to margin tissue, but there was no significant change in the level of this miRNA in plasma samples of the patients compared to control serum (P-value= 0.1865) (Figure 1). In addition, our results do not show any changes between miR-196a expression profiles in both tissue and plasma samples of the CRC patients (Figure 2).

Regarding miR-132 expression, our results showed that the expression level of miR-132 was down-regulated in tumor tissues (P-value= 0.0055). But, no significant changes were detected in the expression of this miRNA in plasma samples of the patients (P-value= 0.8274) (Figure 3).

Our other results on miR-134 showed that the expression level of this miRNA was down-regulated in tumor tissues (P-value<0.0001). However, no significant changes were detected in the expression of this miRNA in plasma samples (P-value= 0.8264) (Figure 4). The expression ratio of these miRNAs was not associated with the clinicopathological features of the patients. However there was significant correlations regarding the expression of miR-196a (P-value = 0.0305) and miR-146 (P-value < 0.0001) in tumor tissue and serum of the same patients (Table 3).

Discussion

Nowadays, the term liquid biopsy is used to represent the study circulating cells and other cell components including circulating DNA, miRNA, micro-vesicles, and exosomes (9). In liquid biopsy, body fluids will be used as surrogate tissues to provide information on cancer target tissues. Observations regarding extracellular miRNA in relation to CRC are mainly operated in circulating blood, plasma, and serum (28). Due to the identification of new markers for early diagnosis and reduction in mortality rate, several miRNAs associated either with malignancy and directly with tumor size of CRC have been studied (29–31). In the present study, the expression patterns of miR-196a, miR-132, miR-146, and miR-134 in plasma and tissue samples of CRC patients provided the evidence on the application of the appropriate method for CRC diagnosis and prognosis.

In recent studies, the over-expression of miR-196 in several malignancies including CRC has been observed (32). Furthermore, the up-regulation of miR-196 in colon cancer tissues and its pro-oncogenic effects in CRC has been demonstrated (23,32). In a study, it has been claimed that miR-196 over-expression participate in CRC occurrence. There have also been meaningful associations made between the expression levels of miR-196 with staging, lymph node, and distant metastasis, while there were not any link with the depth of invasion, size, location, differentiation, gender, and age in CRC patients (33). But, our results do not show any changes between miR-196a expression profiles in both tissue and plasma samples of the CRC patients.

One study suggested that miR-132 has a tumor-suppressing effect by enhancing apoptosis and by inhibiting cancer cell proliferation and migration (34). For instance, increased expression of miR-132 suppressed the proliferation and colony formation in pancreatic cancer cells (35) and reduced the incidence of colitis-associated tumors (35). In accordance with these findings, Zheng et al. also stated the down-regulation of miR-132 in CRC patients with distant metastases which was in

association with adverse prognosis in CRC patients and more aggressive tumor phenotypes (24). In addition, several studies reported that cell proliferation, differentiation, apoptosis, metabolism, and growth can be modulated by miR-132 (36,37). In the current study, we observed the reduced expression of miR-132 in tumor tissue samples. Thus, decreased expression of this miRNA in CRC patients may indicate its importance in inhibiting tumor growth. All together this finding and other previous findings indicate that miR-132 is a tumor suppressor agent (38).

Studies concerning miR-146 role in the initiation and progression of CRC are controversial. In a study by Pizzini et al. down-regulation of miR-146 in the metastasis tumor tissue compared to primary CRC tissue has been reported. While in a study by Ahmed et al, decreased expression of miR-146 in the stool of CRC patients in comparison to normal subjects has been reported (39,40). In addition, in a study by Wang et al. miR-146 expression reduction in cancer tissue was observed which was in association with the TNM stage (41). These results suggest the miR-146 involvement in colorectal carcinogenesis and cancer pathogenesis. Our results showed the overexpression of miR-146 (P-value= 0.0002) in tumor tissues in comparison to margin tissue. This refers to miR-146 as a marker to distinguish between tumor tissue and healthy tissue on the basis of expression. While miR-134 is up-regulated in the stool of CRC patients, but to the best of our knowledge, the relationship between miR-134 and CRC remains unclear (39,42). In our patients the expression levels of miR-134 was down-regulated in tumor tissues. This miRNA might as well have a tumor growth inhibition effect in colorectal carcinoma.

Another interesting finding of our study was the statistically significant correlation of the tissue expression of miR-146 and miR-196a with serum expression levels of them that should be considered in selecting an appropriate biomarker for screening CRC. Improving screening protocols is depended on developing new low-cost and non-invasive cancer biomarkers. Usage of liquid biopsy through extracellular miRNAs in circulation by a noninvasive blood test could potentially be useful as a screening test for CRC due to 3 main reason; 1) differential circulating miRNA profiles between normal controls CRC patients and presence of miRNAs dysregulation in tumor tissues and plasma of the patients (29,43,52,53,44–51); 2) deduction of the circulating levels of specific miRNAs after tumor resection (29,43); and 3) differential miRNA expression profiles between colorectal tumor and normal tissues (29,54–56). There are lots of potentials in this new era since some of the applications of classical biopsy that can be replaced by liquid biopsy such as

early diagnosis, estimating the risk of metastasis and relapses, tumor staging, and monitoring therapy (9).

According to the obtained results, the efficacy of liquid biopsy techniques in CRC detection is very limited. Thus, we suggest that the tissue biopsy for the diagnosis of CRC may provide more appropriate information about the status of patients in comparison to liquid biopsy techniques. The identification of new biomarkers that provide less invasive, less dangerous and less expensive but more informative screening methods will remain one of the most important goals of cancer studies. Therefore, further investigations regarding the miRNA expression of different profiles in tissue and plasma samples of CRC will be helpful in understanding their role as biomarkers for early diagnosis of CRC and as agents for monitoring this disease.

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Tables & Figures

Table 1. Clinicopathological characteristics of the CRC patients.

Sex	Age	Lymph node metastasis	Stages
9 male 8 female	37-74 Mean: 58.06 SD= 11.96	Positive=6 Negative=10	Stage 1=1 Stage 2=9 Stage 3=5 Stage 4=1

Table 2. Primer sequences.

Primer	Sequence
u6 stem loop	**GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATAT**
U6 Forward	**GCTTCGGCAGCACATATACTAAAAT**
U6 Reverse	**CGCTTCACGAATTTGCGTGTCAT**
hsa-miR-146a-5p stem loop	**GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCCT**
hsa-miR-146a-5p Forward	**CGTGCTGTGACCTATGCTG**
hsa-miR-146a-5p Reverse	**CCAGTGCAGGGTCCGAGGTA**
hsa-miR-196a2-5p Stem loop	**GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCAAC**
hsa-miR-196a2-5p Forward	**CGAGCTGGTGACCTATGCTG**
hsa-miR-196a2-5p Reverse	**CCAGTGCAGGGTCCGAGGTA**
hsa-miR-132-5p Stem loop	**GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTAAC**
hsa-miR-132-5p Forward	**GCTGGTGACCGTGGTCGTT**
hsa-miR-132-5p Reverse	**CCAGTGCAGGGTCCGAGGTA**
hsa-miR-134-5p Stem loop	**GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCCTC**
hsa-miR-134-5p Forward	**AGTGGATTGTGACTGGTGG**
hsa-miR-134-5p Reverse	**CCAGTGCAGGGTCCGAGGTA**

Table 3. The correlations of tissue expression of miRNAs with serum expression levels and clinicopathological parameters. (n.s: non-significant differences; Pv: P-value).

