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ORIGINAL ARTICLE

Comparison of Circulating miRNAs Expression Alterations in Matched Tissue and Plasma Samples During Colorectal Cancer Progression

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Abstract MicroRNAs (miRNAs) have been found to play a critical role in colorectal adenoma-carcinoma sequence. MiRNA-specific high-throughput arrays became available to detect promising miRNA expression alterations even in biological fluids, such as plasma samples, where miRNAs are stable. The purpose of this study was to identify circulating miRNAs showing altered expression between normal colonic (N), tubular adenoma (ADT), tubulovillous adenoma (ADTV) and colorectal cancer (CRC) matched plasma and tissue samples. Sixteen peripheral plasma and matched tissue biopsy samples (N $n = 4$; ADT n = 4; ADTV n = 4; CRC n = 4) were selected, and total RNA including miRNA fraction was isolated. MiRNAs from plasma samples were extracted using QIAamp Circulating Nucleic Acid Kit (Qiagen). Matched tissue-plasma miRNA microarray experiments were conducted by GeneChip® miRNA 3.0 Array (Affymetrix). RT-qPCR (microRNA Ready-to-use PCR Human Panel I + II; Exiqon) was used for validation. Characteristic miRNA expression alterations were observed in comparison of AD and CRC groups (miR-149*, miR-3196, miR-4687) in plasma samples. In the N vs. CRC comparison, significant overexpression of miR-612, miR-1296, miR-933, miR-937 and miR-1207 was detected by RT-PCR ($p < 0.05$). Similar expression pattern of these miRNAs were observed using microarray in tissue pairs, as well. Although miRNAs were also found in circulatory system in a lower concentration compared to tissues, expression patterns slightly overlapped between tissue and plasma samples. Detected circulating miRNA alterations may originate not only from the primer tumor but from other cell types including immune cells.

Keywords microRNA · Plasma · Circulating microRNA · Colorectal cancer · Colorectal adenoma · Microarray · Real-time PCR · Tissue

Introduction

Colorectal cancer (CRC) is one of the most common cancer-related cause of death [1]. The most reliable screening method for early detection of CRC is colonoscopy. For increase the compliance of the patients, alternative biomarkers with high precision and non-invasive approaches for CRC pre-screening is needed. Epigenetic modifications, such as non-coding RNA and DNA methylation alterations [2] are important mechanisms of colorectal cancer and may play a crucial role in carcinogenesis [3]. MiRNAs are small non-protein-coding RNA molecules that regulate gene expression post-transcriptionally. MiRNAs have been shown to be dysregulated in several cancer types including CRC [4].

To date, our research group has identified systematic alterations of miRNAs in colorectal cancer development [5]. We reported 1733 mature miRNAs with altered expression in healthy, adenoma (highlighted to tubular and tubulovillous adenoma) and colorectal cancer samples using two different methods (microarray and real-time PCR). Beside the

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described miRNAs in precancerous and malignant lesions, we have also identified less known miRNAs with altered expression between different diagnostic groups. Furthermore, altered levels of miR-3591, miR-4506, miR-31 and miR-187 were identified in plasma and same expression tendency could be observed also in matched tissue samples.

MiRNAs can be detected in different body fluids such as saliva, plasma or urine [6]. During colorectal cancer development, release of nucleic acids (DNA, mRNA, miRNA) can be observed in free circulating, protein and exosome bound form [7]. Due to the stability of miRNAs, they can be specific prescreening markers in plasma samples, once their sensitivity and specificity is proven [8].

Previous reports showed that almost 200 miRNAs were presented in peripheral plasma of pregnant females [9]. The number of expressed miRNAs between the different patient groups is nearly balanced, although less miRNAs were observed in plasma samples compared to matched tissue specimens (approximately 300–330 miRNAs/samples group). Leidinger et al. found that average number of detected miRNAs in control plasma samples was 331 and the overall number of detected miRNAs in plasma of lung cancer patients was between 264 and 364 from 1205 analyzed miRNAs [10].

Origin of circulating cancer-associated miRNAs detected in peripheral blood is not clear whether they can be derived from tumor cell death and lysis or they are actively secreted by tumor cells. In this study, evaluation of circulating miRNA alterations in parallel to the local tissue changes and in pre-neoplastic colorectal alterations has been performed.

