Analysis of miRNA alterations in the development of colorectal cancer

Ph.D. thesis

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1. INTRODUCTION

Colorectal cancer is the third most common cancer type and the fourth leading cause of cancer-related death globally. Each year, 1.2 million new cases and 600 000 deaths of colorectal cancer have been identified. It is also a serious public health problem in Hungary registering 9000 new cases and 5000 deaths each year. Adenomas are the most common neoplastic lesions located in the large intestine, however, only few epidemiological data are available in the literature. Molecular pathogenesis of colorectal cancer is very heterogeneous. Neoplastic lesions that evolving in the normal colonic mucosal cells may cause by genetic and epigenetic alterations such as hypermethylation in the promoter region of selected genes. All these processes can influence the steps of carcinogenesis. Genetic instability increases in parallel with growing number of mutations through development.

Several epigenetic processes are involved in the development of colorectal cancer. A new epigenetic regulatory element become known in the family of non-coding RNAs due to the discovery of miRNAs.

MicroRNAs are short (19-24 nucleotide-long), highly-conserved, single-stranded RNA molecules, which can also regulate tumor progression. Posttranscriptional gene silencing as the regulatory mechanisms of miRNAs can occur via two different ways: through target mRNA degradation and translational repression. MiRNAs have an effect on tumor development and progression, and several types of cancer are characterized by altered expression of miRNAs.

They can act as oncogene (oncomiR) or tumorsuppressor thus regulate gene expression involved in tumor progression. Tumor-specific miRNAs have been detected in whole blood, serum, plasma and other bodyfluids due to their stable form and the resistance to RNase enzymes. According to recent hypotheses circulating miRNAs can be released into the bloodstream passively originating from tissue damages. Plasma- or serum-derived miRNAs may be useful in diagnostic fields as liquid biopsy markers, by the use of this technique, it is possible to eliminate direct sampling from tumor tissues due to the application of circulating miRNAs, thereby expression patterns of cell-free nucleic acids are traceable. MiRNA content in body fluids serve information about emitting cells or the origin of recipient cells or its heterogeneity and level of malignancy.

2. AIMS

The aims of my Ph.D. work were summarized in the following points:

- Identifying miRNA expression pattern in colorectal cancer and in tubular andtubulovillous adenomas compared to healthy colon tissues by using global microarray technology;
- Mapping miRNA-mRNA interactions by *in silico* prediction methods, then confirmation of the results with mRNA expression analysis from the same tissue sample sets;
- Immunohistochemistry measurements of a selected protein to validate the hypothetical miRNA-mediated mRNA degradation;
- Identifying the miRNA expression changes in plasma by microarray, then validating the results with real-time PCR methods;
- To examine whether the expression patterns of circulating miRNAs in plasma samples are correlating with the miRNA expression levels of matched colon tissues in the different patient groups;
- Comparing the efficiency of commercially available miRNA and total RNA isolation methods on FFPE colon tissue samples by specifying the quality and quantity features.

3. MATERIALS AND METHODS

During our experiments, we studied colonic biopsies obtained during routine colonoscopy examinations and matched plasma samples. Formalin-fixed, paraffin-embedded (FFPE) surgically removed tissues were analyzed for the comparison experiments of isolation methods.

3.1. Identifying miRNA expression profiles in colorectal cancer and adenoma tissue samples

3.1.1. miRNA expression analysis performed by GeneChip miRNA 3.0 array

MiRNA microarray measurements were performed on fresh frozen colonic biopsy samples from 20 colorectal cancer, 20 normal colon, 11 tubular and 9 tubulovillous adenoma tissues. Total RNA including miRNA fraction was isolated from biopsy samples using High Pure miRNA Isolation Kit. RNA yield was quantified with Qubit 1.0 fluorometer (ThermoFisherScientific, USA), RNA integrity was evaluated using 2100 Bioanalyzer (Agilent, Technologies, USA). The miRNA expression profiles were examined by GeneChip miRNA 3.0 arrays (Affymetrix, USA). Probe cell intensity files of microarrays were analyzed with Expression Console Software (Affymetrix). GeneChip miRNA 3.0 array contains probe sets for 1733 mature miRNAs. Values of $p<0.05$ were considered as statistically significant with a $\log FC > |1|$.