Gene	Tissue Expression vs. Serum Expression	Tissue Expression vs. Age	Tissue Expression vs. Sex	Tissue Expression vs. Lymph Node Metastasis	Tissue Expression vs. Stage
miR-134	n.s	n.s	n.s	n.s	n.s
miR-132	n.s	n.s	n.s	n.s	n.s
miR-196a	Significant (Pv= 0.0305)	n.s	n.s	n.s	n.s
miR-146	Significant (Pv < 0.0001)	n.s	n.s	n.s	n.s

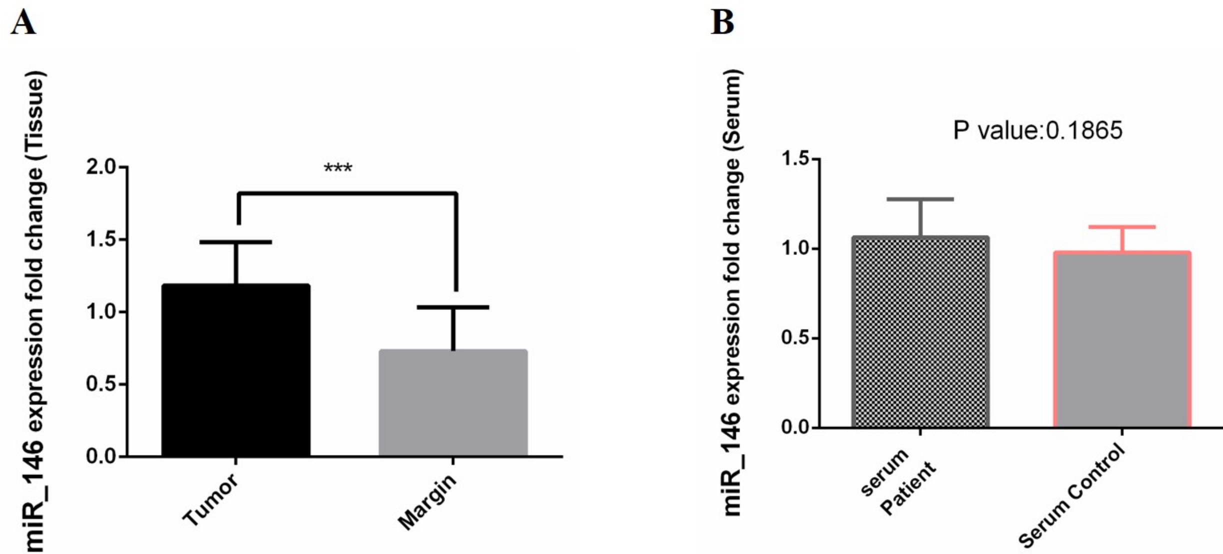

Figure 1. (A) Expression level of miR-146 in tumor and marginal tissues. (B) Expression level of miR-146 in serum of the patients with CRC. The expression of the U6 gene was measured in each sample as the housekeeping gene. ***P <0.001 in comparison with margin group.

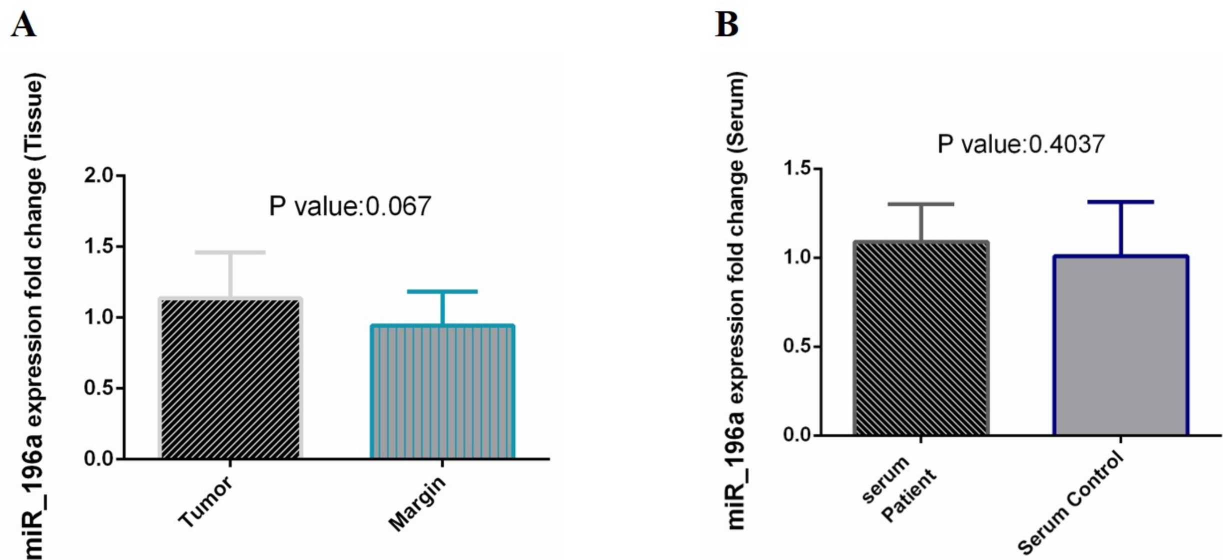

Figure 2. (A) Expression level of miR-196a in tumor and marginal tissues. (B) Expression level of miR-196a in serum of the patients with CRC. The expression of the U6 gene was measured in each sample as the housekeeping gene.

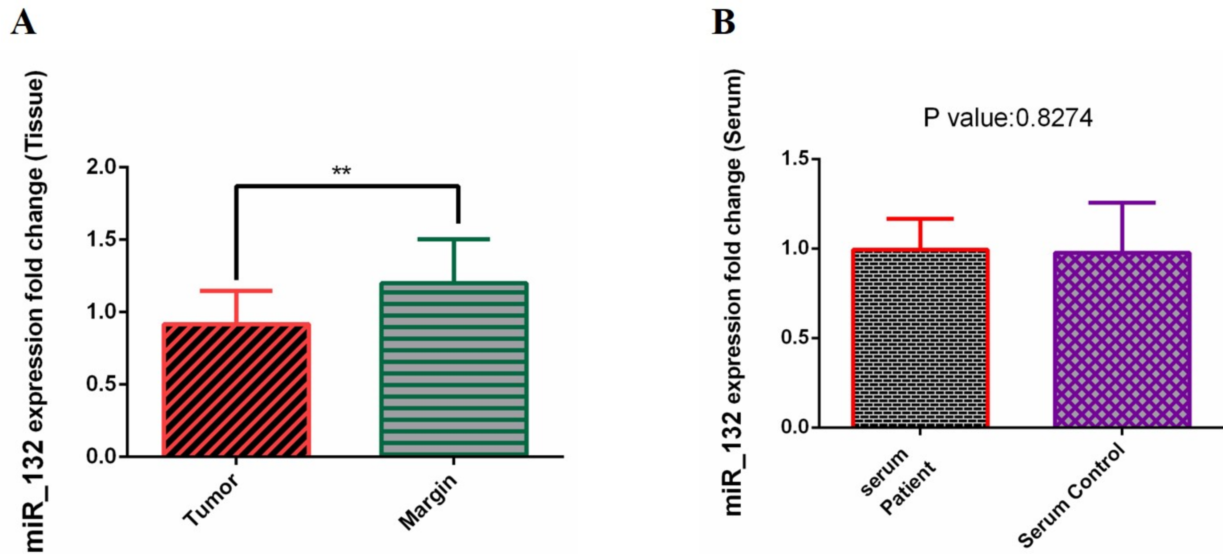

Figure 3. (A) Expression level of miR-132 in tumor and marginal tissues. (B) Expression level of miR-132 in serum of the patients with CRC. The expression of the U6 gene was measured in each sample as the housekeeping gene. **P <0.01 in comparison with margin group.