The aim of the present study was to perform a global miRNA microarray analysis of tubular, tubulovillous adenoma, colorectal adenocarcinoma and normal plasma samples followed by a real-time PCR validation. Furthermore, miRNA expression pattern changes of parallel tissue samples were also examined from the same patients. Our investigations were focused on the question that the detected miRNAs in plasma fraction originated from tumor tissues as a tumor-specific alteration or they are part of a host immune system response for the disease.

Materials and Methods

Clinical Samples

The study was approved by the local ethics committee (Semmelweis University Regional and Institutional Committee of Science and Research Ethics; Nr.: ETT TUKEB 116/2008; 23,970/2011) and written informed consent was provided by all patients. Patients did not receive chemo- or radiotherapy prior to sample collection.

MiRNA Isolation from Plasma Specimens

Peripheral blood samples were drawn into 6 ml K3EDTA Vacuette tubes (Greiner Bio-One Gmbh, Cat.No. 456038) and were centrifuged at 1350 rpm for 12 min at 24 °C (room temperature). The supernatants were then centrifuged again with the same parameters. The plasma samples were stored at −20 °C until use. The miRNA fraction was extracted by the QIAamp Circulating Nucleic Acid Kit (Qiagen, Cat. No 55114) with the following modified protocol based on our previously published study [5]: 1 ml of plasma was mixed with 100 μl proteinase K (Qiagen, Cat. No. 55114) and 800 μl ACL buffer (Qiagen, Cat. No. 55114) and was incubated at 60°C for 30 min. Thereafter 1800 μl ACB Buffer (Qiagen, Cat. No. 55114) and 3000 μl isopropanol were added to the mix and the tube was gently inverted a few times. Tubes were then incubated at −20°C overnight. The mixture was filtered through the column followed by washing steps with 2*1 ml 70% and 1 ml absolute ethanol. MiRNA samples were eluted in 30 μl puffer AVE (Qiagen, Cat. No.55114) and was stored at −20 °C.

Isolation of Total RNA Including miRNA from Matched Fresh Frozen Biopsies

Total RNA including miRNA fraction was isolated from biopsy samples using the High Pure miRNA Isolation Kit (Life Science Roche, Cat. (No.05080576001) according to the manufacturer's instruction, after homogenization with MagNALyser instrument (Life Science Roche, Cat.No. 03358976001) for 2 min, 6000 rpm using the MagnaLyser Green Beads (Cat.No. 03358941001). RNA integrity was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Cat. No. G2940CA) and RNA yield was quantified using RNA Assay Kit on Qubit 1.0 fluorometer (ThermoFischer Scientific Cat. No. Q32852).

Tissue and Plasma miRNA Microarray Expression Profiling by GeneChip miRNA 3.0 Array

MiRNA expression profiles were analyzed using GeneChip miRNA 3.0 arrays (Affymetrix, USA, Cat. No.902413). 1 μg of total RNA including miRNA from tissue and the total amount of eluted miRNA fraction from plasma samples were biotin-labeled using the Flashtag Biotin HSR RNA Labeling Kit (Affymetrix, Cat. No.901911). Samples were hybridized for 16 h in GeneChip Hybridization Oven 645 (Affymetrix, Cat. No. 00–0331), then were washed and stained using GeneChip Fluidics Station 450 (Affymetrix, Cat. No. 00–0079) with the FS450_0002 fluidics protocol and scanned with GeneChip® Scanner 3000 7G (Affymetrix, Cat. No.00–0210).

Statistical Analysis

Probe cell intensity files of microarrays were analyzed by Expression Console Software (Affymetrix). The robust multichip averaging (RMA) algorithm was used for background correction, normalization and probe level summarization. GeneChip miRNA 3.0 arrays contain probe sets of 1733 mature miRNAs. Values of $p < 0.05$ were considered statistically significant with a logFC > |0.5|.

Real-Time Quantitative PCR Array Analysis from Plasma Specimens

Total amount of eluted miRNA fraction from plasma was reverse transcribed using the miRCURY LNA™ Universal RT cDNA Synthesis Kit (Exiqon, Cat. No. 203301). The cDNA template was then amplified using the microRNA Ready-to-Use PCR, Human Panel I + II (Exiqon) in 384 well plates according to the manufacturer's instruction. The qPCR reactions were run on a LC480 thermocycler (Life Science Roche) using the thermal-cycling parameters recommended by Exiqon (Denaturation at 95 °C 10 min, 45 amplification cycles at 95 °C 10s 60 °C 1 min). The amplification curves were analyzed by LightCycler 480 Software (Life Science Roche, Cat. No.04994884001). From the 768 wells, 742 miRNA primer sets were used for miRNA expression profiling, the remaining wells contained interplate calibrator oligonucleotides, spike-in control oligonucleotides and empty wells. SNORD49A was used for normalization. The relative quantification of miRNA expression levels was performed using delta delta Cp method.