3.1.2. Real-time quantitative PCR array analysis from fresh frozen tissue specimens

RT-PCR validation was done on four pooled tissue samples (with equal quantity (ng) of RNA from each samples in each group) according to the analyzed diagnostic groups (normal, CRC, tubular adenoma, and tubulovillous adenoma). 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 onLightCycler 480 System (Life Science Roche).

3.1.3. mRNA expression profiling by Human Transcriptome Array 2.0

Twenty colon biopsies (7 normals, 2 tubular and 4 tubulovillous adenomas, and 7 tumors) were selected for mRNA expression analysis using a GeneChip Human Transcriptome Array 2.0 (Affymetrix) according to the manufacturer's instructions.

3.1.4. *In silico miRNA-mRNA target prediction*

Three miRNAs were selected: miR-31 has the highest fold change in normal vs. adenoma and normal vs. CRC comparison; miR-4417 and miR-497 were one of the continuously upregulated or downregulated miRNAs in adenoma-carcinoma transition. Their mRNA targets were predicted using five algorithms: TargetScan, miRanda, PICTAR2, RNAHybrid and miRWalk on miRWalk 2.0 platform. Using DAVID tools (The Database for Annotation, Visualization and Integrated Discovery v6.7) we acquired pathway enrichment from gene ontology.

3.1.5. Immunohistochemistry of cyclin D1

Immunohistochemistry analysis for cyclin D1 was performed on formalin-fixed and paraffin-embedded tissue samples from normal (n = 15) adenoma (n=15) and CRC (n = 10) patients, thus the expression changes in protein level could be comparable with miRNA and mRNA expression results.

3.2. Analysis of miRNA expression profiles in matched plasma samples

3.2.1. MiRNA microarray expression profiling by GeneChip miRNA 3.0 Array

Our experiments were performed on plasma samples from healthy (n=4), tubular adenoma (n=4), tubulovillous adenoma (n=4) and colorectal cancer patients. The miRNA fraction was extracted with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany) using a modified protocol. MiRNA expression profiles were studied using GeneChip miRNA 3.0 arrays. RNA yield was quantified with RNA High Sensitivity Assay Kit on Qubit 1.0 fluorometer (ThermoFischer Scientific). Probe cell intensity files of microarrays were analyzed by Expression Console Software (Affymetrix).

3.2.3. Real-Time Quantitative PCR Array Analysis

PCR was performed by miRCURY™ Universal RT microRNA PCR (Exiqon, Denmark) methods. In the case of plasma samples, SNORD49A was used for normalization. The qPCR reactions were run on LC480 thermocycler (Roche Applied Science).

3.3. Comparison of the RNA recovery efficiency in the case of miRNA and mRNA isolation protocols

3.3.1. RNA extraction with different isolation protocols

RNA purification methods were performed with four different methods according to the manufacturers' instructions. MiRCURY™ RNA Isolation Kit and High Pure miRNA Isolation Kit can separate small RNAs, especially miRNAs. The High Pure RNA Paraffin Kit (Roche, Germany) and MagNA Pure 96 Cellular RNA LV Kit (Roche, Germany) are able to isolate total RNA fraction.

3.3.2. Quantity and quality check of purified RNA molecules

The concentration of RNA fraction was quantified with Qubit® 1.0 fluorometer using Qubit High Sensitivity RNA Assay according to the manufacturer's instructions. RNA integrity was assessed with 2100 BioAnalyzer (Agilent Technologies, USA) microcapillary electrophoresis system using the Agilent RNA Pico 6000 kit according to the manufacturer's instructions.

3.3.3. Real-Time PCR analysis

The miRCURY™ Universal RT microRNA PCR (Exiqon, Denmark) protocol is a two-part protocol consisting of a first-strand cDNA synthesis, followed by a real-time PCR amplification. In the reverse transcription reactions, 40 ng isolated RNA samples were used.

3.3.4. Reference miRNAs

According to preliminary knowledge, relative expression of interesting miRNAs is influenced by the applied reference miRNA. For relative quantification of gene expression, U6 small nuclear RNA (U6), small nuclear RNA 49A (SNORD49A), small nuclear RNA 38 (SNORD38) and miRNA-490-3p (Hsa-miR-490-3p) reference RNA were applied as endogenous controls to compare the delta Cp values between normal and tumor groups.