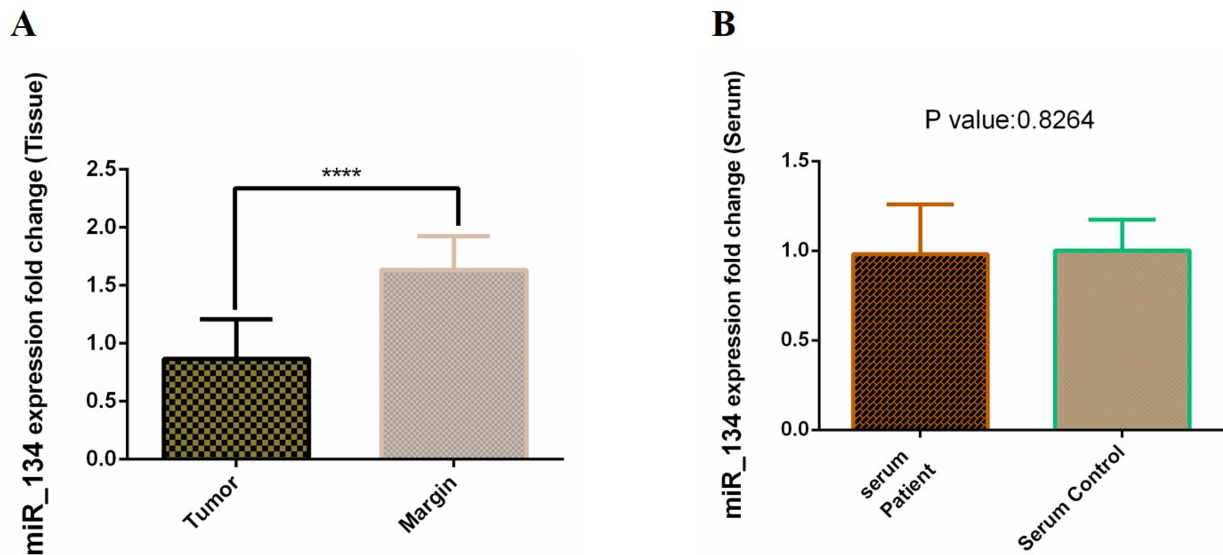

Figure 4. (A) Expression level of miR-134 in tumor and marginal tissues. (B) Expression level of miR-134 in serum of the patients with CRC. The expression of the U6 gene was measured in each sample as the housekeeping gene. ***P <0.001 in comparison with margin group.

CHAPTER 7

Prognostic & Predictive Roles Of Liquid Biopsy In Renal Cell Carcinoma

Prognostic and Predictive Roles Of Liquid Biopsy In Renal Cell Carcinoma

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Research Ongoing

Abstract

Liquid biopsy is very advantageous as it provides not only noninvasive assessment of prognosis of cancers but also detection of tumor specific mutations in circulating DNA. Liquid biopsy helps to improve the real-time monitoring of tumor associated changes and malignancy signatures at the cellular level in peripheral blood of cancer patients. Here, we designed a study to compare informativeness of different fractions of liquid biopsy in RCC cell line and in-vivo models as well as in patients. We harvested our cells in culture and applied them to produce cell line derived xenografts. We extracted Evs and WBC from these models and tested the RNA expression and DNA mutations. We later on moved to our patient part and compared EV DNA with Cell-Free DNA on a mutational basis. Our results revealed that the EV fraction of circulating blood and tissues contained mutated DNA. Patient results however demonstrated that, one mutation in SETD2 gene (chr3:47164191, c.1932_1933delTA) was present in tumor, cfDNA and EV. PBRM1 mutation (chr3: 52637698, c.3219C>A) was also detected in cfDNA of another patient and verified in the tumor tissue, but was not present in the EVs of the patient. Altogether one can expect that by escalating experience of new findings could place EV-DNA in regular clinical practice for RCC cases.

Introduction

The most common form (85-90%) of all kidney cancers which also contributes to 2.4-4% of all human cancers, is renal cell carcinoma (RCC) [1-3]. Almost 300000 new cases of RCC are reported each year [1]. Different histological types of RCC include clear cell RCC (ccRCC), chromophobe RCC, papillary RCC and other RCCs types [4, 5]. Localized ccRCC is mainly treated with surgery [6]. Treatment options for ccRCC were greatly limited until recently, due to its resistance to standard cancer treatments such as chemotherapy and radiation [7]. Clinical use of genetic information obtained by sequencing DNA isolated from RCC tumors has been hampered due to the existence of extensive heterogeneous mutational profiles in RCC tumors.. Furthermore, early diagnosis of RCC is difficult; since the routine imaging tests are not able to distinguish between small RCC tumors and benign lesions [1]. Liquid biopsy is a non-invasive tool which can potentially help early diagnosis of prognosis of RCC through analysis of circulating tumor DNA opening new avenues for genome-based precision medicine in RCC [8, 9].

Used biofluids can be blood, urine, saliva, sputum, etc. The biofluids compartments that can be used for liquid biopsy analysis are Circulating Tumor Cells, Circulating Cell-Free Nucleotides, Extra-Cellular Vesicles, Tumor Educated Platelets, WBC Fraction and RBC Fraction.

In spite of early identification of circulating cell-free DNA (cfDNA) in 1948, its utilization as a liquid biopsy for cancer biomarker detection has only recently been applied [10]. Oncogenic Tumor DNA, which may contribute to the pool of circulating cell-free tumor DNA (ctDNA), has also been detected in EVs circulating in the plasma of cancer patients [11-13]. Considerable interest has been generated about the analysis of diagnostically and therapeutically informative Tumor DNA sequences in WBC, EVs/exosomes and even platelets [11, 14, 15].

It has been suggested that the use of a "biomarker panel" can improve sensitivity and specificity in comparison to single markers [16]. However, there are questions that remained to be answered such as which compartment holds promises for more actionable/detailed information. Here, we designed a study to compare informativeness of different fractions of liquid biopsy in cell line and in-vivo models as well as in patients. Following this route, we aimed to identify and validate most suitable biofluid fraction(s) for the detection of tumor material (mutation and transcript).

Materials and Methods

Cell Culture Conditions

The established renal cell cancer cell line 786-O derived from human renal cell cancer was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured according to the recommendations of ATCC in the appropriate cell culture media and were incubated at 37C in humidified air with 5% CO2 [17].

Extracellular Vesicle (EV) extraction from cells

We collected EVs from 786-O line by collecting the conditioned media. EV-depleted media was obtained by overnight ultracentrifugation at 100,000 × g of RPMI medium (ThermoFisher) supplemented with 1% penicillin. After 72 hours incubation of cells in EV-depleted medium, cell culture media was collected and EVs were isolated. For Extracellular vesicles extraction we stablished a protocol based on ultracentrifugation [18]. Our adapted protocol however was as follows. Briefly, conditioned medium was centrifuged at 10,000 × g for 70 min at 4 °C. The Pellet was washed with PBS and was centrifuged at 10,000 × g for 70 min at 4 °C for a second time. Pellet was washed with RLT Plus buffer from AllPrep kit and the extraction was followed immediately.

