



MicroRNAs and their role for T stage determination and lymph node metastasis in early colon carcinoma

Melanie Rammer¹ · Gerald Webersinke¹ · Sophie Haitchi-Petnehazy² · Eva Maier² · Hubert Hackl³ · Pornpimol Charoentong³ · Theodora Malli¹ · Maria Steinmair² · Andreas L. Petzer⁴ · Holger Rumpold^{4,5}

Received: 20 June 2017 / Accepted: 12 September 2017 / Published online: 13 November 2017
© Springer Science+Business Media B.V. 2017

Abstract

Worldwide, colon cancer is among the most common cancer entities. Understanding the molecular background is the key to enable accurate stage determination, which is crucial to assess optimal therapy options. The search for preoperative biomarkers is ongoing. In recent years, several studies have proposed a diagnostic and prognostic role for miRNAs in cancer. Aim of this study was to evaluate miRNA expression patterns correlating with tumor stage, especially lymph node metastasis, in primary colon carcinoma tissue. Screening was accomplished using GeneChip[®] miRNA v3.0 arrays (Thermo Fisher Scientific, Waltham, MA, USA) and validated via TaqMan[®] qPCR assays (Thermo Fisher Scientific, Waltham, MA, USA) to investigate miRNA expressions in 168 FFPE and 83 fresh frozen colon carcinoma samples. Regarding lymph node status, analyses displayed no significantly differential miRNA expression. Interestingly, divergent expression of miR-18a-5p, miR-20a-5p, miR-21-5p, miR-152-3p and miR-1973 was detected in stage pT1. Although miRNAs might not represent reliable biomarkers regarding lymph node metastasis status, they could support risk assessment in stage T1 tumors.

Keywords Biomarkers · Colonic neoplasms · Lymphatic metastasis · MicroRNAs · Risk assessment

Abbreviations

Adj. p Adjusted p value
BID BH3 interacting domain death agonist

BNIP2 BCL2 interacting protein 2
CCND1 Cyclin D1
CDC42 Cell cycle division 42
CO Control
Corr. p Nested F corrected p value
COX-2 Cyclooxygenase 2
CRC Colorectal cancer
EIF5A2 Eukaryotic translation initiation factor 5A2
FC Fold change
FFPE Formalin-fixed paraffin-embedded
hnRNP A1 Heterogeneous nuclear ribonucleoprotein A1
IQR Interquartile range
KRAS KRAS proto-oncogene, GTPase
miRNA MicroRNA
PDCD4 Programmed cell death 4
PGE2 Prostaglandin E2
PIK3R3 Phosphoinositide-3-kinase regulatory subunit 3
PTEN Phosphatase and tensin homolog
qPCR Quantitative real-time polymerase chain reaction
SPRY2 Sprouty RTK signaling antagonist 2

Electronic supplementary material The online version of this article (doi:10.1007/s10585-017-9863-9) contains supplementary material, which is available to authorized users.

✉ Holger Rumpold
holger.rumpold@vlkh.net

¹ Laboratory for Molecular Biology and Tumor Cytogenetics, Department of Internal Medicine I: Medical Oncology, Hematology and Gastroenterology, Ordensklinikum Linz, Barmherzige Schwestern, Linz, Austria

² Department of Pathology, Ordensklinikum Linz, Barmherzige Schwestern, Linz, Austria

³ Division of Bioinformatics, Biocenter, Medical University of Innsbruck, Innsbruck, Austria

⁴ Department of Internal Medicine I: Medical Oncology, Hematology and Gastroenterology, Ordensklinikum Linz, Barmherzige Schwestern, Linz, Austria

⁵ Present Address: Internal Medicine II: Medical Oncology, Hematology, Gastroenterology and Rheumatology, Academic Teaching Hospital Feldkirch, Carinagasse 47, 6807 Feldkirch, Austria

TERT	Telomerase reverse transcriptase
TGFBRII	TGF- β receptor II
TGF- β	Transforming growth factor beta
TNM staging	Tumor-node-metastasis staging
TSP-1	Thrombospondin
TU	Tumor
UICC	Union for international cancer control
VIM	Vimentin
ZNF217	Zinc finger protein 217
Δ Cq	Delta threshold cycle

Introduction

Colon cancer is the third most common cancer worldwide [1]. Metastasis, which is the major cause of colon cancer related mortality, evolves from a complex multistep process in which lymph nodes often are affected prior to other organs and contribute to further dissemination of cancer cells to additional sites [2]. Accurate staging, using the tumor-node-metastasis (TNM) system (UICC, 7th edition), is crucial to determine treatment options and prognosis [3]. The differentiation of UICC stage III (tumors positive for lymph node metastasis) from high and low risk stage II (negative for lymph node metastasis, with or without risk factors like T4) has a major impact on prognosis, treatment benefit and therapy regimen options [4]. Furthermore, early treatment start was associated with a higher benefit [5]. This is also the case in other cancer entities (e.g. gastric, rectal or breast cancer), where the clinical assessment of tumor stage allows their neoadjuvant treatment, providing significant benefits for the patients [6–9]. A further rationale for neoadjuvant treatment is the higher treatment compliance compared to adjuvant treatment, and tumor shrinkage and cell death might reduce the risk of cell shedding during operation [10]. Another important argument in favor of neoadjuvant treatment concepts is that it allows for tumor chemosensitivity testing, which might have implications for duration and type of therapy. Due to this, preoperative systemic treatment has recently been an issue also in colon cancer. Several studies aim to show efficacy and safety for the neoadjuvant treatment with capecitabine and oxaliplatin, for example, and interim findings are promising [11–13]. Future clinical concepts for enhancing efficacy of multimodal treatment in colon cancer could include the option of timing systemic therapy before resection of the tumor. The prerequisite for this, however, is an accurate staging to separate high risk stage II and stage III from low risk stage II and stage I disease to prevent overtreatment. Based on biopsy results or imaging techniques, this is, unfortunately, challenging. Additional biomarkers to facilitate a more precise staging prior to surgical excision are necessary, in order to enable earlier treatment. Furthermore, the molecular differences between colon

carcinomas extending into superficial epithelial layers (T1; tumor invades submucosa) presenting lymph node metastasis and those invading deep into the intestinal wall (T4) without lymph node metastasis need further investigation. Additionally to the metastatic niche, also early alterations in the primary tumor might contribute to the varying onset of lymph node metastasis [14–17].

