Gender-Dependent Expression of Leading and Passenger Strand of miR-21 and miR-16 in Human Colorectal Cancer and Adjacent Colonic Tissues

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Summary

miRNAs are small regulatory RNA molecules involved in post-transcriptional gene silencing. Their biosynthesis results in the formation of duplex consisting of a leading and a passenger strand of mature miRNA. The leading strand exhibits the main activity but recent findings indicate a certain role of the passenger strand as well. Deregulated levels of miRNA were found in many types of cancers including colorectal cancer. miR-21 and miR-16 were indicated as possible markers of colorectal cancer, however, small attention to gender differences in their expression was paid so far. Therefore, the aim of our study was to investigate the expression of miR-21-5p, miR-21-3p, miR-16-5p and miR-16-3p in human colorectal cancer tissue and compare it to the adjacent tissues taken during surgery in men and women separately. Our results showed an up-regulation of all measured miRNAs in tumor tissue compared to adjacent tissues. As expected, tumors and adjacent tissues exhibited a significantly higher expression of leading miRNAs compared to passenger strand of miR-21 and miR-16. The expression of leading and passenger strand of miR-21 and miR-16 positively correlated exhibiting the highest correlation coefficient in the distal tissue. The expression pattern showed gender-dependent differences, with higher levels of miRNA in men than in women. Our findings indicate a gender-related expression pattern of miRNA, which should be considered as an important factor in generating new prognostic or diagnostic biomarkers.

Key words

Carcinoma • miR-21-5p • miR-21-3p • miR-16-5p • miR-16-3p • Biomarker

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Introduction

MicroRNAs (miRNAs) are short (17-25 nucleotides), single-stranded RNA molecules that belong to a family of small non-coding regulatory RNAs. Their function relies on translation repression or degradation of target mRNA sequences. Specific regulation is based on high complementarity between 2-8 nucleotides called seed sequence and 3´ untranslated region (3´UTR) of target genes. According to genome analysis of 3´UTRs, it has been estimated that more than 60 % of all human protein coding genes are regulated by miRNAs (Friedman *et al.* 2009, Voglova *et al*. 2016).

Biogenesis of miRNA is a complex process that includes the processing of primary miRNA in the nucleus by RNase Drosha into precursor miRNA (pre-miRNA) and subsequent transport into the cytoplasm where pre-miRNA is cleaved by another RNase Dicer and forms a mature duplex (Treiber *et al*. 2012). After maturation, it is necessary to unwind the miRNA duplex and separate each strand. Strand that exerts higher levels is called a leading or guide strand. The fate of the other strand, called the passenger strand, is not fully understood. In contrast to siRNAs, which typically undergo

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an immediate cleavage of the passenger strand in order to liberate the leading strand (Matranga *et al.* 2005), miRNA duplexes at least partially omit this step and the co-existence and functionality of both miRNA strands has been reported frequently (Choo *et al.* 2014). Some authors suggest a tissue-dependent mechanism of strand selection. While in some tissues a passenger strand is subjected to degradation, in others it can be accumulated and biologically active (Ro *et al*. 2007).

miRNAs have the capacity to modulate many cellular pathways, including cell proliferation, differentiation and survival or apoptosis, which are frequently deregulated in the tumor cells. In fact, changes in miRNA levels were reported in a variety of human cancers, including colorectal cancer (CRC) (Bandrés *et al.* 2006, Slaby *et al.* 2007).

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, with an increasing level of incidence and mortality mainly in middle-East Europe and South America (Torre *et al.* 2015). The process of CRC development consists of a multistep pathway that involves the deregulation of many oncogenes and tumor suppressors including miRNAs (Vogelstein *et al.* 1988, Nagel *et al.* 2008, Johnson *et al.* 2005).