Animal models

We have established orthotopic xenografts of RCC. 6-8 week-old female YFP-SCID mouse models (Laboratory Animal Center of McGill university, Montreal, Canada) aged 6-8 weeks were used for the experiments.. For orthotopic injection, the mice were anesthetized with isoflurane. A small incision was made along the left flank of the mouse. Opening the abdominal cavity, the left kidney exposed. An ultra-fine needle attached to a 10µl Hamilton syringe was used to inject cells mixed with matrigel. The needle was inserted into the lower pole of the kidney and advanced until the needle's point reached just below the renal subcapsule. One million viable cells were slowly injected (volume: 10µl). Later the needle was slowly removed and a sterile cotton tip was used to prevent local bleeding and leakage of tumor cells. After injection, the abdominal wall was closed with a re-absorbable suture and the skin secured with surgical staples. Tumor growth and metastatic disease progression was monitored weekly. The mice were sacrificed after sufficient tumor growth and tissue sample was taken and stored at -80C. Blood sample was taken from the

samples via the inferior vena cava (IVC) using 3.8% sodium citrate as anticoagulant. Blood samples were centrifuged and plasma and buffy coat had been stored separately at -80c following routine instructions [19]. All in vivo experiments were performed according to the Animal Use Protocol (AUP) approved by the Institutional Animal Facility Care Committee and following Guidelines of the Canadian Council of Animal Care (CCAC).

WBC and EV extraction from animal models

EVs were collected from plasma of the sample that was previously stored in -80 degrees. The ultracentrifuge protocol followed has been previously described. WBC and RBC were separated using Ammonium Chloride Solution (STEMCELL Technologies) based on the optimized protocol. After Collection of the Pellets of EVs and WBC extraction was performed without storage.

RNA & DNA extraction

Primarily we were interested in using a single isolation method that can be used for simultaneous isolation of DNA and RNA form the same sample. The AllPrep DNA/RNA/miRNA Universal kit (QIAGEN, Germany) was used to extract both RNA and DNA from cells and EVs from cells as well as from tissue, WBC and EVs originated from plasma of the mouse sample. RNA and DNA concentration was primarily tested with MBI biodrop (Montreal Biotech Inc.). Later QC analysis was performed which was quite promising for quality and amount of isolated RNA. For DNA however, the yield was lower.

Quantitative Real-Time Polymerase Chain Reaction

Equal amount of RNA (20 ng/μl) was used for real-time qPCR analyses. Reverse transcription was done using the QuantiTect Reverse Transcription Kit for RT-qPCR (QIAGEN). Real-time polymerase chain reaction was run using the Real-Time PCR System previously described by Perron et. al [20]. and Luna Universal qPCR Master Mix (Biolab) according to the manufacturers' specifications. Primers were diluted to 10X You can find the primer sequences in Table1. The adapted PCR Protocol was as follows: 50 ºC for 2 minutes, 95 ºC for 10 minutes, followed by 45 cycles of 95 ºC and 60 ºC both 1 minute in total. Polymerase chain reactions were performed in

triplicate. All the information regarding the materials of the PCR reaction can be found in Table 2.

Digital Droplet PCR

We also have developed validated protocols to characterize different fractions of liquid biopsies for tumor DNA. We designed mutations-specific primers, gblocks and probes for two mutations which are present in 786-O cell line (see below).

VHL: Deletion (Frameshift), Chromosome 3, Alteration: TG to T; Chr 3:10183839-10183841 (GRCh37).

TP53: Point mutation, Chromosome 17, Altered allele: G to A (rs121912651); Chr 17: 7577538-7577539 (GRCh37)

DNA samples from all study phases were stored at -20 ºC until ddPCR analysis. Samples derived from cell line and Xenograft sample were processed according to instructions provided by BioRad, as described by Taylor et. al [21]. To correct for sampling error and to increase detection rate, samples were analyzed with at least two replicates. Primers used for ddPCR can be found in Table 1.

DNA isolation from tissue and plasma

The study was performed with Plasma samples obtained from RCC patients available through McGill University RCC Biobank. The study was written informed consent was obtained from all patients and ethical approval was obtained from the ethical committee of the University of McGill University. DNA from tissue was extracted using DNeasy Blood & Tissue kit (Qiagen). Cell free DNA from individual samples was extracted from blood using the Isolation protocol from QiaSeq All-in-one kit (QIAGEN, Germany). EVs from Plasma of the entitled patients was obtained as previously described. DNA was extracted from these EVs using AllPrep DNA/RNA/miRNA Universal kit (QIAGEN, Germany) following instructions provided by the manufacturer.

Optimized Targeted NGS Assay

Riazalhosseini's Lab has previously optimized a targeted NGS assay that is able to interrogate mutational status of coding regions as well as exon intron boundaries of 17 genes relevant to diagnosis, patient survival, and response to treatment in RCC Patients. The entitled genes are as

follows: COL11A1, DMD, VHL, NFE2L3, CDKN2A, TP53, NF2, KDM5C, BAP1, MET, SETD2, PTK7, KDM6A, ATM, PBRM1, ATP9B and TRRAP. NGS libraries are prepared using the NxSeq® AmpFREE library kit from Lucigen for Tissue samples and the IDT kit for cfDNA and DNA from EVs. Libraries are multiplexed before capture and enriched for genes of interest using custom lock down probes. Generated libraries were then sequenced on an Illumina NovaSeq instrument to a minimum depth of 2000X coverage for tissue samples. However the depth of coverage for cfDNA and EV-DNA was much higher. We aimed for 50 million sequencing reads per sample of cfDNA and EV-DNA (around 200,000X of our panel).

Bioinformatic analysis

For the processing of the sequencing data and call variants, GenPipes DNASeq High Coverage pipeline that has been developed by the Canadian Centre for Computational Genomics was used. The pipeline has been previously optimized for to the panel used. The pipeline is capable of calling insertion/deletions as well as single nucleotide variants (SNVs). Furthermore; manual inspection of mutations was done to verify called variations. Furthermore, variants were called in tumor, cfDNA and EV-DNA individually in order to be compared.

Results

In this study we investigated the presence of tumor-associated RNA and DNA in EVs tumor bearing animals in order to model similar conditions in human patients affected by RCC.

Detection of VEGF and LOX transcripts in cell line-derived EVs and tissue of animal models

Nucleic acid isolation from cells and from EVs secreted from 786-O cells in culture media was our first step. For our first target which was RNA, we designed RT-PCR primer pairs for VEGF and LOX mRNAs. We tested the human-specificity of these primers for detection of target mRNAs conducting a RT-PCR experiment to ensure that positive amplification signal obtained in EVs isolated from tumor-bearing mice is not due to amplification of mouse RNA. Tested RNA samples for our preliminary study were RNA isolated from 786-O cells, RNA isolated from EVs secreted by 786-O cells and RNA isolated from mouse cells as negative control for the PCR. GAPDH was our housekeeping gene. We detected VEGF and LOX transcripts in the cells and in the EVs

secreted from cells. Later we moved on with our mouse sample. Tested RNA samples were as follows: RNA isolated from 786-O cells. RNA isolated from tumor tissue of the cell line-derived xenograft, RNA from EVs and RNA from WBC taken from Plasma of the xenograft. cDNA synthesis time was adapted through several tests. Our results demonstrated that VEGF and LOX transcripts are present in the tissue and in the cell-line but not in the EVs from plasma and not in the WBC (Figure 1).