Results

MiRNA Expression in Plasma Samples from Different Diagnostic Groups

MiRNA microarray analysis was performed on plasma samples from patients with tubular adenoma or tubulovillous

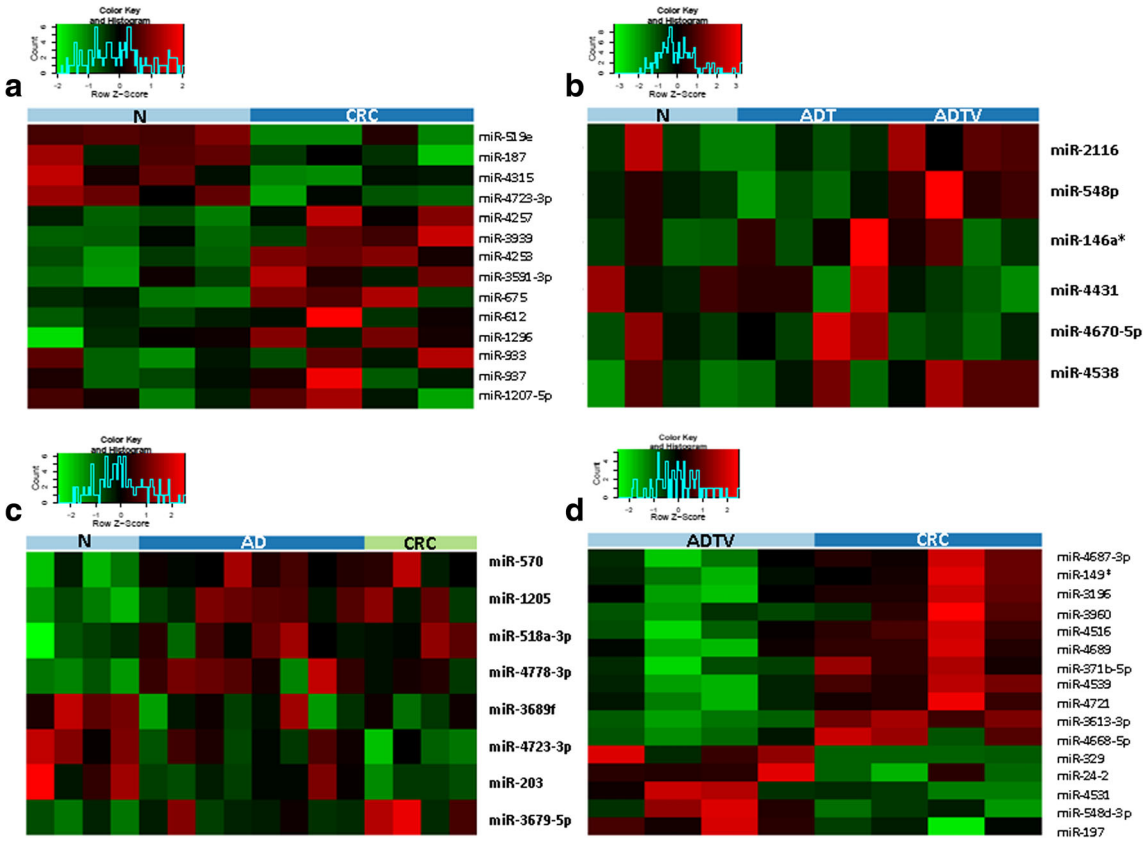

Fig. 1 Altered miRNA levels in different patient groups in plasma samples. (**a**): Heatmap of the significantly dysregulated miRNAs in plasma between N and CRC samples. Red color means upregulation, green color means downregulation. Rows represent the miRNAs, columns represent the samples. (**b**): miRNA expression between adenoma subtypes and normal samples (**c**): miRNA expression alterations in normal plasma compared to neoplastic lesions (**d**): miRNA expression alteration in plasma from patients with tubulovillous adenoma compared to colorectal cancer. (N = Normal; AD = Adenoma; ADT = Adenoma tubular; ADTV = Adenoma tubulovillous; CRC = Colorectal adenocarcinoma)

adenoma or colorectal cancer. Expression Console Software was used to determine the presence of miRNAs based on expression intensity numbers. In healthy controls 306 miRNAs were expressed, while 334 miRNAs were presented in plasma of adenoma patients, and 321 miRNAs were observed in CRC samples. In terms of the number of expressed miRNAs there were no significant differences between the analyzed clinical groups.