MicroRNAs (miRNAs) are small (18–24 bases), non-coding, regulatory RNA molecules. Aberrant miRNA expression associated with cancer was first described by Calin and colleagues [18]; since then, miRNAs have been a major subject of cancer research. Several groups proposed miRNA expression profiles able to distinguish colorectal cancer from normal tissue as well as specific tumor stages and metastasis [19–22]. However, the distinction of lymph node positivity from negativity in primary lesions was not discussed extensively.

In this work we investigated miRNA expression in early colon carcinoma stages excluding distant metastasis. Along with comparing formalin-fixed paraffin-embedded (FFPE) as well as fresh frozen primary tumor tissue from lymph node negative with lymph node positive tumors, the question of T stage dependent miRNA expression was addressed. This was achieved with miRNA screening in test samples and validation via qPCR in a large cohort.

Materials and methods

Tissue samples

FFPE primary tumor tissue samples from 168 patients as well as fresh frozen primary tumor and corresponding benign colon tissue specimens from 83 patients, who underwent surgical excision due to colon cancer without neoadjuvant treatment prior to surgery at the hospital Barmherzige Schwestern Linz between 2007 and 2014 and between 2011 and 2014, respectively, were collected. Clinical information and diagnosis including TNM (tumor-node-metastasis) staging according to UICC criteria were accessed from the pathological and medical records and are listed in Table 1 [3]. Patients with known hereditary colon cancer, other histological types than adenocarcinomas and distant metastases at time of diagnosis and collection were excluded. FFPE tissue was chosen, as it represents a standard method for long-term storage and therefore a substantial set of samples were available. Furthermore, FFPE tissue facilitates determination of tumor portion. Hence, for each FFPE specimen the standard protocol of hematoxylin and eosin staining was performed on adjacent slices and the tumor fraction was marked by pathologists to ensure that only tissue with a tumor content of more than 80% was included for RNA isolation [23]. Additionally, frozen tissue specimens were

Table 1 Clinicopathological parameters of all patients (all M0)

	FFPE (n = 168)		Frozen (n = 83)			
	n	%	n	%		
Median age at diagnosis (years/range)	72	22–95	71	31–89		
Gender						
Male	95	57	50	60		
Female	73	43	33	40		
Tumor location						
Right (cecum, ascending, hepatic flexure)	90	54	20	24		
Left (sigmoid, descending, splenic flexure)	70	42	43	52		
Transverse	8	5	20	24		
T category						
pT1	19	11	0			
pT2	27	16	0			
pT3	95	56	70	84		
pT4	27	16	13	16		
N category						
Negative	100	60	50	60		
Positive	68	40	33	40		
Histological grade						
G1	20	12	3	4		
G2	106	63	51	61		
G3	42	25	29	35		
<i>KRAS</i>						
WT	25	15	61	18	22	62
Mutation ^a	16	9	39	11	13	38
n/a	127	75	54	65		
MSS/MSI status						
MSS	10	6	77	20	24	83
MSI	3	2	23	4	5	17
n/a	155	92	59	71		
Treatment						
Fluorouracil-based (therefrom with Oxaliplatin)	27 (13)	16	9 (5)	5		
Capecitabine-based (therefrom with Oxaliplatin)	23 (4)	19	20 (5)	12		
Best supportive care	107	63	51	30		
n/a	2	1	3	2		

FFPE formalin-fixed paraffin embedded, *n* quantity, WT wild type, *n/a* not available, MSS microsatellite stable, MSI microsatellite instable

^aFollowing mutations in *KRAS* were detected: c.G35A, c.G38A, c.A183T, c.G34A n=1, c.G34T n=1, c.G35C, c.G35T, c.G38A, c.32_37dupCTGGTG

available from the hospital's tumor bank for further validation of specific miRNAs in pT3 and pT4 (regarding pT1 the sample number was not sufficient for analysis). Ethical approval for this retrospective study was granted from the hospital's ethics committee.

RNA isolation

Total RNA was extracted from two to five 7–10 µm slices of FFPE material using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific,

Waltham, MA, USA) according to the manufacturer's instructions with the modification of agitated protease digestion at 50 °C for 20–40 min.

PeqGOLD TriFast™ (VWR, Radnor, PA, USA), an optimized guanidinium thiocyanate-phenol-method, was applied for total RNA isolation from fresh frozen samples according to the manufacturer's instructions with overnight RNA precipitation. Tissue homogenization was achieved through automated mechanic disruption with the gentleMACS™ Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany).

RNA concentration and purity were determined via photometric measurement. Additionally miRNA quality was assessed in the array quality control workflow.

miRNA array

GeneChip[®] miRNA arrays v3.0 (Thermo Fisher Scientific, Waltham, MA, USA) were conducted from 20 nodal negative and 20 nodal positive samples (test samples), investigating expressions of 1733 human mature miRNAs in each sample. According to the manufacturer's instructions, 300 ng RNA was biotinylated using the FlashTag[™] Biotin HSR RNA Labeling Kit and hybridized to the chip, which was stained, washed and scanned.

qPCR

TaqMan[®] miRNA qPCR assays (Thermo Fisher Scientific, Waltham, MA, USA) were performed with the Applied Biosystems 7900HT Fast Real-Time PCR System according to the manufacturer's instructions, using 10 ng total RNA for cDNA synthesis with the TaqMan[®] microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The real-time PCR was conducted in triplicates in 10 μ L reactions utilizing the TaqMan[®] Fast Advanced Mastermix (Thermo Fisher Scientific, Waltham, MA, USA) with the appropriate cycler protocol. Detailed information on TaqMan[®] miRNA qPCR assays is provided in Supplemental Table 1. RNU6B was used for normalization.

Statistical analyses

Array normalization (RMA: Robust Multi-Array Average) and quality control were carried out by use of the expression console software (Thermo Fisher Scientific, Waltham, MA, USA). Statistical analyses were performed using the statistical software environment R/Bioconductor (<http://www.r-project.org>) and the packages limma (linear models for microarray data) and affy [24–26]. Only human mature miRNAs with an IQR > 0.1 (interquartile range) and a standard detection quality ($p < 0.06$) were included in the analyses. The adjusted p value (adj. p) for multiple hypothesis testing was determined using the Benjamini–Hochberg method. Nested F was applied as post-hoc analysis for parallel testing correction (corr. p).