We Later Confirmed our results testing our samples with Luciferase Primers through RT-PCR. Luciferase is another specification of our cell-line since the cells contain luciferase as an add-gene. We evaluated abundance of luciferase mRNA levels following 30 and 60 minutes of cDNA synthesis. Interestingly Luciferase expression was 14 times more in the tissue compared to the cell line itself (Figure 2). Our results of three tumor-specific mRNA targets confirm that while these molecules are present in tumors (xenografts) they are not detectable in mouse WBC and EVs circulating in the mouse blood .

Detection of tumor DNA mutations in circulating EVs of tumor-bearing mice

Our Second Target was DNA. To explore the presence of tumor DNA in circulating EVs we designed standard PCR and digital droplet PCR (ddPCR) assays to screen for specific VHL and TP53 mutations in soluble and cellular fractions of the circulating blood and in tissue of mice harboring 786-O xenografts. We confirmed the specificity and robustness of the assays for mutation detection through digital droplet PCR using pre-designed gblocks that served as synthetic controls for mutations of interest. We then moved forward to use materials extracted from different compartments of the mouse samples. We isolated the soluble fraction of plasma EVs and white blood cells along with samples of solid tumor tissues from kidney of the mice. We also used the DNA from 786-O cells as a positive control for our assay.

Notably, ddPCR analysis revealed that the EV fraction of circulating blood and tissues contained mutated DNA. This might suggest that cancer cell derived material is systemically distributed in tumor-bearing mice. However, the levels of DNA regarding to the mutation in both compartments were markedly different. The amount in the Tissue was closer to the cell line (~3000 copies per 20microliter well). For EVs however the median copies per 20microliter well was 7.2 and 5.1 for VHL and TP53, respectively (Figures 3, 4).

Detection of specific mutations in cfDNA and in EVs from Plasma of selected RCC Patients

We studied the known mutations and their frequencies in our patient samples. These mutations were the ones that were found in tumors previously (Table 3). As shown in the table we found mutations on chromosome 3 and chromosome X. Allele frequencies in cfDNA and in EV were respectively close. We found also some mutations that were present in both EV and cfDNA but were not detected in tumor previously. Although allele frequencies are low for some of them but they might be real changes that are detectable through liquid biopsy since there are some variants present in both EV and cfDNA at AFs of ~15% that were not called in tumor. One mutation in SETD2 gene (chr3:47164191, c.1932_1933delTA) was present in tumor, cfDNA and EV. PBRM1 mutation (chr3: 52637698, c.3219C>A) was also detected in cfDNA of another patient and verified in the tumor tissue, but was not present in the EVs of the patient. Moreover, there were other mutations discerned in cfDNA as well as in the EVs which were not present in the tissue of the same patients. However, thorough examination is needed to confirm them as genuine mutations. Additional information regarding these mutations can be found in Table 3.

We also evaluated exon coverage by gene, and generated charts comparing each of the 2 compartments of our 3 patient samples. There are regions that are captured better in EV-derived DNA than in cfDNA, and vice versa. Genes studied in this part were COL11A1, DMD, VHL, NFE2L3, CDKN2A, TP53, NF2, KDM5C, BAP1, MET, SETD2, PTK7, KDM6A, ATM, PBRM1, ATP9B and TRRAP respectively. Figures 5 shows each gene compared on the basis of Ev-DNA (blue lines) and cfDNA (green lines). We however summarized the average coverage per gene in a bar graph comparing the different samples of patients that were used for this study (Figure 6). In all of the entitled genes the average coverage of cfDNA is higher than Ev-DNA in all the patient.

Regarding how well the hybridization worked we studied the percentage of the sequencing reads that were mapped to our target region. Table 4 shows the percentages of on- and off- target of the sequencing reads within or without our target genes. The on-target rate is typically low ranging from 5.15% to 11.10% with both cfDNA and EV-derived DNA.

Discussion

Renal cell carcinoma (RCC) diagnosis and therapy selection are often based on analysis (mainly histological) of tumor specimens obtained through surgery or invasive tumor biopsies. Information of somatic genetic mutations in RCC tumors have shown promise for personalized medicine [5]. However, RCC tumors have a high genetic heterogeneity [22]. A striking example that illustrates intertumoral heterogeneity in kidney cancer specimens was a study which demonstrated extensive heterogeneous mutational profiles in 26 out of 30 tumor samples from four renal cell carcinoma patients [23]. The liquid biopsy is a less invasive method of accessing actionable genomic information in solid tumors, that can help overcome heterogeneity-driven false negative results of genetic analysis of bulk tumors. While promising, liquid biopsy analysis has not yet been fully described with regards to various blood fractions in patients with kidney tumors [23]. Due to the low abundance of tumor-associated materials in liquid biopsies, we need to determine the liquid biopsy compartment which enable higher sensitivity as a biomarker for renal cancer. Therefore, we designed a study to compare liquid biopsy (blood in this study) in compartment level with tissue biopsy. We also tried to determine which nucleic acid types is more informative regarding presence of RCC.

In the first part of this project, we aimed to optimize liquid biopsy screening assays by identifying the most informative liquid biopsy fraction in terms of tumor detection in renal cancer. Therefore, we planned to screen for known mutations, identified in RCC cell lines in our lab's previous studies, and interrogate them in liquid biopsies procured from mice harboring the same cells as xenograft after the optimization procedure.

Due to the lack of solid knowledge about nucleic acid content of EVs in RCC, we investigated the presence of both DNA and RNA molecules in EVs. Therefore, we needed to develop assays for the detection of cancer-associated DNA and RNA in the cell line as well as in different compartments of our xenograft models. As a model to use for protocol calibration, we used 786-O cell line, which is a well-established RCC line, with known RCC-related DNA mutations (VHL and TP53 mutations), and two selected mRNA expressions (VEGF, LOX). Notably, our cell line has also been tagged with a luciferase-expressing construct, providing the possibility of tracking tumor RNA by interrogating luciferase mRNA (LUC). We also had access to tissue and plasma

from xenograft models that have been previously generated using this cell line. Protocols for analysis of different materials was set according to relevant techniques described such as ddPCR and RT-qPCR for genome or transcriptome analyses.
For the RNA part; we tested RNA samples from the cells, EVs from cells, tumor tissue of the xenograft, WBC from the mouse harboring the tumor and EVs extracted from the Plasma of our RCC- Xenograft model. For the entitled RT-qPCR; human specific GAPDH primer was used to test the housekeeping gene, and performance of the assay. We detected mRNA expressions in the cell line, the EVs from cell line and the tumor tissue of our xenograft models for RCC. We however didn't find any tumor-released transcripts in WBC and EVs from Plasma of our animal Model. For the DNA part; we tested DNA samples from the cells, tumor tissue of the xenograft, WBC and EVs extracted from the Plasma of our RCC- Xenograft model through ddPCR. We managed to detect VHL frameshift and TP53 mutations in the tissues and EVs from Plasma of our Xenograft samples but we didn't find those mutations in the WBC of the entitled mice. We confirmed our results with repeats and with the cell line as a positive control. Therefore, our experiments using xenograft models of RCC show that tumor-associated DNA, investigated through analysis of VHL and TP53 mutations, is present in blood stream in forms of soluble cfDNA as well as EV cargo. These observations bring forward the idea that circulating EVs may represent a promising reservoir of tumor DNA emitted from tumor cells into the circulation.
Moving from in-vitro and in-vivo studies to patient samples, we focused our analyses on soluble cfDNA and EV compartments of patient blood samples, based on our promising results for these compartments in the in vivo experiments.
PCR-based approaches are classic methods for cfDNA assessment, when targeting hotspot mutations. Nowadays higher sensitivity for the detection of point mutations in cfDNA as well as other informative materials obtained from liquid biopsy is possible by using digital droplet PCR approaches. Other methods of analysis that are frequently being used include targeted and whole genome sequencing technologies. These methods are particularly helpful for detecting non-hotspot mutations that may be actionable. In the second part of this project we compared results of our liquid biopsy approach in capturing tumor information between EVs and cfDNA isolated from plasma RCC patients. Given that genomics data of tumor tissues of these patients have already been generated by our lab, we were also able to compare results of our EV based liquid biopsy analysis to the genomic background of tumor tissues and investigate the sensitivity and specificity

of our liquid biopsy assay. A targeted NGS assay that is cable of interrogating mutational status of coding regions as well as exon intron boundaries of 17 genes was previously optimized in our lab. Through this assay, we studied the known mutations and their frequencies in 3 patient samples. We detected mutations in chromosome 3 and chromosome X.