4. RESULTS

4.1. Microarray-based identification of expressed miRNAs in colorectal tissue samples

Along the colorectal adenoma-carcinoma transition, based on our microarray profiling 19 continuously upregulated or downregulated miRNAs were selected. Expression elevation could be observed in the case of miR-4417, which was upregulated 2.8-fold in adenoma compared to normal samples, moreover, the expression level was further elevated (1.9-fold) in CRC samples. Eight (without miR-378 variants) downregulated miRNAs showed approximately 29-60% lower expression in adenomas compared to healthy controls, and reduction was 34-66,8% lower in carcinoma samples. Twenty-three miRNAs (11 downregulated and 12 upregulated) were significantly differentially expressed in healthy normal colonic tissue vs. precancerous and neoplastic lesions. The highest miRNA expression alteration was observed in the case of miR-31 showing eight-fold higher expression both in adenoma and in CRC tissues compared to normal samples. 24 miRNAs showed characteristic differences between adenoma and colorectal cancer samples. Only five miRNAs were upregulated in CRC compared to adenoma.

MiR-489 expression showed the greatest difference between adenoma subtypes with a > 4.5-fold increase in tubulovillous adenoma samples. Colorectal cancerous samples were investigated based on Dukes stages, the majority of miRNAs showed overexpression in Dukes D stage samples. Most of the differentially expressed miRNAs in the adenoma-CRC comparison were upregulated in adenomas compared to CRC samples. Therefore, we focused on these adenoma-specific miRNA groups and selected those ones which are upregulated in adenomas but after significantly downregulated in CRC patients. Expression levels of 4 miRNAs (miR-182, miR-183*, miR-96, and miR-34a) showing the highest overexpression in adenoma samples are decreased 29% or higher in carcinoma tissues compared to adenoma samples. In order to confirm the detected miRNA alterations between patient groups performed by microarray, real-time PCR analysis was done.

4.1.1. *In silico* target mRNA prediction and validation on Human Transcriptome Array 2.0

Three miRNAs were selected: miR-31 has the highest fold change in normal vs. adenoma, normal vs. CRC comparison; miR-4417 and miR-497 were one of the continually upregulated or downregulated miRNAs in adenoma-carcinoma transition. mRNA targets were predicted on miRWalk 2.0 platform *in silico*. Based on the Human Transcriptome Array 2.0 mRNA expression results, downregulated mRNA targets were selected and inverse expression patterns were visualized. Based on the pathway analyses, the predicted target genes participate in pathways related to cancer progression in 10% moreover, transduction pathways like PI3K-Akt and MAP Kinase are affected. GO analysis was also conducted on these miRNAs revealing transcription regulations and cell proliferation.

4.1.2. Immunohistochemistry of cyclin D1

A predicted target mRNA of miR-497 was selected and the suspected miRNA-mRNA functional connection was investigated in protein level. The stromal protein expression was low, but significantly ($p<0.05$) increased in adenomas. Heterogenic, significantly ($p<0.05$) increased nuclear cyclin D1 expression was detected in epithelial compartment of adenomas and CRCs. Significant ($p<0.05$) protein level alteration of cyclin D1 was detected in stroma and epithelial cells along normal-adenoma and adenoma-CRC transition.

4.2. Microarray results of matched tissue and plasma samples

MiRNA microarray analysis was performed on plasma samples from patients with tubular adenoma or tubulovillous adenoma or colorectal cancer. 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. Results were compared with the numbers of expressed miRNA in matched tissue biopsy samples. We found that 350-500 miRNAs had positive values in colon tissues independently from patient groups, and in plasma samples 150-450 miRNAs were detected. 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. In miRNA microarray experiments, 14 miRNAs showed significantly altered expression between CRC and normal plasma samples. The majority of the significant changes were

detected in the above-mentioned comparison. In tubular adenoma vs. normal comparison, no significant expression alterations could be measured, in contrast, two miRNAs (miR-2116, miR-548p) were found to be upregulated in the 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 ($p<0.05$). MiR-4723-3p, miR-203, and miR-3689f were downregulated during CRC progression. Sixteen miRNAs discriminating tubulovillous adenoma from CRC were also determined from which 11 showed significantly higher expression in CRC samples.

4.2.1. MiRNA expression validation with Real-Time PCR Panel in plasma samples

In order to confirm the microarray data, miRCURY Human Panel real-time PCR (Exiqon) was applied using the same plasma samples. From the 14 significantly altered miRNAs in N vs. CRC comparison detected by microarray analysis, only miR-187, miR-612, miR- 1296, miR-933m, 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.