Changes in miR-21-5p expression are clearly associated with CRC and other types of cancers. Several groups reported up-regulated miR-21-5p expression in CRC tumor tissue or in cancer cells. The high expression of miR-21-5p correlates with the clinical stage, lymph node metastasis and distant metastasis presence (Faltejskova *et al.* 2012, Dong *et al.* 2014). miR-21 was confirmed to target several tumor suppressor genes in CRC cells, including *pdcd4* (Asangani *et al*. 2008), *tiam1* (Cottonham *et al*. 2010) or *spry* (Sayed *et al.* 2008). Post-transcriptional suppression of these genes induces cell proliferation and migration, invasion activity and the forming of metastasis.

miR-16 was suggested by some authors as a miRNA suitable for the normalization of miRNA measurement (Peltier and Latham 2008, Song *et al.* 2012, Zhu *et al.* 2012), but recent evidences indicate its role in tumorigenesis. miR-16 was identified as a negative regulator of multiple oncogenes e.g. *k-ras* in colorectal cancer (You *et al.* 2016), oncogene *Wip1* in mammary cells (Zhang *et al.* 2010) and apoptosis suppressor *FEAT* in a variety of cancer cells (Liang *et al.* 2015). miR-16 was implicated as a possible marker of the colorectal cancer (Diamantopoulos *et al.* 2017).

Despite the existence of many studies that focus on the expression and role of the leading strand of miR-21 and miR-16 in CRC, little is known about the expression pattern of their passenger strands. Moreover, small attention to gender differences in miR-21 and miR-16 expression was paid so far. Therefore, the aim of our study was to investigate the expression of leading and passenger strand of miR-21 and miR-16 in human colorectal cancer tissue and compare it to the healthy adjacent tissues in male and female patients separately.

Methods

The study includes 53 patients of both genders undergoing surgery for CRC (27 males and 26 females, average age 68 ranging from 47-86 years). All patients were exposed to a standard hospital practice with lights on from 6:00 a.m. to 9:00 p.m. (The First Surgery Department, University Hospital, Comenius University, Bratislava). The experimental protocol was explained to each patient and informed consent was obtained. The experimental protocol was approved by the Ethics Committee. Histopathological examinations were performed by a hospital pathologist. Tissue samples taken during the surgery were collected from the tumor as well as from the proximal (≥10 cm above the tumor) and distal (≥2 cm under the tumor) parts of the resected colon. The surgery was conducted between 10:00 a.m. and 1:00 p.m. Tissue samples were collected into liquid nitrogen and then stored at -80 °C until further processing. Details about the characteristics of the patients included in the study are listed in Table 1.

Mature miRNA was extracted from the tissue samples using RNAzol (MRC, USA) according to the manufacturer's instructions. cDNA was synthesized with miScript II RT (Qiagen, Germany). Detection of relative miRNA expression was performed by real-time PCR with the MiniScript SYBR Green PCR Kit (Qiagen, Germany) and the StepOnePlus TM Real-Time PCR Systems (Applied Biosystems, USA) using universal primer miScript Universal Primer (Qiagen, Germany) and specific primers with the following sequences: miR-21-5p 5′-TAGCTTATCAGACTGATGTTGA-3′, miR-21-3p 5′-CAACACCAGTCGATGGGCTGT-3′, miR-16-5p 5′-TAGCAGCACGTAAATATTGGCG-3′, miR-16-3p 5′-CCAGTATTAACTGTGCTGCTGA-3′. The 20 µl PCR reaction mixture included 0.06 µl of RT product. Reactions were incubated at 95 °C for 15 min, followed by 35-45 cycles: 94 °C for 15 s, 55 °C for 30 s

and 70 °C for 30 s. The specificity of PCR reaction was validated by melting curve analysis. The expression of *U6* was used as a normalization control of gene expression, with the following sequences: sense 5′-GCTTCGGCAGCACATATACTAA-3′, antisense 5′-AAAATATGGAACGCTTCACGA-3′. Ct (threshold value) at 35 cycles was set as assay sensitivity. Relative standard curve method was used to calculate relative miRNA concentration. Calibration curve was prepared from the sample with the high concentration of measured miRNA.