Genes studied in this part were COL11A1, DMD, VHL, NFE2L3, CDKN2A, TP53, NF2, KDM5C, BAP1, MET, SETD2, PTK7, KDM6A, ATM, PBRM1, ATP9B and TRRAP respectively. As mentioned previously, in all of the entitled genes the average coverage of cfDNA is higher than Ev-DNA in all the patients. This could be due to library quality since according to the library profiles; we had better library yields for cfDNA as compared than for EV-DNA. This could be explained by lower amount of DNA isolated from EVs as compared to the amount of cfDNA. There were some variants in some genes such as TRRAP, ATM, ATP9B, SETD2, BAP1, PTK7, NFE2L3, MET and TP53 that were present in both EV-DNA and cfDNA that were not present in tumor of the P10 and P20 patient. To confirm the obtained results and to find the possible reasons of these variants not being detected in tumor, further investigation of these variants is needed. Regarding the exon coverage we could see that there were some regions that EV-DNA capture was better than cfDNA capture. This might be useful for identifying which genes/regions may be more enriched in EV vs cfDNA. Also this is important for evaluating if the capture works efficiently with one or both sample types.

Although our study compared the two sources of DNA, our hypothesis was that the sequencing of Ev-DNA can reveal the real-time evaluation of tumor dynamics. The fact that in our patients the results showed little differences between cfDNA and EV-DNA regarding the coverage may thus ring a bell that EV-DNA might constitute a reliable non-invasive biomarker in the disease course. Despite these potentialities, the use of liquid biopsy fragments such as EV-DNA and even cfDNA in routine clinical practice has still been burdened by costs, heterogeneity in methodology and results from studies that could be influenced by the noise caused by normal tissue cfDNA. For these reasons, the level of evidence regarding this specific issue is not yet considered as efficient to recommend the use of EV-DNA in routine clinical practice. However, one can expect that by escalating experience of new findings and testing different analyzing methods regarding cfDNA and EV-DNA; the aforementioned barriers could be addressed. Additionally, this could place EV-DNA in regular clinical practice for RCC cases. This opens up an era through which we may use

liquid Biopsy and its inclusive biomarkers as a mirror to reflect the diagnosis in the earliest possible time, as well as to monitor the patient's response to therapy.

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Tables & Figures

Table 4: Primers used for RT-qPCR and ddPCR

RT-qPCR Primers	VEGF	Forward	CACACAGGATGGCTTGAAGA
		Reverse	AGGGCAGAATCATCACGAAG
	LOX	Forward	GGCTAAACTCATCCATACTGTGGT
		Reverse	TTTCTTACCCAGCCGACCAA
	LUC	Forward	CGGAAAGACGATGACGGAAA
		Reverse	CGGTACTTCGTCCACAAACA
ddPCR Primers	VHL	Forward	GCCCGTATGGCTCAACTT
		Reverse	TACCTCGGTAGCTGTGGAT
	TP53	Forward	TATCTCCTAGGTTGGCTCTGAC
		Reverse	CAGTGTGATGATGGTGAGGATG

Table 2: RT-qPCR Reaction Compartments

LUNA Master Mix	$5\mu l$
10X Primer Mix	$0.5\ \mu l$
RNAse free H_2O	$4.5\ \mu l$
cDNA	$1\ \mu l$
Total V	$11\ \mu l$

Table 3: Variants found in Tumor

Patient	Genes Mutated	Location	Codon Change	Impact	AF in Tumor	AF in cfDNA	AF in EV
P6	KDM5C	chrX: 53222024	c.4047-6G>C	Splicing region	12.70%	0.00%	0.00%
P10	SETD2	chr3: 47164191	c.1932_1933delTA	Frameshift	21.00%	3.30%	3.50%
P20	PBRM1	chr3: 52637698	c.3219C>A	Frameshift	14.64%	6.30%	0.00%
	KDM5C	chrX: 53222447	c.4381_4383delCGG	Frameshift	9.30%	0.00%	0.00%

Table 4: Percentages of on- and off- target of the sequencing reads

	Ev-DNA P10	Ev-DNA P20	Ev-DNA P6	cfDNA P10	cfDNA P20	cfDNA P6
On Target	8.41%	6.32%	5.15%	11.10%	9.99%	8.27%
Off Target	91.59%	93.68%	94.85%	88.90%	90.01%	91.73%

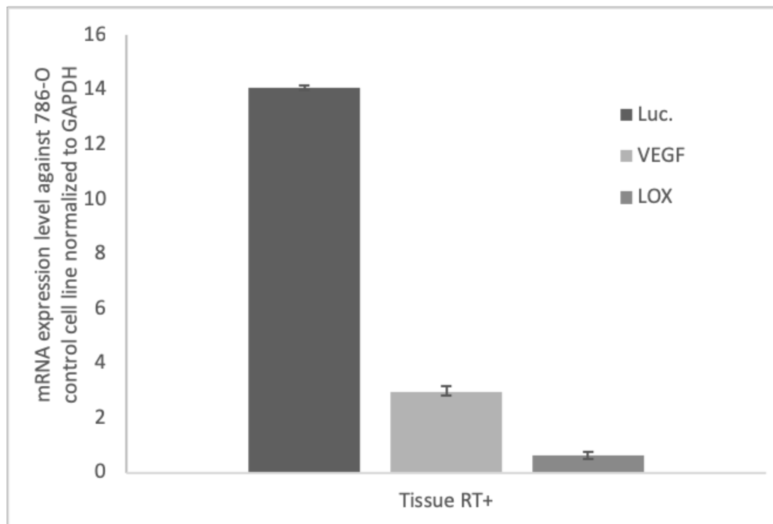

Figure 1: Luciferase, VEGF and LOX expression levels in the 786-O control cell line and Tissue RT+. Gene expression levels are normalized to the 786-O control cell line. GAPDH gene expression was used to normalization as a housekeeping gene (n=3; error bars, ± s.d.).

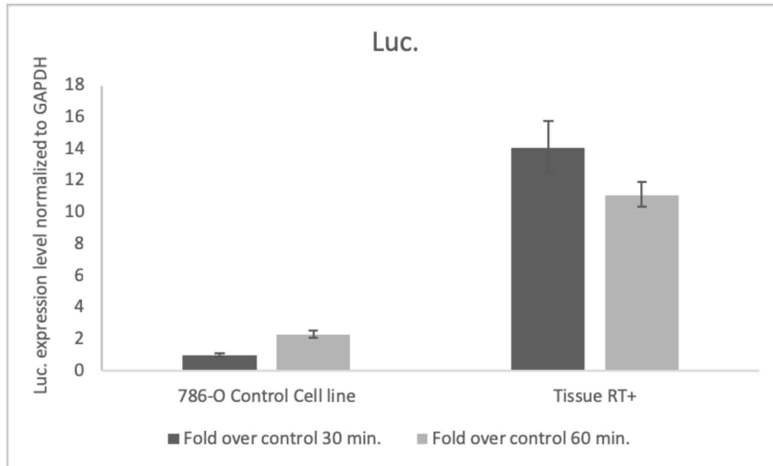

Figure 2: Luc. values are normalized to the 30 min. cDNA synthesis of 786-O control cell line. GAPDH gene expression was used to normalization as a housekeeping gene (n=3; error bars, ± s.d.).