The expression alterations were determined between different diagnostic groups (Fig. 1). In miRNA microarray experiments, 14 miRNAs showed significantly altered expression between CRC and normal plasma samples (Fig. 1a). The majority of the significant changes were detected in the abovementioned comparison. Relatively low fold changes could be detected in plasma samples (most of the described miRNA changes were between 1 and 1.5-fold) however, miR-933 was almost 1.6-fold upregulated in CRC samples compared to normals. When adenoma and normal samples were compared (Fig. 1b.), no significant expression alteration between tubular adenoma and normal samples could be measured, however, two miRNAs (miR-2116, miR-548p) were found to be upregulated in tubulovillous histological type of adenoma compared to the normal group. Differentially expressed miRNAs between neoplastic lesions and healthy samples were also identified. Eight miRNAs showed characteristic differences between these patient groups ($p < 0.05$). Only three miRNAs were upregulated in CRC and adenoma compared to normal controls (Fig. 1c). MiR-4723-3p, miR-203 and miR-3689f were downregulated during CRC progression. Sixteen miRNAs discriminating tubulovillous adenoma from CRC were also determined (Fig. 1d) from which 11 showed significantly higher expression in CRC samples.

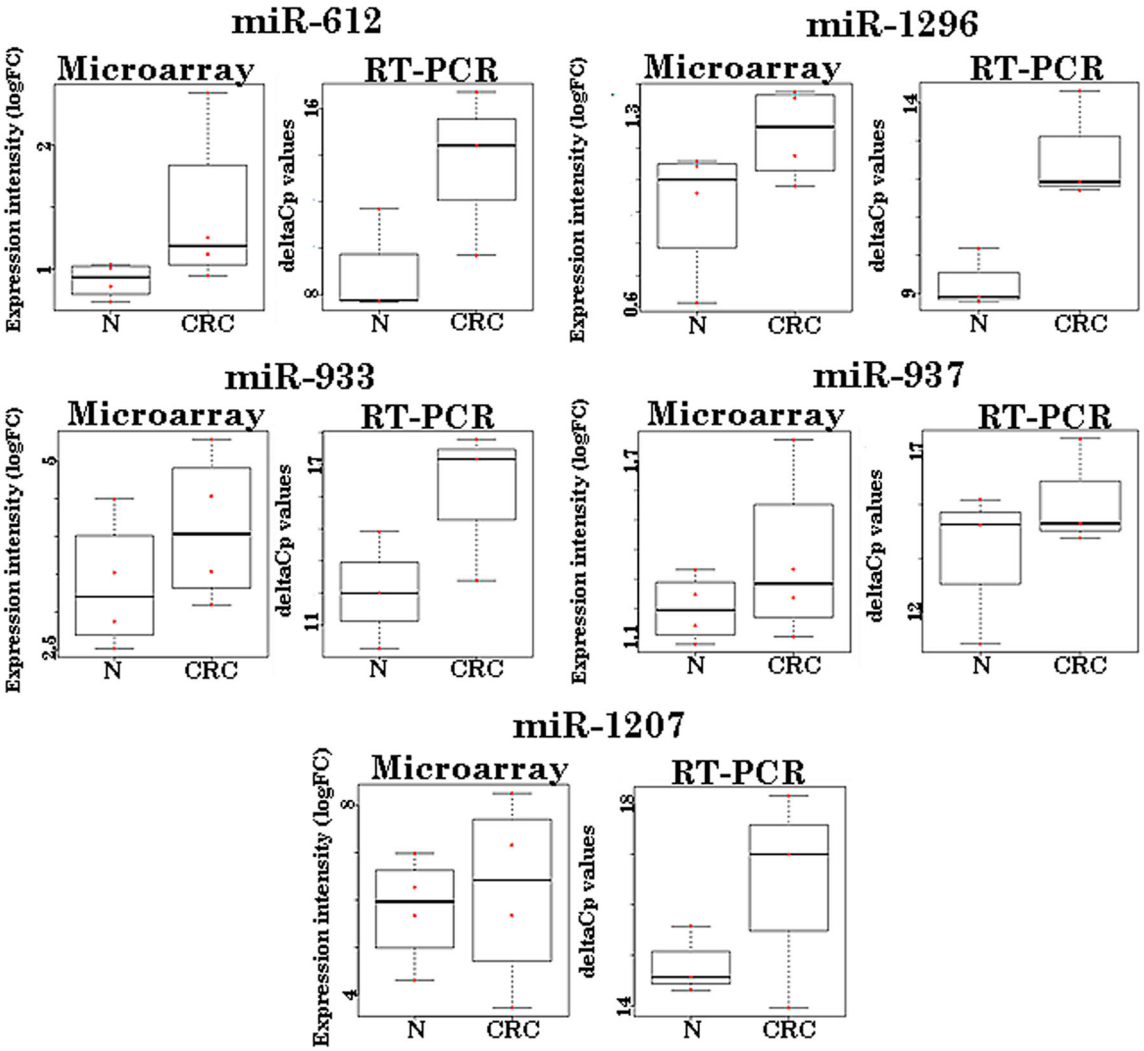

