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ORIGINAL ARTICLE

Altered miR-21, miRNA-148a Expression in Relation to *KRAS* Mutation Status as Indicator of Adenoma-Carcinoma Transitional Pattern in Colorectal Adenoma and Carcinoma Lesions

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Abstract

Sporadic colorectal cancer (CRC) is a fatal disease, mostly known as the silent killer, due to the fact that this disease is asymptomatic before diagnosis in advanced stage. Screening and the early detection of CRC and colorectal adenoma (CRA) by non-aggressive molecular biomarkers' signature is useful for improvement of survival rate in CRC patients. To achieve such a goal, a better understanding of distinct molecular abnormalities as candidate biomarkers in CRC development is crucial. In this study, seventy-five archived FFPE CRC samples, including colorectal adenocarcinoma, adenomatous polyps (adenoma), and adjacent non-neoplastic mucosa were collected for the investigation by Sanger sequencing at the DNA level and by real-time PCR at the RNA level. The results of the *KRAS* mutational analysis have shown that the majority of somatic mutations in the *KRAS* affect only one codon, mainly codon 12(p.G12D) with low frequency in adenomas (13.3%) versus CRCs (36%). The results of dysregulated epigenetic changes of miR-21 clearly showed upregulation of expression in colorectal adenocarcinoma, compared to non-neoplastic mucosa, in colorectal adenoma vs non-neoplastic mucosa: ($p < 0.001$) and in CRC versus adenoma ($p < 0.001$); while miR-148a expression were significantly downregulated in CRC, compared to non-neoplastic mucosa, in colorectal adenoma vs non-neoplastic mucosa, and in adenoma vs CRC ($p < 0.001$). Our findings support the important role of miR-21 in stages I–II of CRC, and the *KRAS* G12D mutant, and differential miR-148a expression, in advanced stages of CRC.

Keywords Sporadic CRC · miR-21 · miR-148a · The *KRAS* G12D mutant · CRC development

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Introduction

Colorectal cancer (CRC) is mainly a prevalent and lethal form of gastrointestinal cancer, among which roughly 70% of all cases are considered sporadic (Yamagishi et al. 2016). Colorectal cancer survival is extremely relying on the stage of disease at the diagnosis. The early detection is crucial, as, the earlier stage patients (TNM stages I–II) have a higher chance of survival than advanced-stage patients (TNM stages III–IV) (Chuang et al. 2016). An overwhelming majority of colorectal tumor cases (Approximately 95%) are ultimately regarded as adenocarcinoma (AC) (Soto et al. 2016), progressing from a benign colorectal adenoma to a malignant carcinoma with even metastatic dissemination (Adenoma-carcinoma sequence) (Enquist et al. 2014). The molecular carcinogenesis of sporadic CRC is very complex and multifarious, and resulted from a series of genetic and epigenetic instability during CRC development (Grady and Carethers 2008). The genetic alterations in cancer cells, such as oncogenic mutations in the *KRAS*, are responsible for the reactivation of the RAS/MAPK kinase pathway (Armaghany et al. 2012), cancer initiation (Margetis et al. 2017), progression (Boutin et al. 2017), and failure to respond to EGFR inhibitors in colorectal cancer (Knickelbein and Zhang 2015). In addition, the significance of the genetic perturbations, as contributing factors in CRC genesis, epigenetic perturbations, such as altered expression levels of various miRNAs has also been demonstrated newly, as a major driver of CRC initiation and progression (Colussi et al. 2013).

MicroRNAs (miRNAs) are described as endogenous short, single-stranded RNAs, which epigenetically regulate the gene expression, based on the post-transcriptional process (Colussi et al. 2013). In the literature, most notably reported that abnormal expressions of key miRNAs, such as miRNA-21, miRNA-148a, miRNA-143, miRNA-145, and several other miRNAs have consistently involved in CRC, compared with non-cancerous tissue (Sun et al. 2016; Baltruskeviciene et al. 2017). The central role of integrated-signature miRNAs has been also reported contributing to CRC development (Eslamizadeh et al. 2018). Early detection and therapeutic intervention programs for CRCs and adenoma polyps in patients with increased risk of malignant transformation have contributed to the downward trends in CRC incidence and mortality (Lionis and Petelos 2011). For early detection and treatment enhancement of CRC, the molecular understanding of CRC development is crucial (Konda et al. 2014). Furthermore, miRNAs can be considered as a potential diagnostic, therapeutic, and prognostic molecular biomarkers in colorectal cancer (Price and Chen 2014). As two of the most researched and extensively studied miRNAs, miR-21 and miRNA-148a have a high degree of validity, and thus exhibit huge diagnostic potential as two biomarkers for early detection of CRC (Takahashi et al. 2012; Hibino et al. 2015; Penget al. 2017).