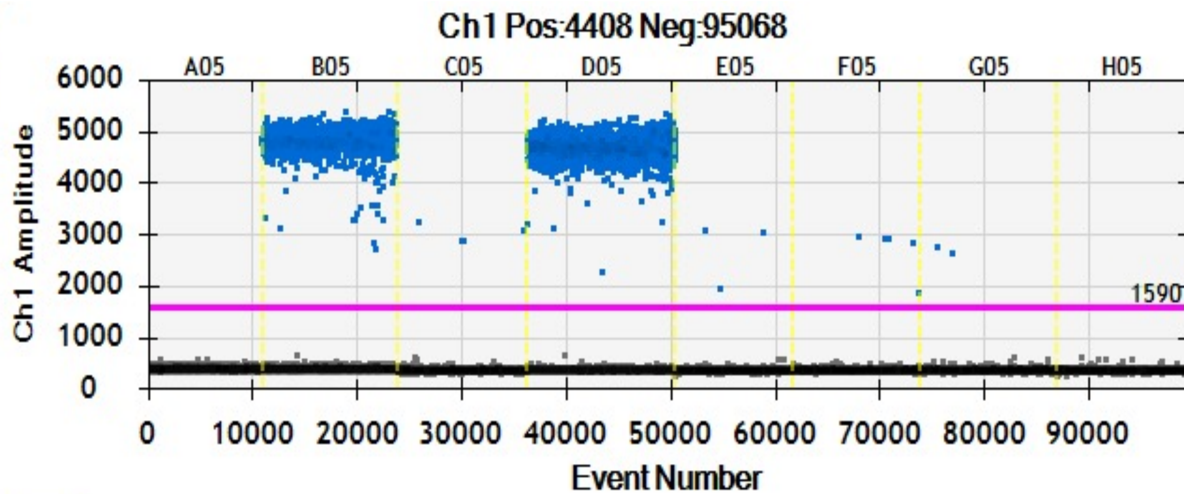

Figure 3: VHL mutation in Xenografts. A05: WBC, B05: Tissue, C05: EV, D05: 786-O cell line, E05 & F05 & G05: Replicates of EV, H05: NTC.
Concentrations: 0, 157, 0.38, 259, 0.31, 0.48, 0.27, 0.
Copies Per 20uL Well: 0, 3140, 7.6, 5180, 6.2, 9.6, 5.4, 0.

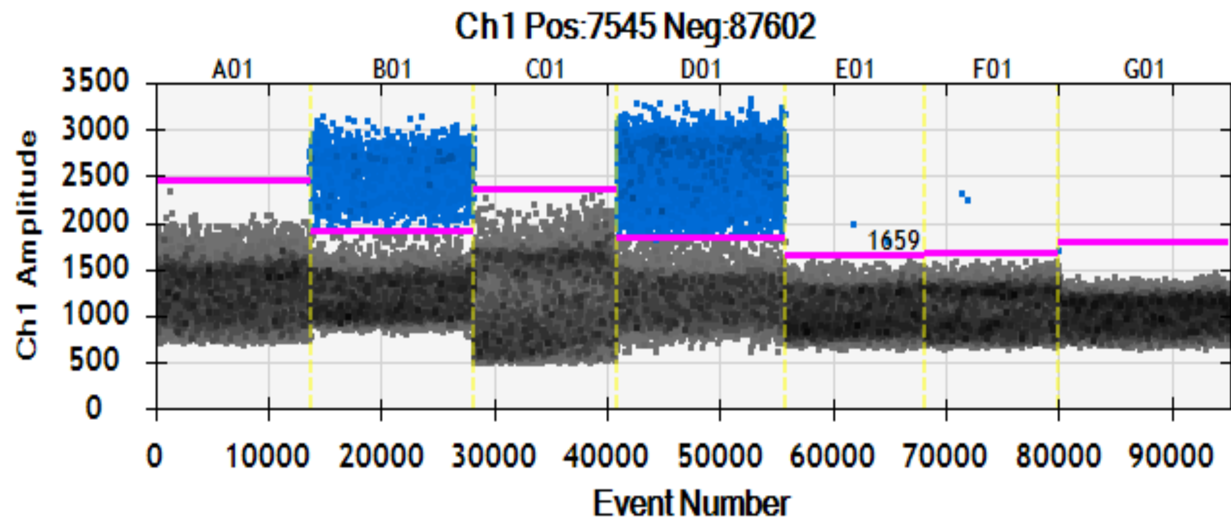

Figure 4: TP53 mutation in Xenografts. A01: WBC, B01: Tissue, C01: EV, D01: 786-O cell line, E01 & F01: Replicates of EV, G01: NTC.
Concentrations: 0, 195, 0, 517, 0.19, 0.3, 0.
Copies Per 20uL Well: 0, 3900, 0, 10340, 3.8, 6, 0.