For qPCR analysis, RNU6B served as endogenous control. Delta threshold cycle (Δ C_q) values were calculated by the SDS2.2.2 software (Thermo Fisher Scientific, Waltham, MA, USA) and relative expression values (fold change, FC) were calculated as $2^{-\Delta\Delta C_q}$, using the comparative C_q method [27]. Welch's T test, paired T test or Welch's ANOVA and Tukey-HSD/Tukey–Kramer post-hoc analysis were used to determine significance ($p < 0.05$, $\alpha = 0.05$). Survival analysis

was conducted using the Kaplan–Meier method (log rank, $p < 0.05$), for which median was used to stratify for high and low expression.

Results

Lymph node metastasis

Expressions of 1733 human mature miRNAs were investigated in 40 test samples (FFPE tissue) with equal lymph node status and T stage distribution (all M0) via miRNA array screening. Comparison of 20 lymph node negative (stage I/II) and 20 node positive samples (stage III) displayed no miRNA with a significant differential expression [criteria: fold change (FC) > 1.5, adj. $p < 0.05$] (Supplemental Table 2). This was consistent with lymph node status investigation in two RNA deep sequencing data sets (The Cancer Genome Atlas, TCGA) of colorectal cancer ($n = 255$, $n = 323$) (Supplemental Fig. 1) [28].

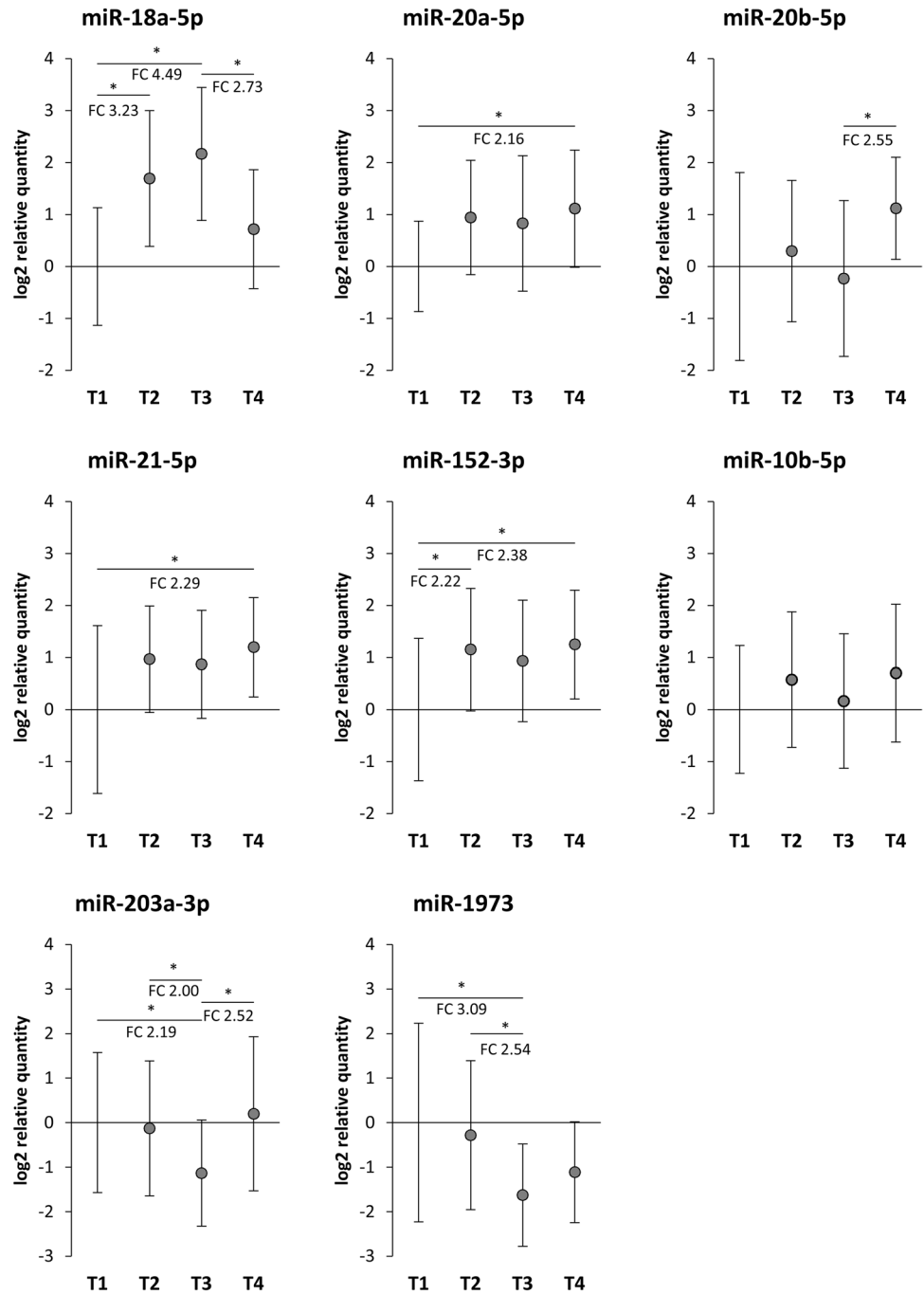
To validate these array findings, for four miRNAs (miR-18a-5p/-20a-5p/-20b-5p/-203a-3p), which displayed differences between lymph node negative and positive samples at analysis without p value adjustment and the highest average expression levels within this group, qPCR was conducted in test and 128 validation FFPE tissue samples. Additionally, two miRNAs (miR-378a-3p/-422a) displayed divergent expression in test samples regarding lymph node metastasis within T stages and were therefore also investigated in 128 validation FFPE tissue samples regarding lymph node status in general. qPCR data exhibited no significant expression differences of these six miRNAs regarding lymph node status.

T stage comparison

Comparing T stages (acc. to UICC criteria), irrespective of nodal status, revealed several differentially expressed miRNAs according to array screening (nested F test, corr. $p < 0.1$, FC > 1.5) (Supplemental Table 3). Of these, the six miRNAs (miR-10b-5p/-21-5p/-152-3p/-378a-3p/-422a/-1973) additionally displaying average expression values $A > 2.00$ were selected for validation via qPCR. Furthermore, qPCR data from the four miRNAs, available from the investigation regarding lymph node metastasis, were also analyzed regarding T stage dependent expression.