Fig. 2 Validation of differentially expressed miRNAs by real-time PCR in normal vs. CRC comparison in plasma. Expression changes are visualized by box-plots. Left boxes represent the expression intensity (logFC) values of microarray data: (is described on (axis Y)). Right boxes show the deltaCp values (Y axis) of RT-PCR data. (N = normal; CRC = colorectal adenocarcinoma)

MiRNA Expression Validation with Real-Time PCR Panel

In order to confirm the microarray data, miRCURY Human Panel real-time PCR (Exiqon) were applied using the same plasma samples. Plates contained a limited number of assays for altogether 742 mature miRNAs. From the 14 significantly altered miRNAs in N vs. CRC comparison detected by microarrays, only miR-187, miR-612, miR-1296, miR-933 m, miR-937, miR-1207, miR-146a and miR-675 were represented on the PCR plate. Except for miR-675, the above-mentioned miRNAs showed signals by RT-PCR, moreover, the expression tendencies measured by microarrays in plasma samples correlated well with RT-PCR results (Fig. 2).

Plasma-Specific miRNA Expression in Matched Tissue Samples

Using matched tissue samples, miRNA expression profiling was performed and miRNAs showing altered expression in plasma fraction were selected. Expression profiles of these miRNAs were different in tissue samples. In normal vs. CRC comparison, miRNAs had the same expression tendency in case of miR-612, miR-1296, miR-933, miR-937 and miR-1207 showing upregulation in CRC compared to normal samples both in tissue and plasma (Fig. 3). In normal vs. adenoma comparison, similar expression tendencies were not observed in tissue sample pairs (Fig. 4). MiR-3689f, miR-4723-3p and miR-203 were highly expressed in normal plasma compared