Figure 5: Exon coverage by gene; Ev-DNA vs cfDNA

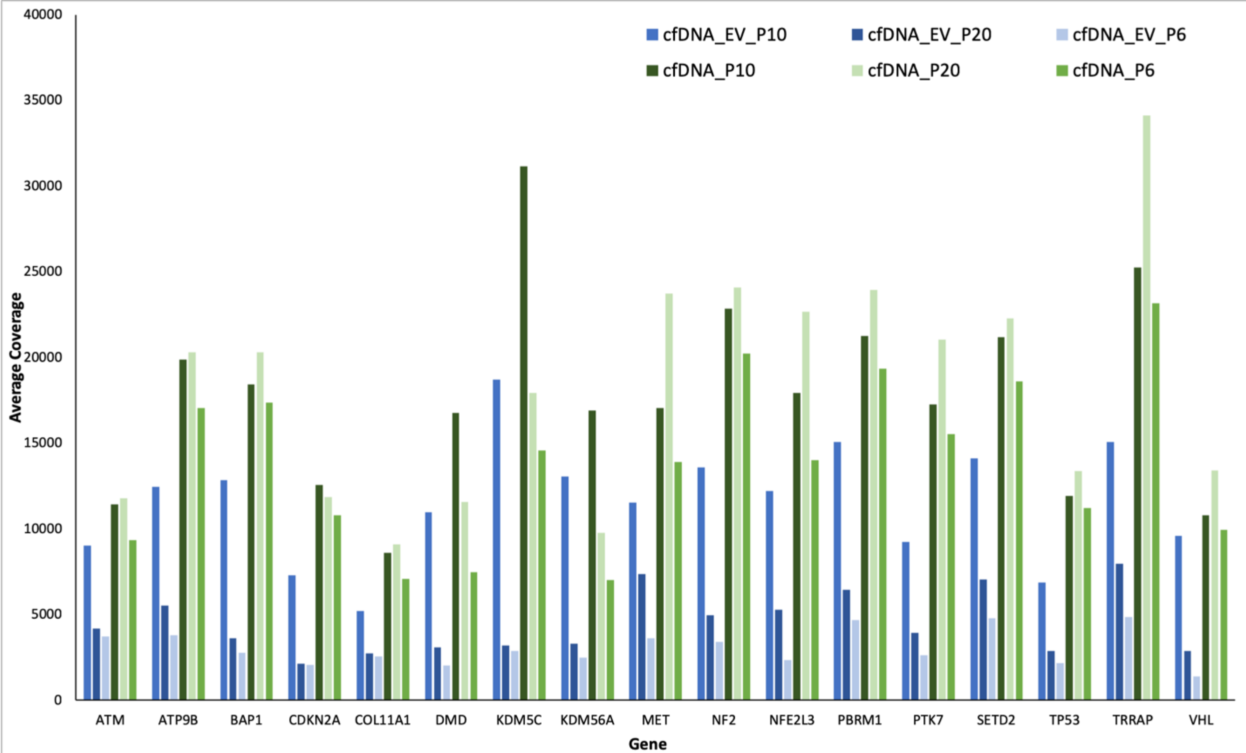

Figure 6: Average coverage per gene

CHAPTER 8

General Discussion & Future Prospective

General Discussion & Future Prospective

Developing a non-invasive tool for biomarker detection and discovery with higher sensitivity and lower complications compared to invasive biopsy methods, seems vital. Liquid biopsy is an approach that gained considerable attention recently. Advantages of this technique is that it is noninvasive and reliable causing lower risk & lower trauma for the patient. Temporal measurements of tumor burden and early evidence of recurrence or resistance during treatment can be discovered by liquid biopsies which are associated with significantly less morbidity [1]. As a result of its minimal invasive nature, liquid biopsies can be used more frequently during treatment period to provide a personalized snapshot of disease. Unlike tissue biopsies that are only obtained from one tumor region, liquid biopsy may better reflect the genetic profile of all tumor sub-clones present in a patient [2]. Traditional techniques, which are used routinely now, play vital roles in tumor diagnosis; however, for therapeutic guidance, the specificity of traditional serum biomarkers is unsatisfactory. Imaging techniques on the other hand cannot be used for 'real-time' detection due to economic concerns and exposure to the radiation [3-6]. In contrast, liquid biopsy can be considered a harmless and cheap strategy for early tumor diagnosis, recurrence detection and therapeutic monitoring [7]. The use of such non-invasive methods for diagnosis can also revolutionize the cancer treatment and therapy.

The most important and difficult task regarding liquid biopsy is mutation detection. Numerous techniques can be used for this purpose including ddPCR and NGS [8,9,10]. Focusing on NGS technologies; studies revealed that these techniques can be applied to obtain a more comprehensive view of the entire genomic regions. Approaches which involve deep sequencing are as follows; AmpliSeq, Safe-Seq, CAPP-Seq and TAm-Seq [11-14]. Elegantly these technologies make NGS a tool that provides broader opportunities to distinguish personalized cancer gene maps and characterize personalized medicine approaches. Moreover, Detection and usage of cancer-specific features may elevate a critical dimension for new biomarker discovery. Liquid biopsies are excellent sources for diagnostic tests that can also be used to monitor patient drug therapy responses [15, 16]. Peripheral blood can provide various forms of liquid biopsies which can be used for purification of circulating nucleic acids. For example, approximately less than 0.5% of the cell-free nucleic acids can provide circulating tumor DNA that could be released into the

bloodstream through necrosis or apoptosis of tumor cells or directly by primary tumor or circulating tumor cells (CTCs) [15, 17].
Regarding this aim however, strong quantitative computational analysis is essential. Hence these algorithms serve calculate which combination of biomarkers yields the highest sensitivity and specificity. To this end, various computational aspects are needed to be taken into account such as; (1) secure and hierarchically structured data storage, (2) innovative machine learning models that use various bio-source combinations and (3) standardized bioinformatics protocols [18]. Subsequently these will provide a biological proof for the functionality of the detected biomarkers. It can be inferred that moving from a single to multi-marker concept will be an important progress in this field. This project also had a special focus on liquid biopsy compartments such as Extracellular Vesicles specially Exosomes. EVs are present in blood and in different types of body fluids where can be isolated and stored for many years [19]. Because of the lack of accurate isolation and detection methods, few biomarkers in the EVs have been implemented into clinical practice, in spite of the fact that these molecules are excellent source of cancer biomarkers. Developing sensitive capture platforms makes it possible to introduce the novel EV based biomarkers into the clinical practices in the future. Following the introduction of NGS and ddPCR, it is likely to reduce the operating costs and time of circulating cancer biomarker detection.

Conclusion

Liquid biopsy provides a noninvasive and most importantly real-time method which serves as an alternative to traditional ‘solid biopsy’. Despite numerous advantages of this aspect, several limitations remain to be overcome in order to for liquid biopsy to reach its full potential. These include lack of consensus regarding the capture and detection methods, insufficient grounded proof for evidence-based medicine studies and the difficulties encountered to obtain informative sequencing data are some of a few challenges that remain unsolved.
Liquid biopsy enables the assessment of circulating molecules in various biological fluids to detect biomarker in a non-invasive manner and to assess prognosis and treatment failure in early stages. Liquid biopsy is an efficient tool for monitoring therapeutic response and recurrence besides its use for early detection & distant metastasis detection. Liquid biopsy is also crucial in planning personalized cancer treatment. Whereas new evidences revealed the real clinical value of these

non-invasive cancer biomarkers in circulation, the advances in their detection, development and related technologies are growing rapidly in recent years.

Conclusively, the assessment of the main approaches as well as alternative methods to implement and develop liquid biopsy in the clinical settings requires a strong effort through a broad range of scientific and technology competencies which can open a new era in medical research and lead to a radical breakthrough with great transformative impact. Combining the study of bio-sources obtained from Cell-free Nucleic acids, CTCs, EVs and TEPs with other materials may enable next generation liquid biopsy frameworks with enhanced sensitivity and specificity for cancer detection. The key to this success will be to combine the knowledge of biological properties of generation and spread of liquid biopsy with technologies with cutting-edge sensitivity for capture and analysis of genomic cargo of liquid biopsies.

This thesis aims to add information to an important matter that needs to be further discussed in order to be utilized in precision medicine; which is to use circulating tumor material. This will enable the earliest diagnosis of the disease as well as real time monitoring before and after treatment due to the abundant knowledge that it can provide about tumors. Cancer derived biomarkers with diagnosis, prognosis and therapy-predictive information can be found in the populations circulated compartments and therefore may be a promising platform to overcome the existing problems of liquid biopsy-based approaches.

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Scientific Products

Potential prognostic and predictive roles of liquid biopsy in colorectal carcinoma; With A Special Focus on Circulating DNA and Exosomes
*__Maralani M__, *Galvano A, *Castiglia M, Barraco N, Incorvaia L, Fanale D, Gristina V, Silvestris N, Bazen V, Russo A. (Submitted)

The role of microRNAs in cancer regulation
Maralani M, Baradaran B, Khalil Hajiasgharzadeh, Peeters M. (Submitted)

Expression profiles of miR-196, miR-132, miR-146a and miR-134 in human colorectal cancer tissues in accordance with their clinical significance: comparison regarding KRAS mutation
M Maralani, D Shanehbandi, M Asadi, S Hashemzadeh, Kh Hajiasgharzadeh, B Baradaran, M Peeters. (Submitted)

The different profiling of microRNA-based tissue biopsy and liquid biopsy for colorectal cancer disease screening and detection
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