Table 1. Association of miRNAs expression with clinicopathological features, age and gender of patients with colorectal cancer.

	Number (%)	**miR-21-5p**		**miR-21-3p**		**miR-16-5p**		**miR-16-3p**	
		Mean ± SEM	P	Mean ± SEM	P	Mean ± SEM	P	Mean ± SEM	P
Gender									
Male	27 (51)	39.06 ± 11.13	**0.024**	37.42 ± 19.58	**0.004**	28.88 ± 13.74	**0.067**	29.01 ± 16.32	**0.041**
Female	26 (49)	9.66 ± 2.31		5.11 ± 2.61		6.77 ± 2.22		7.54 ± 3.21	
Histological grade									
G<2	11 (21)	40.74 ± 21.63	ns	58.62 ± 43.15	ns	39.82 ± 30.44	ns	53.73 ± 41.95	ns
G≥2	42 (79)	19.95 ± 5.25		10.73 ± 3.91		12.30 ± 4.44		10.01 ± 2.62	
Clinical stage									
I-II	26 (49)	33.81 ± 11.34	ns	35.24 ± 19.68	ns	26.79 ± 14.13	ns	30.15 ± 18.45	ns
III-IV	27 (51)	15.12 ± 4.41		7.29 ± 2.91		9.56 ± 3.52		9.01 ± 3.04	
T (tumor invasion)									
T1-T2	4 (8)	18.06 ± 6.51	ns	12.06 ± 6.64	ns	15.81 ± 8.36	ns	16.07 ± 9.03	ns
T3-T4	49 (92)	24.78 ± 6.51		22.07 ± 10.91		18.19 ± 7.76		19.19 ± 9.61	
N (nodal status)									
N0	27 (51)	32.55 ± 11.13	ns	33.89 ± 18.95	ns	25.80 ± 13.64	ns	28.90 ± 17.71	ns
N1-2	26 (49)	15.75 ± 4.41		7.59 ± 3.02		9.93 ± 3.64		9.37 ± 3.14	
M (distant metastasis)									
M0	40 (75)	25.83 ± 7.77	ns	24.44 ± 13.10	ns	20.32 ± 9.31	ns	21.41 ± 11.59	ns
M1	13 (25)	19.53 ± 8.19		11.23 ± 5.70		10.92 ± 6.33		11.32 ± 5.47	
Tumor localization									
C18 (colon)	31 (59)	23.10 ± 8.40		25.52 ± 15.52		25.06 ± 12.06		25.97 ± 15.18	
C19 (rectosigmoid junction)	7 (13)	22.47 ± 12.18	ns	6.26 ± 3.91	ns	6.70 ± 3.40	ns	6.02 ± 3.27	ns
C20 (rectum)	15 (28)	27.93 ± 12.18		18.07 ± 11.48		8.72 ± 3.71		11.32 ± 5.68	
Age									
≤68	26 (49)	15.12 ± 5.04	**0.040**	7.14 ± 3.06	ns	8.18 ± 3.58	**0.025**	7.41 ± 2.62	ns
>68	27 (51)	33.18 ± 10.71		33.30 ± 18.25		27.48 ± 13.57		29.13 ± 16.38	

miRNAs expression is given in arbitrary units. Statistical evaluation was performed by Mann-Whitney *U*-test in all cases except of expression related to tumor localization that was evaluated by Kruskal–Wallis analysis; P – statistical difference between groups, ns – non significant.

Statistical analysis

The Kruskal-Wallis analysis of variance and *post hoc* test – Dunn's multiple comparisons test – were used for evaluation of differences between tumor, proximal and distal tissues. Mann-Whitney *U*-test was used for the determination of gender differences in miRNA expression. Linear regression analysis was used to determine the correlation between leading and passenger strands of miR-21 and miR-16.

Results

Summary of relationships between expression of studied miRNAs in colorectal cancer tissue and clinicopathological features, age and gender of patients is given in Table 1. Expression of leading strand miR-21 and miR-16 was significantly higher in older patients with the age above 68 compared to younger patients (P<0.05, Mann-Whitney *U*-test, Table 1).