Investigating T stage dependent expression differences of ten miRNAs via qPCR in test and validation samples (both FFPE tissue) revealed significances for following miRNAs (Fig. 1). Stage pT1 displayed significantly lower expression ($\alpha = 0.05$, FC > 2) of miR-18a-5p compared to pT2 and pT3, miR-20a-5p versus pT4, miR-21-5p versus pT4, miR-152-3p versus pT2 and pT4, and significantly

Fig. 1 miRNA expression in T stages. Graphs depict qPCR results of miRNAs showing significant differential expression regarding T stage comparison in FFPE samples (miR-18a-5p/-20a-5p/-20b-5p/-203a-3p in 19 pT1, 27 pT2, 95 pT3 and 27 pT4 samples; miR-10b-5p/-21-5p/-152-3p/-1973 in 19 pT1, 27 pT2, 30 pT3 and 27 pT4 samples). miR-18a-5p/-20a-5p/-21-5p/miR-152 display lower expression and miR-1973 higher expression in stage pT1. Endogenous control: RNU6B, relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method [27], x-axis: compared groups, y-axis: log₂ relative quantity, error bars depict standard deviation, asterisks indicate significance according to Welch’s ANOVA and Tukey post-hoc test ($\alpha=0.05$) and FC > 2



higher expression of miR-203a-3p and miR-1973 compared to pT3. The highest miRNA down- and up-regulation in pT1 was detected for miR-18a-5p (4.49-fold) and miR-1973 (3.09-fold), respectively. For miR-20b-5p and miR-203a-3p significantly decreased expression was observed in pT3 compared to pT4, while miR-18a-5p displayed an increase in pT3 versus pT4. miR-10b-5p, miR-378a-3p and miR-422a did not show significant expression differences in T stage comparison.

Lymph node status within T stage

Analyses of qPCR data regarding lymph node expression differences within T stages displayed aberrant expression for miR-378a-3p and miR-422a in stage pT3 and for miR-203a-3p in stage pT4 (Supplemental Fig. 2). Based on power calculation results, the expression of these three miRNAs was analyzed in additional FFPE as well as frozen tissue samples to investigate nodal metastasis-associated

expression in greater detail. miR-378a-3p and miR-422a were investigated in 65 FFPE and 70 frozen stage pT3 samples and for miR-203a-3p 13 frozen stage pT4 samples could be obtained. Regarding expression of miR-378a-3p and miR-422a in FFPE tissue, the differences couldn't be confirmed in the enlarged cohort. Results of the frozen samples displayed a marginal decrease in lymph node positive samples (Supplemental Fig. 3). miR-203a-3p displayed no significant expression difference.

Tumor versus non-tumor colon tissue

Additionally, stage pT3 and pT4 tumor tissue was compared to corresponding non-tumor colon tissue regarding miR-378a-3p/-422a and miR-230a-3p expression, respectively. This revealed a significantly higher expression of miR-378a-3p and miR-422a in control tissue compared to stage pT3 tumor tissue (Fig. 2). For miR-203a-3p no significant expression variation could be observed.

Histological grade, KRAS mutation status, survival

Investigation of miRNAs expression pattern correlation with histological grade displayed no significant differences (Supplemental Fig. 4). According *KRAS* mutation status, no significantly aberrant miRNA expression was detected (Supplemental Fig. 5). Survival analyses did not display a correlation of miRNA expression levels with survival (Supplemental Fig. 6).

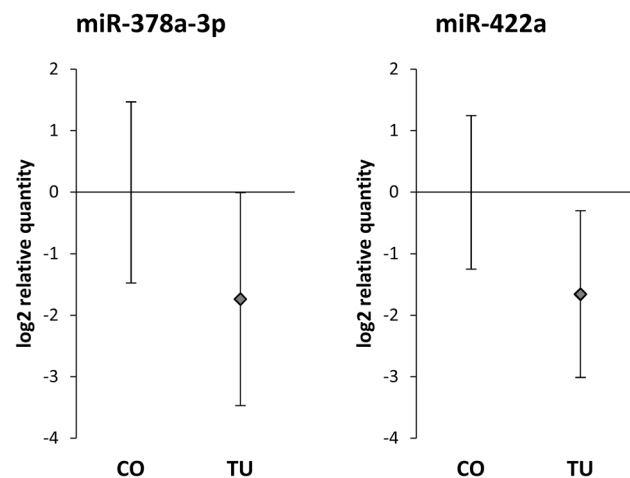


Fig. 2 Tumor (TU) versus control (CO) tissue of frozen samples. Graphs picture results of comparison between tumor (n=69) and corresponding non-tumor (n=69) colon tissue in frozen samples. Both, miR-378a-3p and miR-422a, display a significantly lower expression in tumor tissue with a 3.33 and a 3.14-fold decrease, respectively. Endogenous control: RNU6B, relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method [27], x-axis: compared groups, y-axis: log2 relative quantity, error bars depict standard deviation, significance according to paired *T* test ($p < 0.05$)

Discussion

Accurate stage determination is crucial to assess colon cancer patients' outcome and treatment options. Lymph node metastasis has an important prognostic value and often constitutes the step prior to increasing tumor malignancy such as distant spread [2, 29]. The deeper the primary tumor invades surrounding colon tissue layers, the greater the likelihood for lymph node metastasis [30]. Nevertheless, in some cases tumors are found reaching deep into adjacent tissue layers, but without exhibiting detectable lymph node metastasis. Furthermore, some localized tumors already infiltrate lymph nodes despite their presumably lower malignancy potential. Reasons for this inconsistency might be found in the primary tumor. The small regulatory miRNAs gained importance as possible biomarkers in various malignancies regarding development and progression [31]. Aberrant expression was also reported in colorectal cancer metastasis [22]. This prompted us to investigate whether specific miRNAs affect the primary tumor's initial invasive potential, especially regarding lymph node metastasis.

In this study we analyzed the expression of 1733 human mature miRNAs in a test cohort of 40 colon cancer patients and validated a set of miRNAs in a larger cohort of FFPE samples. As FFPE constitutes the standard method for long-term sample storage, a substantial amount of samples was available and furthermore, detailed information on tumor content could be provided. Additionally, fresh frozen colon cancer samples (available from the hospital's tumor bank established 2010) were analyzed regarding the expression of selected miRNAs for further validation.