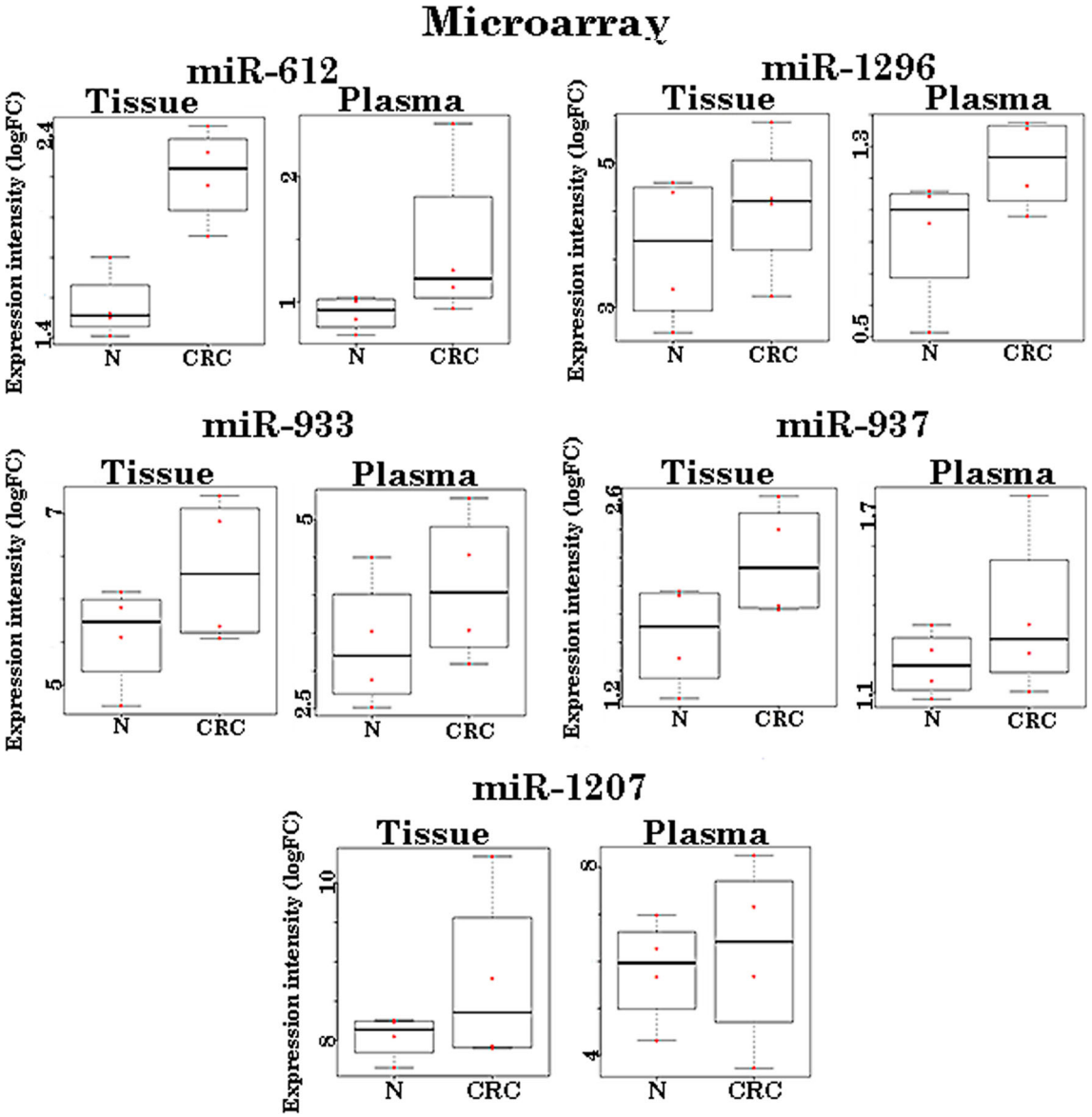

Fig. 3 The most stable miRNAs in plasma showing the same expression pattern in matched tissue pairs. Expression intensity (logFC) is represented on axis Y of the box-plots. (N = normal; CRC = colorectal adenocarcinoma)

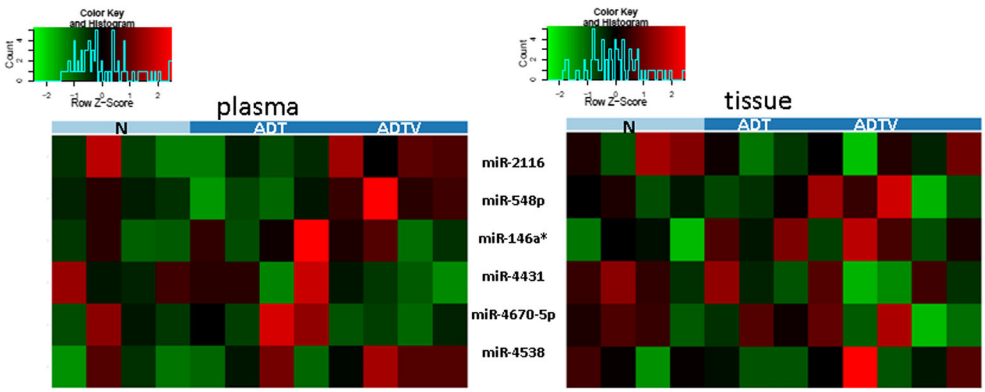

Fig. 4 MiRNA expression alteration in plasma (left) in normal vs. adenoma samples and their expression in tissue (right). (N = Normal; ADT = Adenoma tubular; ADTV = Adenoma tubulovillous)

to neoplastic samples; however, these expression levels were lower in healthy tissue pairs (Fig. 5). MiR-548d-3p was the only miRNA which showed the same expression alteration in adenoma vs. CRC comparison both in tissue and plasma (Fig. 6). Downregulated miRNAs in tubulovillous adenoma in case of plasma were also found to be underexpressed in tissue sample pairs as well (Fig. 6.).

Discussion

Circulating miRNAs have many advantageous characteristics to be used as potential biomarkers: resistance to RNase activity, long-term storage and multiple freeze-thaw cycles. Moreover, miRNA sequences are conserved across species and their circulation levels have been associated with different diseases or pathological stages [11].