Expression of leading strand of miR-21 and miR-16 was several thousand times higher in comparison with expression of passenger strand. Ct values of samples measured with the same dilution were as follows: tumor – miR-21-5p Ct 16±0.6, miR-21-3p Ct 29±0.4; proximal tissue – miR-21-5p Ct 23±0.5, miR-21-3p Ct 33±0.5; distal tissue – miR-21-5p Ct 22±0.6, miR-21-3p Ct 32±0.4 and tumor – miR-16-5p Ct 21±0.5, miR-16-3p Ct 30±0.4; proximal tissue – miR-16-5p Ct 25±0.4, miR-16-3p Ct 34±0.5; distal tissue – miR-16-5p Ct 25±0.5, miR-16-3p Ct 34±0.4.

Expression of miR-21-5p, miR-21-3p, miR-16-5p and miR-16-3p showed significant up-regulated levels in tumor tissue compared to both adjacent proximal and distal tissues (P<0.001, Kruskal-Wallis, Fig. 1A).

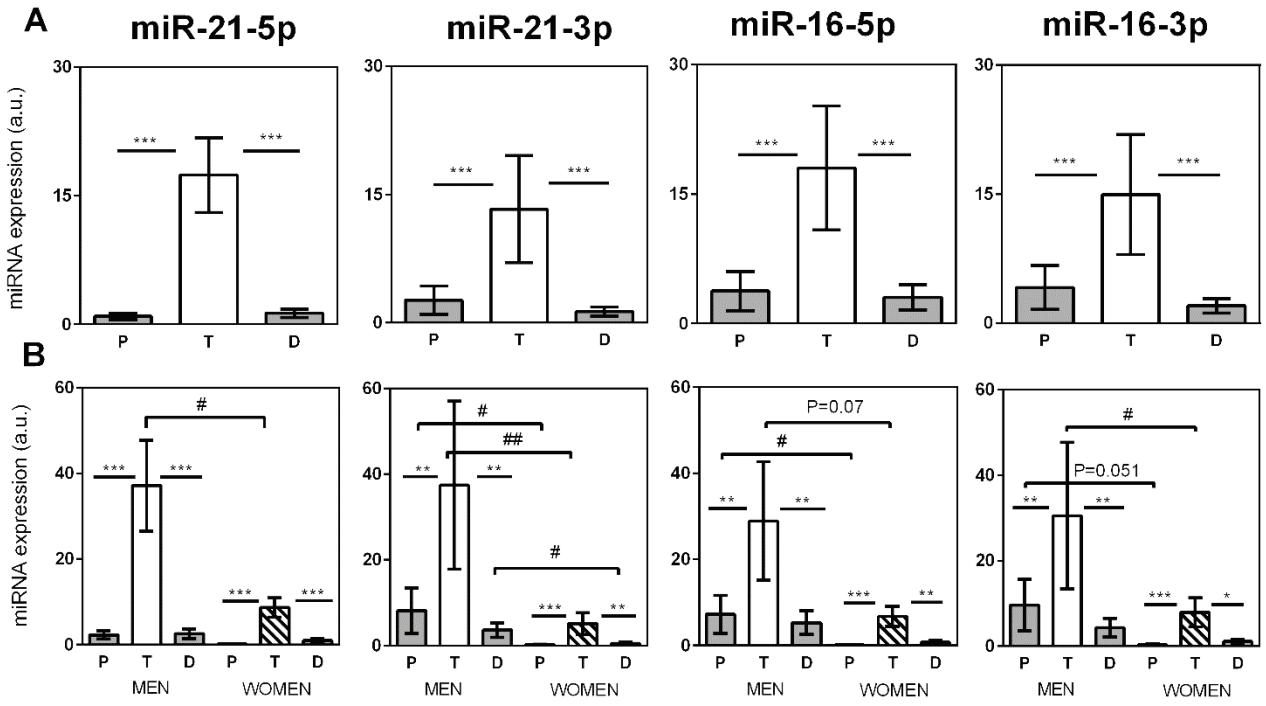

Fig. 1. Expression of leading and passenger strands of miR-21 and miR-16 in tumor and adjacent proximal and distal tissues of all patients (**A**). Gender-dependent differences in miRNAs expression in tumor and adjacent tissues (**B**). Values are presented as arithmetic means ± SEM. P – proximal tissue, T – tumor tissue, D – distal tissue, * P<0.05, ** P<0.01, *** P<0.001 Kruskal-Wallis analysis; # P<0.05, ## P<0.01, Mann-Whitney *U*-test; a.u. – arbitrary units.

After dividing patients according to the gender, differences in miR-21-5p, miR-21-3p, miR-16-5p and miR-16-3p expression between men and women in tumor and adjacent tissue were observed as well (Kruskal-Wallis, Fig. 1B). Men showed a significantly higher expression of miR-21-5p, miR-21-3p and miR-16-3p in tumor tissue than women (P<0.05, Mann-Whitney *U*-test, Fig. 1B). The same pattern in miR-21-3p and miR-16-5p expression was preserved in proximal non-tumor tissue with significant up-regulation in men compared to women (P<0.05, Mann-Whitney *U*-test, Fig. 1B). In the distal tissue a significant difference between men and

women was observed in miR-21-3p expression (P<0.05, Mann-Whitney *U*-test, Fig. 1B).

Expression of leading and passenger strand of miR-21 and miR-16 positively correlated in all analyzed tissues. Correlation equations and coefficients of leading and passenger strand of miR-21 were as follows: in tumor y=57.09x-345.32 (R=0.755, P<0.0001), in the distal tissue y=57.804x-9.246 (R=0.836, P<0.0001) and in the proximal tissue y=119.54x+5.4517 (R=0.613, P<0.0001). Correlation equations and coefficients of leading and passenger strand of miR-16 were as follows: in tumor y=0.4876x-2.5658 (R=0.893, P<0.0001), in the distal tissue y=0.3716x+1.2948 (R=0.950, P<0.0001) and in the proximal tissue y=0.6258x-0.695 (R=0.933, P<0.0001; in all cases linear regression analysis was used, data are not shown). Expression of miR-21 and miR-16 leading and passenger strand showed the highest correlation in the distal tissue.