Comparing lymph node positive with lymph node negative primary colon cancer samples, no miRNA displayed significant expression alteration in array screening data. For a more thorough investigation of this issue, we compared our data with two RNA deep sequencing data sets from the TCGA to explore mutually differentially expressed miRNAs [28]. Supporting the first results, the analyses displayed no common aberrantly expressed miRNAs regarding lymph node comparison in all three data sets (Supplemental Fig. 1). qPCR verification was conducted for the miRNAs, which displayed differences (significance without *p* value adjustment) between the two groups and the highest average expression, in order to validate array findings in a larger cohort. The validation confirmed the initial screening displaying uniform expression in lymph node comparison. Most of the previous findings regarding miRNA expression in lymph node metastasis were evaluated in cohorts including rectal cancer and more advanced TNM stage IV, i.e. exhibiting distant metastasis. Slaby et al., Xiong et al. and Huang et al. reported

upregulation of miR-21 and miR-137, respectively, to be associated with lymph node positivity, while Wang et al. described decreased expression of miR-195 in lymph node positive tumors [32–35]. These reported expression differences could not be detected in our colon cancer sample pool without distant metastasis.

Rectal cancer is prevalently distinguished from colon cancer, because the two carcinoma types differ not only in prognosis, but also in therapy options—e.g. neoadjuvant treatment is applied in certain stages of rectal cancer [8, 36, 37]. Furthermore, also regarding miRNA expression several reports described differences between colon and rectal cancer, e.g. increased miR-31/-1973 and decreased miR-126 expression in rectal compared to colon cancer [38, 39]. Slattery et al. reported an association of aberrant miR-21 expression with mortality in rectal cancer but not in colon cancer [40]. However, reports are not entirely consistent, since Nielsen et al. didn't observe such correlation in stage II rectal cancer, but they found a positive correlation of miR-21 expression with short disease-free survival in stage II colon cancer [41]. Because of the reported inconsistent effect of miRNAs and their seemingly different functions in various cancer types, a stage specific regulatory potential might explain the varying results. Therefore, separate analysis of miRNA expression in rectal and colon cancer should be considered.

Another question we addressed was miRNA expression variation between T stages. Interestingly, we detected several miRNAs with differential expression in stage T1 compared to the other T stages. miR-18a-5p, miR-20a-5p, miR-21-5p and miR-152-3p were significantly decreased in stage T1, while miR-1973 showed a higher expression. Currently, several parameters are investigated for risk assessment of T1 tumors (differentiation grade, lymphovascular invasion etc.) and the discussion whether their oncologically correct excision represents a necessity to avoid recurrence or constitutes an evitable burden for patients is ongoing [36, 42–44]. These miRNAs might provide supporting information as additional factors in decision-making.

miR-18a-5p and miR-20a-5p belong to the miR-17~92 cluster. In previous reports their elevated expression was detected in colon adenocarcinoma and colorectal cancer (CRC) compared to non-tumor control or adenoma tissue and was associated with adenoma to carcinoma progression [45–48]. Correspondingly, miR-18a targets for instance comprise anti-angiogenic factor thrombospondin 1 (TSP-1) or tumor suppressors involved in transforming growth factor beta (TGF- β) signaling, namely SMAD2 and SMAD4 [49, 50]. Similarly, also miR-20a-5p has been shown to target SMAD4 and TGF- β receptor II (TGFBR2) within this pathway important in mediating cell proliferation [50, 51]. Additionally miR-20a-5p involvement in preventing apoptosis via targeting pro-apoptotic BH3 interacting domain death

agonist (BID) and BCL2 interacting protein 2 (BNIP2) has been documented [52, 53]. No association of miR-20a-5p and miR-18a-5p expression to clinicopathological parameters has been reported in the literature [47]. However, miR-18a-5p has also been shown to induce apoptosis in colon cancer cells via targeting heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) and was linked to a decreased mortality hazard in colorectal cancer [40, 54]. Additionally, Humphreys et al., observing reduced cancer cell growth in association with high miR-18a levels, detected the cell cycle control gene cell cycle division 42 (CDC42) as target and proposed a regulatory function for this miRNA in colorectal cancer within the oncogenic miR-17~92 cluster [55]. Interestingly, although in our results miR-18a-5p displayed significant decrease in stage T1 compared to T2 and T3, significance could not be detected regarding the comparison stage T1 versus T4. Results even displayed a significant decrease in stage T4 compared to T3. These findings underline the reported versatile function of miR-18a and suggest that low miR-18a-5p and miR-20a-5p expression could be linked to lower malignant potential. The miRNAs might only be involved in regulation of initial steps in tumor progression (e.g. invasion beyond the submucosa), while their role in further tumor progression (excluding distant metastasis) is limited.

Regarding miR-152, previous studies indicated tumor suppressor attributes in CRC, where phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3) has been identified as target [56, 57]. This, however, is contradictory to the results from our cohort, since we detected decreased expression in stage T1. On the other hand, also lack of significant expression variation in colon cancer has been described [58]. Because of this variability and the relatively low number of literature findings, this miRNA should be further investigated to elucidate its expression pattern. Also the distinct analysis in colon and rectal cancer should be taken into account.

miR-21 presented decreased expression in pT1 cases of our cohort. It represents one of the highly investigated miRNAs displaying aberrant expression in carcinoma tissue. The majority of reports attributed oncogene properties to miR-21. Its expression was found to be elevated in different carcinoma types as well as in metastatic tissue and was associated with a short disease-free survival [33, 41, 59–62]. Documented miR-21 targets comprise tumor suppressors e.g. phosphatase and tensin homolog (PTEN), sprouty RTK signaling antagonist 2 (SPRY2), BCL2, apoptosis regulator (BCL2) or programmed cell death 4 (PDCD4), many of them represent tumor suppressor genes involved in regulation of cell cycle or apoptosis and for some even effect on chemotherapy response has been observed [33, 63–65]. Furthermore, involvement in inflammation has been reported. Accumulation of prostaglandin E2 (PGE2), which is induced

by pro-inflammatory cyclooxygenase 2 (COX-2) due to persistent inflammation, has been shown to increase miR-21 expression, which in turn caused further decrease of tumor suppressor PDCD4, thereby facilitating cancer progression [66]. This supports the role of miR-21 as important biomarker and therapy target in many carcinoma types. On the other hand, several groups could not detect significant aberrant expression, neither among CRC subgroups nor in association with clinicopathological parameters [40, 67, 68]. Also lack of connection with survival has been documented, similar to the results observed in our cohort [40]. This underlines the complexity of the miRNA network also within cancer progression.