4.2.2. 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. In normal vs. CRC comparison, miRNAs had the same expression tendency in the 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. In normal vs. adenoma comparison, similar expression tendencies were not observed in tissue sample pairs. MiR-3689f, miR-4723-3p, and miR-203 were highly expressed in normal plasma compared to neoplastic samples; however, these expression levels were lower in healthy tissue pairs. MiR-548d-3p was the only miRNA which showed the same expression alteration in adenoma vs. CRC comparison both in tissue and plasma.

4.3. RNA quantity and integrity of the different isolation methods on FFPE samples

FFPE samples are easier to handle in the clinic, thus miRNAs isolated from FFPE samples were also investigated. The four isolation methods resulted different RNA yields. The recovery of miRNA isolation methods was represented in lower range compared to total RNA isolation procedures. As it was expected, the miRNA isolation methods (miRCURY™ RNA Isolation Kit, High Pure miRNA Isolation Kit) resulted in short nucleotide length RNA molecules between 20 and 200 nt. Electropherograms of total RNAs showed that the samples contains short, degraded fragments.

4.3.1. Mirna profiling on Exiqon PCR Panels from the RNA samples purified by different isolation methods

Our results confirmed that more miRNAs were expressed on Panel I and in the case of all isolation methods, the number of expressed miRNAs on Panel II were found to be lower. The number of expressed genes was higher in the case of the samples which were purified by miRNA isolation methods compared to samples were purified by total RNA isolation protocols. Considering the detected number of miRNAs in each samples, the Roche isolation method showed the best results from the miRNA isolation protocols. Twice as much miRNAs gave sign altogether in healthy and tumor samples in the case of short RNA isolation protocols compared to samples isolated with total RNA purification methods. In general, higher Cp values were shown in the case of total RNA isolation protocols that possibly indicate the lower concentration of miRNAs in total RNA extracted samples.

4.3.2. Comparative expression analysis of selected colon specific miRNAs

For relative quantification of miRNA expression, reference short RNAs (U6, SNORD38B, SNORD49A) and a selected miR-490-3p were chosen for comparative analysis. To investigate the question, whether the selection of different miRNA for normalization steps have an influence on the miRNA expression between clinical groups, two individual colorectal cancer-specific miRNAs (miR-21 és miR-34) were selected based on the literature references to evaluate the expression differences resulting from purification variations and housekeeping reference genes. The raw Cp values of miRNAs isolated with miRNA purification protocols were significantly lower, thus showed stronger signals than Cp values of miRNAs conducted by total RNA isolation methods. On the other hand, ΔΔCp differences between clinical groups were altered among the different normalization methods. On the basis of normalization with

miR-490-3p, miR-21 and miR-34 were upregulated in tumor samples after all isolation methods. These tendencies were less pronounced if normalization was performed with SNORD38B.

5. CONCLUSIONS

Most of the miRNA profiling studies aim to compare only miRNA expression patterns between normal and cancerous samples. In my PhD work, a large number of samples from adenoma patient groups were analysed beside CRCs, furthermore, miRNA expression alterations were identified in patient groups confirmed with different techniques. On the basis of my experiments, adenoma-specific miRNAs were identified, which showed the highest expression level in this patient group. My results showed that expression level of miRNAs is more elevated in adenoma compared to CRC samples. A group of miRNAs were identified which can discriminate between the adenoma subgroups (tubular and tubulovillous).

In order to examine the biological function of miR-497, protein experiments were conducted, thereby the posttranscriptional inhibition of the predicted target, Cyclin D1 protein was proved with immunohistochemistry methods. According to the observation of plasma samples, miRNA expression alterations were described between the analysed patient groups. Expression of miR-187, miR-612, miR-1296, miR-933, miR-937, miR-1207 and miR-146a was correlated in tissue and plasma samples in case of normal vs. CRC.

Moreover, in my PhD work different miRNA and total RNA isolation methods were compared on formalin-fixed, paraffin embedded colon tissues. Based on my results, the miRNA content in samples were isolated with total RNA isolation protocols were lower compared to the short RNA isolation methods. The frequently recommended reference miRNAs showed high standard deviations between patient groups in our experiments, therefore, miR-497 was applied as a candidate reference miRNA which showed the lowest standard deviation between healthy and CRC samples in the present study.

During the analysis of patient groups, it is an important aspect to apply the optimal reference miRNA, since the miRNA expression is highly influenced by the normalisation steps.

6. PUBLICATIONS

6.1. Publications related to the Ph.D. dissertation

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