Discussion

Our study clearly demonstrated gender-dependent differences in miR-21-5p and miR-16-5p expression. There is a strong implication that miR-21-5p and miR-16-5p are expressed more in men compared to women. The study was also focused on differences in the leading and passenger strand of miR-21 and miR-16 that also showed gender-dependent regulation of expression.

We confirmed previous results describing significantly up-regulated expression of miR-21-5p in CRC human tissue samples (Slaby *et al.* 2007, Dong *et al.* 2014). *In situ* hybridization assay on formalin-fixed paraffin embedded tissue samples revealed that miR-21 is predominantly expressed in stromal parts of colorectal cancer tumours. High expression of miR-21 in stage II colon cancer patient group correlated with shorter disease-free survival and miR-21 expression was found higher in male than in female patients (Nielsen *et al*. 2011). This finding is supported by the present study.

Expression analysis of the passenger strand miR-21-3p in CRC tissue and adjacent tissues revealed high correlation with the leading strand miR-21-5p despite lower levels of miR-21-3p expression compared to miR-21-5p in all analyzed tissues. Our results are in agreement with studies that identified co-expression and cross-targeting of several miRNAs 5p and 3p strands, including miR-21 in colon cancer cells (Choo *et al.* 2014). Passenger strand miR-21-3p has been studied mainly in ovarian cancer cells. miR-21-3p was shown to mediate processes leading to ovarian cancer resistance to chemotherapeutics. High levels of miR-21-3p increase cisplatin resistance by targeting NAV3 (Pink *et al.* 2015). Higher expression of miR-21-5p compared to miR-21-3p was identified on a panel of cancer cell lines and inhibition of miR-21-5p or miR-21-3p resulted in a significant decrease in ovarian and prostate cancer cell proliferation and invasion (Baéz-Vega *et al.* 2016). Overexpression of miR-21-3p but not leading strand miR-21-5p activates, by an unknown mechanism, the transcription of L1CAM expression in renal-, endometrial- and ovarian carcinoma-derived cell lines. L1CAM is a molecule that promotes cell motility, invasion and metastasis formation and it is frequently expressed in different types of human cancers and is associated with poor prognosis (Doberstein *et al.* 2014). Our findings indicate gender dependent regulation of miR-21-3p expression that was not studied previously.