Little evidence is available on differential expression of miR-20b-5p in colon or colorectal cancer. Elevated expression of miR-20b-5p in CRC and association with targeting phosphatase and tensin homolog (PTEN) was suggested [69]. However, also its down regulation was detected in various colorectal cancer types [70]. Since we detected elevated expression in stage pT4, which represents the stage with the deepest invasion of the primary lesion into the surrounding tissue layers, our results rather suggest an oncogenic potential of miR-20b-5p in colon cancer.

For miR-203a-3p our results did not show a clear correlation with tumor progression. A significantly lower expression was detected in stage pT3. Chiang et al. detected inverse correlation with pT stage (T2 + 3 versus T4) and tumor size and reported decreased expression of miR-203 in colorectal cancer (without distant metastasis) compared to control [71]. This could not be confirmed in our results, as stage pT4 colon cancer displayed no significant difference compared to matched non-tumor tissue. A tumor suppressive function of miR-203 is described in various cancer types, and has recently also been shown in colorectal cancer by Deng et al. [72–75]. In line with this, zinc finger protein 217 (ZNF217) and eukaryotic translation initiation factor 5A2 (EIF5A2), promoting proliferation (the former) and invasive potential (both), have been documented as miR-203 targets [75, 76].

miR-1973 expression displayed inverse correlation with pT stage in our cohort. Literature reports are inconclusive, as along with elevated expression in CRC also aberrant expression comparing colon and rectal cancer was observed, thus suggesting a separate investigation for colon and rectal carcinoma [38, 39, 77]. Predicted targets for miR-1973 include cyclin D1 (CCND1) and PIK3R1, both associated with oncogenic potential in colon cancer [78–81].

To thoroughly address possible lymph node status correlation with miRNA expression, we also investigated alterations within T stages. Initial detection of elevated miR-378a-3p and miR-422a expression in stage T3N0 FFPE tissue compared to stage T3 with lymph node metastasis

prompted us to further investigate these miRNAs in an enlarged cohort as well as in frozen samples. Stage T3 represents an interesting cohort, since a large number of colon cancer cases are diagnosed at that stage and prognosis is difficult to determine due to high variability. However, analyses of the additional FFPE tissue specimens did not confirm these differences. Only in the frozen tissue samples a marginally lower expression of miR-378a-3p and miR-422a in T3 tumors with lymph node metastasis was detected. Some reports discovered decreased expression of miR-378a-3p and miR-422a in lymph node positive specimens but, in contrast to our cohort, they included patients with distant metastases or rectal cancer [20, 82, 83]. However, Li et al., who investigated FFPE tissue, did not show significant differences for miR-378a-3p, similar to our findings in FFPE specimens [84]. Corresponding to decreased miR-422a and miR-378a-3p levels in metastasis, telomerase reverse transcriptase (TERT) and vimentin (VIM)—both associated with invasion—have been documented as targets, respectively [82, 85].

Consistent with our findings in frozen colon tumor and corresponding control tissue, miR-378a-3p and miR-422a were reported to be down-regulated in CRC tumor specimens [20, 82–84, 86, 87].

No correlation of miRNA expression with survival, *KRAS* mutation status or histological grade was detected in ten miRNAs analyzed via qPCR. Although high miR-21 and miR-18a expression levels have been associated with unfavorable outcome/survival in colon cancer, our results are in line with reports observing no such connection [40, 59, 88]. Low miR-378a expression has been reported to correlate with mutated *KRAS*, which could not be confirmed in our cohort [89]. Variation in cohort distribution might account for differing results, as our cohort included colon cancer without distant metastasis.

Although thoroughly investigating a large cohort, a limitation of this study is the small number of T1 samples due to the low detection frequency of this stage.

In conclusion, our results suggest that miRNAs are not suitable as early biomarkers in colon biopsy samples to determine lymph node metastasis prior to surgery in our population. T stage comparison revealed differential expression of miR-18a-5p, miR-20a-5p, miR-21-5p, miR-152-3p and miR-1973 in early tumor development, i.e. stage pT1 tumors. These miRNAs might serve as additional parameters for deciding on the necessity of oncologically correct tumor excision, thus we propose their validation in a larger colon cancer cohort.

Funding This project received a research grant from “Krebshilfe Oberösterreich”.