In the present study, we have carried out a high-throughput screening of circulating microRNA expression alterations by microarray analysis in plasma samples from patients with two subtypes of colorectal adenoma (tubular, tubulovillous) and carcinoma, then our data were validated by real-time PCR. We found several miRNAs showing the same expression tendency in plasma using two different methods.

Remarkable downregulation of miR-519e ($p < 0.05$) was observed in CRC compared to healthy samples in plasma. In HCT116 colon carcinoma cells miR-519e was upregulated after SN38 treatment (topoisomerase I inhibitor) [12]. Tambyah et al. also characterized serum miRNA profile after a H1N1 virus infection and found that miR-519e was able to distinguish infected patients from healthy individuals [13]. Downregulation of miR-519e in CRC can refer to its tumor suppressor function which was described by Zhou et al. [14]. However, the underexpression of miR-519e found by microarray analysis could not be verified by RT-PCR in our study.

In plasma samples, we found five upregulated (miR-612, miR-1296, miR-933, miR-937 and miR-1207) miRNAs in CRC compared to normal controls both by microarray and RT-PCR methods, and these expression alterations could be observed in tissue pairs, as well. Interestingly, Sheng et al. reported the downregulation of miR-612 in CRC tissue samples compared to normal [15]. It was described in breast cancer that miR-612 inhibit phosphorylation of myosin II and cell invasion [16]. Furthermore, miR-1296 was found to be upregulated in CRC plasma samples in a previous study [17] and our results also confirmed its underexpression. Elevated concentration of miR-1296 was also observed in plasma samples of patients with cardiovascular disorders [18], however, in gastric cancer, miR-1296 could have a tumor suppressor function, as described by Shan et al.

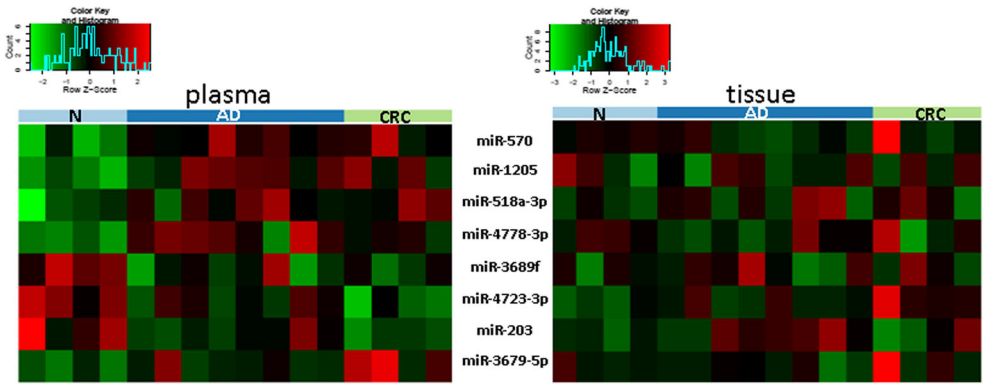

Fig. 5 MiRNA expression alteration in plasma (left) in normal vs. neoplastic lesion comparison and their expression in tissue samples (right). (N = Normal; AD = Adenoma; CRC = Colorectal adenocarcinoma)

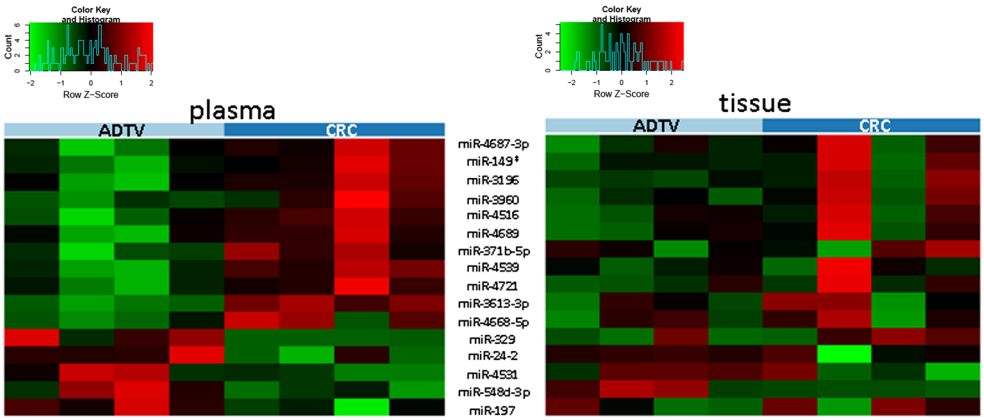

Fig. 6 MiRNA expression pattern between adenoma subtypes and normal plasma samples (left) and their expression in tissue samples (right). (ADTV = Adenoma tubulovillous; CRC = Colorectal adenocarcinoma)