We determined up-regulated levels of both strands of miR-16 in CRC tissue when comparing with healthy adjacent tissues. Similar results were observed by Diamantopoulos *et al.* (2017) who reported a significant up-regulation of miR-16 in CRC tissue compared to adjacent tissue in a study involving 182 patients. A higher expression of miR-16 was associated with a worse survival rate of patients. On the other hand, there are results reporting down-regulation of miR-16 in CRC tissue compared to the adjacent tissue (Qian *et al.* 2013, Xiao *et al.* 2014). Down-regulated levels of miR-16 in CRC tissue were associated with advanced TNM stage and a poor histological grading stage. Moreover, low levels of miR-16 together with miR-15a better correlate with survival rates than those of miR-15a or miR-16 expression alone (Xiao *et al.* 2014).

There is also uncertainty in studies describing miR-16 expression in other types of cancers. Expression of miR-16 in ovarian cancer tissue was found to be down-regulated (Bhattacharya *et al.* 2009). On the other hand, up-regulated expression of miR-16 in ovarian cancer compared to non-cancerous tissues has been reported. Expression of miR-16 significantly positively correlated with CDC25C, a critical molecule that triggers entry to mitosis (Miles *et al.* 2012). The serum level of miR-16 in ovarian patients and control patients were similar and did not show any difference (Meng *et al.* 2015).

Our results indicate gender-related differences that may be responsible for the variability frequently reported in miRNA measurements. Male patients

exhibited significantly higher expression of miR-21-5p, miR-21-3p and miR-16-3p in colorectal cancer tissue when compared with female patients. The same results were observed in non-tumor proximal tissue in miR-21-3p and miR-16-5p measurements. Taken together, according to our results expression of miRNAs in tumor and non-tumor tissues is higher in male patients compared to female patients.

Gender differences in miRNA expression were reported in several diseases, mostly in those with some gender-related predisposition. Regulation of miRNA may be influenced by gender-dependent factors, such as sex steroid hormones or X-linked genes. In particular, estrogen nuclear receptors regulate the biogenesis of specific miRNA – miR-30a by inhibiting pri-miRNA synthesis, or promoting miR-23b, miR-27b and miR-24-1 accumulation in breast cancer cells (Paris *et al.* 2012). The lupus-associated miRNA- cluster miR-182, miR-31 and miR-148a was found overexpressed in the splenocytes derived from female mice (Dai *et al.* 2013). A study that focused on the analysis of female and male familial breast cancer tissues identified significantly up-regulated levels of miR-152 and miR-497 in male breast cancer compared to female (Pinto *et al.* 2014). The expression profile of serum miRNAs in healthy human men and women subjects revealed that 90 miRNAs were present both in women's and men's serum, while 10 and 1 miRNAs were expressed only in men and women subjects, respectively. Men's serum-specific miRNAs were miR-100, miR-184 and miR-923 while women's serum-specific miRNA was miR-222 (Chen *et al.* 2008).

To conclude, we provide evidence supporting gender-specific expression of miRNAs in human colorectal cancer tissue. Our results implicate that the gender-related expression pattern of miRNAs should be considered as an important factor in generating new prognostic or diagnostic biomarkers and the role of both strands of miRNAs should be investigated.

Conflict of Interest

There is no conflict of interest.

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