References

- Torre LA et al (2015) Global cancer statistics, 2012. *Cancer J Clin* 65(2):87–108
- Kawada K, Taketo MM (2011) Significance and mechanism of lymph node metastasis in cancer progression. *Cancer Res* 71(4):1214–1218
- Sobin LH, Gospodarowicz MK, Wittekind C (2009) TNM classification of malignant tumours, 7th edn. Wiley-Blackwell in affiliation with the International Union against Cancer (UICC), Chichester
- Chee CE, Meropol NJ (2014) Current status of gene expression profiling to assist decision making in stage II colon cancer. *Oncologist* 19(7):704–711
- Biagi JJ et al (2011) Association between time to initiation of adjuvant chemotherapy and survival in colorectal cancer: a systematic review and meta-analysis. *JAMA* 305(22):2335–2342
- Kaufmann M et al (2006) Recommendations from an international expert panel on the use of neoadjuvant (primary) systemic treatment of operable breast cancer: an update. *J Clin Oncol* 24(12):1940–1949
- Gillen S et al (2010) Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. *PLoS Med* 7(4):e1000267
- Sauer R et al (2012) Preoperative versus postoperative chemoradiotherapy for locally advanced rectal cancer: results of the German CAO/ARO/AIO-94 randomized phase III trial after a median follow-up of 11 years. *J Clin Oncol* 30(16):1926–1933
- Reim D et al (2015) Clinical research of neoadjuvant chemotherapy for gastric cancer—current and future concepts. *Transl Gastrointest Cancer* 4(2):131–140
- Hendren S et al (2010) Surgical complications are associated with omission of chemotherapy for stage III colorectal cancer. *Dis Colon Rectum* 53(12):1587–1593
- Arredondo J et al (2017) Mid-term oncologic outcome of a novel approach for locally advanced colon cancer with neoadjuvant chemotherapy and surgery. *Clin Transl Oncol* 19(3):379–385
- Karoui M et al (2015) Neoadjuvant FOLFOX 4 versus FOLFOX 4 with Cetuximab versus immediate surgery for high-risk stage II and III colon cancers: a multicentre randomised controlled phase II trial—the PRDIGE 22–ECKINOXE trial. *BMC Cancer* 15:511
- Liu F et al (2016) CapOX as neoadjuvant chemotherapy for locally advanced operable colon cancer patients: a prospective single-arm phase II trial. *Chin J Cancer Res* 28(6):589–597
- Psaila B, Lyden D (2009) The metastatic niche: adapting the foreign soil. *Nat Rev Cancer* 9(4):285–293
- Paget G (1889) Remarks on a case of alternate partial anaesthesia. *Br Med J* 1(1462):1–3
- Hart IR, Fidler IJ (1980) Cancer invasion and metastasis. *Q Rev Biol* 55(2):121–142
- Fidler IJ, Kripke ML (1977) Metastasis results from pre-existing variant cells within a malignant tumor. *Science* 197(4306):893–895
- Calin GA et al (2002) Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 99(24):15524–15529
- Ma Y et al (2012) Candidate microRNA biomarkers in human colorectal cancer: systematic review profiling studies and experimental validation. *Int J Cancer* 130(9):2077–2087
- Faltejskova P et al (2012) Identification and functional screening of microRNAs highly deregulated in colorectal cancer. *J Cell Mol Med* 16(11):2655–2666
- Zhang JX et al (2013) Prognostic and predictive value of a micro-RNA signature in stage II colon cancer: a microRNA expression analysis. *Lancet Oncol* 14(13):1295–1306
- Drusco A et al (2014) MicroRNA profiles discriminate among colon cancer metastasis. *PLoS ONE* 9(6):e96670
- Fischer AH et al. (2008) Hematoxylin and eosin staining of tissue and cell sections. *CSH Protoc* 2008:pdb prot4986
- Gentleman RC et al (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 5(10):R80
- Smyth GK (2005) limma: linear models for microarray data. In: Gentleman R et al (eds) *Bioinformatics and computational biology solutions using R and bioconductor*. Statistics for biology and health. Springer, New York, pp 397–420
- Gautier L et al (2004) affy—analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* 20(3):307–315
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 25(4):402–408
- The Cancer Genome Atlas Network (2012) Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 487(7407):330–337
- Kritsanasakul A et al (2012) Impact of lymph node retrieval on surgical outcomes in colorectal cancers. *J Surg Oncol* 106(3):238–242
- Cserni G (2003) Nodal staging of colorectal carcinomas and sentinel nodes. *J Clin Pathol* 56(5):327–335
- Macfarlane LA, Murphy PR (2010) MicroRNA: biogenesis, function and role in cancer. *Curr Genomics* 11(7):537–561
- Slaby O et al (2007) Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Int Soc Cell* 72(5–6):397–402
- Xiong B et al (2013) MiR-21 regulates biological behavior through the PTEN/PI-3 K/Akt signaling pathway in human colorectal cancer cells. *Int J Oncol* 42(1):219–228
- Huang ZM et al (2009) MicroRNA expression profile in non-cancerous colonic tissue associated with lymph node metastasis of colon cancer. *J Dig Dis* 10(3):188–194
- Wang X et al (2012) Downregulation of miR-195 correlates with lymph node metastasis and poor prognosis in colorectal cancer. *Med Oncol* 29(2):919–927
- Schmoll HJ et al (2012) ESMO Consensus Guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making. *Ann Oncol* 23(10):2479–2516
- Lee YC et al (2013) Differences in survival between colon and rectal cancer from SEER data. *PLoS ONE* 8(11):e78709
- Li X et al (2012) Identification of aberrantly expressed miRNAs in rectal cancer. *Oncol Rep* 28(1):77–84
- Chen Z et al (2012) Differential miRNA expression profiling of rectal and colon cancers using deep sequencing. In: *Proceedings of the 103rd annual meeting of the American Association for Cancer Research, Chicago, IL 2012, vol 72*. AACR, Philadelphia
- Slattery ML et al (2015) An evaluation and replication of miRNAs with disease stage and colorectal cancer-specific mortality. *Int J Cancer* 137(2):428–438
- Nielsen BS et al (2011) High levels of microRNA-21 in the stroma of colorectal cancers predict short disease-free survival in stage II colon cancer patients. *Clin Exp Metastasis* 28(1):27–38
- Nascimbeni R et al (2002) Risk of lymph node metastasis in T1 carcinoma of the colon and rectum. *Dis Colon Rectum* 45(2):200–206
- Sakuragi M et al (2003) Predictive factors for lymph node metastasis in T1 stage colorectal carcinomas. *Dis Colon Rectum* 46(12):1626–1632
- Peravali R, Naem T, Wheeler J (2015) A single tertiary centre experience of T1 colorectal cancers—a retrospective analysis. *Gut* 64:A551