[19].MiR-933 was also upregulated in CRC plasma samples, however, the expression changes of miR-933 were described only in thyroid cancers and melanoma tissues [20–22]. MiR-937 overexpressed in CRC plasma compared to normals. Zhang et al. found that it can be a valuable target for lung cancer therapy by the inhibition of INPP4B gene [23]. These last two miRNAs are not well characterized in the literature. Putative targets of miR-933 and miR-937 participate in pathways related to "Pathway in cancer" (ID: hsa05200) "Colorectal cancer" (ID: hsa05210) and signaling pathways (MAPK ID: hsa04010; ErbB ID: hsa04012) analyzed by KEGG algorithms.

According to histological classification of adenoma samples, higher levels of miR-2116 and miR-548p could be observed in tubulovillous adenoma compared to normal and tubular adenoma plasma samples. Upregulation of miR-570, miR-1205 and miR-518a-3p and downregulation of miR-3689f, miR-4723-3p and miR-203 were also found in adenoma-carcinoma transition compared to healthy controls. Li et al. described that miR-548p has a regulatory relationship with leukemia-related genes in acute myeloid leukemia [24]. MiR-570 is associated with colon and rectal tumor and beside two other miRNAs significantly reduced the hazard of dying [25]. According to the findings of Wang et al., miR-570 could regulate CD274 molecule in tissue. CD274 is usually mutated in gastrointestinal cancers and contributes in cancer development [26]. Upregulated miR-1205 was found to be a member of the PVT1-locus which is enhanced by the binding of p53 tumor suppressor [27].

Characteristic miRNA alterations were observed in plasma samples of patients with adenoma compared to CRC. Twelve of them were upregulated in CRC, while 5 of the above-mentioned miRNAs were downregulated in CRC samples. The most significant alterations could be measured in this comparison in microarray experiments; however, the expression changes could not be verified by RT-PCR. Most of the highly altered miRNAs are less abundant and there is no information about them in literature. However, miR-149* was identified as a circulating prognostic biomarker miRNA in geriatric coronary artery disease by Sayed et al. [28]. It was reported that miR-149 could be a tumor-associated miRNA inducing apoptosis by inhibiting Akt1 and E2F1 in human cancer cells [24]. High concentration of this miRNA in CRC plasma could be the clue of tumor suppressor activity in tissues.

Between normal vs. cancerous patients, the low number of miRNAs with correlating expression alterations detected by different methods can be explained with the low amount of input miRNA extractable from plasma fraction. Chen et al. also observed higher variation in expression of miRNAs between microarray and RT-PCR methods [29]. We also visualized on heatmap those miRNAs showing altered expression in plasma samples between diagnostic groups. The results did not support our original hypothesis that the expression of circulating miRNAs correlates with their alterations in tissue pairs, as similar expression tendencies were not found in plasma and tissue samples. However, some exceptions, such as miR-187, miR-675 and miR-3591-3p which were upregulated in CRC compared to normal samples both in tissue and in plasma. MiR-548d-3p was the only miRNA which showed the same expression alteration in adenoma vs. CRC comparison both in tissue and in plasma. Downregulation of miR-187 in CRC tissue was confirmed by Wang et al., and they revealed that it suppressed CRC cell proliferation and decreased miR-187 expression was closely associated with shorter overall survival [30]. MiR-675 was found to be upregulated in colorectal cancer tissue samples as well [31]. In the case of miR-3591 there was no information about any alteration in the literature. MiR-548d-3p has an influence in progression of breast cancer [32]. Similarly to the findings of Larrea et al., our results also can refer to that circulating miRNA are not exclusively derived from tumor specific tissues [33].

Our results demonstrated that the miRNA expression patterns between plasma and adenomatous and/or cancer tissue samples are only partly overlapping. It follows that cancer-

associated miRNAs in the circulatory system may originate from other types of tissues such as the immune cells or from other metastatic regions far from the primer tumor [33, 34]. Dysregulation of circulating miRNAs may be an important link between defense mechanisms and cancer [11, 35]. Working with plasma samples could be difficult to find the right correlation between different patient cohorts.

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Compliance with Ethical Standards All authors read and approved the final manuscript.

Conflict of Interest The Authors declare that there is no conflict of interest.

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