45. Zhang GJ et al (2014) miR20a is an independent prognostic factor in colorectal cancer and is involved in cell metastasis. *Mol Med Rep* 10(1):283–291
46. Brunet Vega A et al (2013) microRNA expression profile in stage III colorectal cancer: circulating miR-18a and miR-29a as promising biomarkers. *Oncol Rep* 30(1):320–326
47. Yu G et al (2012) Prognostic values of the miR-17-92 cluster and its paralogs in colon cancer. *J Surg Oncol* 106(3):232–237
48. Mazeh H et al (2013) The diagnostic and prognostic role of microRNA in colorectal cancer—a comprehensive review. *J Cancer* 4(3):281–295
49. Mogilyansky E, Rigoutsos I (2013) The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. *Cell Death Differ* 20(12):1603–1614
50. Fuziwarra CS, Kimura ET (2015) Insights into regulation of the miR-17-92 cluster of miRNAs in Cancer. *Front Med* 2(2):64
51. Cheng D et al (2016) MicroRNA-20a-5p promotes colorectal cancer invasion and metastasis by downregulating Smad4. *Oncotarget* 7(29):45199–45213
52. Huang G et al (2017) miR-20a-directed regulation of BID is associated with the TRAIL sensitivity in colorectal cancer. *Oncol Rep* 37(1):571–578
53. Chai H et al (2011) miR-20a targets BNIP2 and contributes chemotherapeutic resistance in colorectal adenocarcinoma SW480 and SW620 cell lines. *Acta Biochim Biophys Sin* 43(3):217–225
54. Fujiya M et al (2014) microRNA-18a induces apoptosis in colon cancer cells via the autophagolysosomal degradation of oncogenic heterogeneous nuclear ribonucleoprotein A1. *Oncogene* 33(40):4847–4856
55. Humphreys KJ, McKinnon RA, Michael MZ (2014) miR-18a inhibits CDC42 and plays a tumour suppressor role in colorectal cancer cells. *PLoS ONE* 9(11):e112288
56. Chen Y et al (2010) Altered expression of MiR-148a and MiR-152 in gastrointestinal cancers and its clinical significance. *J Gastrointest Surg* 14(7):1170–1179
57. Li B, Xie Z, Li B (2016) miR-152 functions as a tumor suppressor in colorectal cancer by targeting PIK3R3. *Tumour Biol* 37(8):10075–10084
58. Takahashi M et al (2012) The clinical significance of MiR-148a as a predictive biomarker in patients with advanced colorectal cancer. *PLoS ONE* 7(10):e46684
59. Schetter AJ et al (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 299(4):425–436
60. Kjaer-Frifeldt S et al (2012) The prognostic importance of miR-21 in stage II colon cancer: a population-based study. *Br J Cancer* 107(7):1169–1174
61. Oue N et al (2014) High miR-21 expression from FFPE tissues is associated with poor survival and response to adjuvant chemotherapy in colon cancer. *Int J Cancer* 134(8):1926–1934
62. Hansen TF et al (2014) Redefining high-risk patients with stage II colon cancer by risk index and microRNA-21: results from a population-based cohort. *Br J Cancer* 111(7):1285–1292
63. Feng YH et al (2012) MicroRNA-21-mediated regulation of Sprouty2 protein expression enhances the cytotoxic effect of 5-fluorouracil and metformin in colon cancer cells. *Int J Mol Med* 29(5):920–926
64. Asangani IA et al (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdc4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* 27(15):2128–2136
65. Corte H et al (2012) MicroRNA and colorectal cancer. *Dig Liver Dis* 44(3):195–200
66. Peacock O et al (2014) Inflammation and MiR-21 pathways functionally interact to downregulate PDCD4 in colorectal cancer. *PLoS ONE* 9(10):e110267
67. Kulda V et al (2010) Relevance of miR-21 and miR-143 expression in tissue samples of colorectal carcinoma and its liver metastases. *Cancer Genet Cytogenet* 200(2):154–160
68. Schee K et al (2012) Clinical relevance of microRNA miR-21, miR-31, miR-92a, miR-101, miR-106a and miR-145 in colorectal cancer. *BMC Cancer* 12:505
69. Zhu J et al (2014) MiR-20b, -21, and -130b inhibit PTEN expression resulting in B7-H1 over-expression in advanced colorectal cancer. *Hum Immunol* 75(4):348–353
70. Yamaguchi T et al (2014) Underexpression of miR-126 and miR-20b in hereditary and nonhereditary colorectal tumors. *Int Soc Cell* 87(1):58–66
71. Chiang Y et al (2011) Aberrant expression of miR-203 and its clinical significance in gastric and colorectal cancers. *J Gastrointest Surg* 15(1):63–70
72. Zhao G et al (2015) miR-203 functions as a tumor suppressor by inhibiting epithelial to mesenchymal transition in ovarian cancer. *J Cancer Sci Ther* 7(2):34–43
73. Zhang X et al (2015) MicroRNA-203 is a prognostic indicator in bladder cancer and enhances chemosensitivity to cisplatin via apoptosis by targeting Bcl-w and survivin. *PLoS ONE* 10(11):e0143441
74. Li J et al (2011) miR-203 reverses chemoresistance in p53-mutated colon cancer cells through downregulation of Akt2 expression. *Cancer Lett* 304(1):52–59
75. Deng B et al (2016) MiRNA-203 suppresses cell proliferation, migration and invasion in colorectal cancer via targeting of EIF5A2. *Sci Rep* 6:28301
76. Li Z et al (2015) MiR-203 suppresses ZNF217 upregulation in colorectal cancer and its oncogenicity. *PLoS ONE* 10(1):e0116170
77. Della Vittoria Scarpati G et al (2014) Analysis of differential miRNA expression in primary tumor and stroma of colorectal cancer patients. *Biomed Res Int* 2014:840921
78. Balcerczak E et al (2005) Cyclin D1 protein and CCND1 gene expression in colorectal cancer. *Eur J Surg Oncol* 31(7):721–726
79. Li L et al (2008) Association between phosphatidylinositol 3-kinase regulatory subunit p85alpha Met326Ile genetic polymorphism and colon cancer risk. *Clin Cancer Res* 14(3):633–637
80. Dweep H, Gretz N (2015) miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat Methods* 12(8):697
81. Hsu JB et al (2011) miRTar: an integrated system for identifying miRNA-target interactions in human. *BMC Bioinformatics* 12:300
82. Zhang GJ et al (2014) MiR-378 is an independent prognostic factor and inhibits cell growth and invasion in colorectal cancer. *BMC Cancer* 14:109
83. Wu X et al (2015) The potential value of miR-1 and miR-374b as biomarkers for colorectal cancer. *Int J Clin Exp Pathol* 8(3):2840–2851
84. Li H et al (2014) Clinical and biological significance of miR-378a-3p and miR-378a-5p in colorectal cancer. *Eur J Cancer* 50(6):1207–1221
85. Qin YZ et al (2015) Screening and preliminary validation of miRNAs with the regulation of hTERT in colorectal cancer. *Oncol Rep* 33(6):2728–2736
86. Wang YX et al (2010) Initial study of microRNA expression profiles of colonic cancer without lymph node metastasis. *J Dig Dis* 11(1):50–54
87. Chang KH et al (2011) MicroRNA signature analysis in colorectal cancer: identification of expression profiles in stage II tumors associated with aggressive disease. *Int J Colorectal Dis* 26(11):1415–1422
88. Motoyama K et al (2009) Over- and under-expressed microRNAs in human colorectal cancer. *Int J Oncol* 34(4):1069–1075
89. Mosakhani N et al (2012) MicroRNA profiling differentiates colorectal cancer according to KRAS status. *Genes Chromosom Cancer* 51(1):1–9