MiR-21, as an excellent example of oncogenic miRNA (oncomiR), modulates oncogenesis, and can promote the malignant transformation through induction of proliferation, cell cycle progression, invasion, and inhibition of apoptosis by repressing PTEN protein expression in CRC cells (Wu et al. 2017).

Recently, there has also been accumulating evidence that miRNA-148a is involved in major carcinogenesis pathways, which by its silencing and potentially other target genes, such as DNMT1, an epigenetic regulator; ERBB3—involved in angiogenesis; CDC25B—cell cycle regulator, and IGF-IR, AKT, MAPK/ERK—cell growth, caused significant inhibition of cell proliferation, migration, invasion, apoptosis, and angiogenesis (Baltruskeviciene et al. 2017). To the best of our knowledge, miR-21 alone was not sufficient for discrimination of tumors or precursor lesions of CRC patients from normal controls, while miR-21-associated combination markers improved the diagnostic accuracy in tissue-based samples (Peng et al. 2017).

Consequently, this study has focused on the potential biomarkers, such as miRNA-21, miRNA-148a signature, and the definitive mutation of the *KRAS,* because of the growing evidence of their importance in the progression of sporadic benign adenomas to malignant carcinomas (initiation), tumor invasion and metastasis (progression) in specific stages of CRC (Liu et al. 2011; Hibino et al. 2015; Palmirotta et al. 2011; Bullock et al. 2013). Accordingly, we hypothesized that miRNA-21, miRNA-148a, and *KRAS*-mutation status together may be able to generate a model of adenoma–carcinoma transitional pattern for early detection. Therefore, for discovery of such a relationship, we performed qRT-PCR, using SYBR-Green I, and DNA sequencing analysis, on adenocarcinomas, adenoma, and paired non-neoplastic colon tissues from different individuals.

Materials and Methods

Patients and Tissue Samples

Sixty tissue samples from colorectal adenocarcinoma and adjacent non-neoplastic mucosa, from patients who underwent surgical excision for CRC, and 15 colorectal adenoma samples from patients were tubulovillous adenomas, who had endoscopic mucosal resection, at Imam Khomeini University Hospital in Ahvaz, were chosen for qRT-PCR and DNA sequencing analysis, between January 2006 and December 2014. The collaborating pathologists in Khuzestan province confirmed the colorectal adenoma and invasive/diffuse type (signet ring) adenocarcinoma colorectal diagnoses. The staging of tumors was determined, according to the American Joint Committee on Cancer TNM (tumor, node, metastases) staging system.

Extraction of Genomic DNA

DNA was extracted from 10-μm-thick FFPE tissues, using the QIAmp DNA FFPE tissue kit (Qiagen, Valencia, California, USA), following the manufacturer's instructions.

DNA Amplification by PCR

In current study, only 25 tumor samples were analyzed for detection of *KRAS* mutations. The *KRAS* primers were as follows: Forward: 5′-TGGTGGAGTATT

TGATAGTGTA-3′, Reverse: 5′-CATGAAAATGGTCAGAGAA-3′. The amplified fragments had 400–600 bp. A 20-µl mixture was prepared for each reaction, including 2 µl PCR buffer (10×), 21 µl (50 mM) MgCl2, 0.4 µl dNTPs (10 mM), 2 µl of each primer (10 µM), 0.4 µl Taq polymerase (5 U/µl) (Qiagen), and 1 µL template DNA. The amplification program was 95 °C for 5 min; 40 cycles of 94 °C for 30 s, 60°C for 30 s, 72ºC for 15 s, followed by the final elongation step at 72 °C for 5 min. PCR products were evaluated by electrophoresis on 1.5% gel. After extracting the single band of the PCR product, the mutation was determined by sequencing technique.

DNA Sequencing Analysis

The products from the Sanger sequencing reaction were analyzed on an automated sequencing machine, ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, CA).

RNA Isolation and Real-Time PCR

For the preliminary Real-time PCR, total RNA was extracted from 5- to 10-micron tissue slices, using the high-pure miRNA Isolation Kit (Roche, Germany), as per the manufacturer's instructions. Due to the low concentration of miRNA in tissue, it was not possible to measure the miRNA concentration and purity, using spectrophotometry; our samples were at or below the limit of detection of the Nano Drop (ND-2000, Thermo Scientific, USA).

One microgram of total RNA was reverse transcribed to cDNA, using the miScript II RT Kit (Qiagen, Inc., Valencia, CA, USA). MiR-21¸ miR-148a, and the reference gene U6 were amplified, using gene-specific stem-loop primers. The cDNA was used for the quantification of miR-21 and miR -148a. Real-time PCR was performed, using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara, USA), according to the manufacturer's protocol. The reactions were carried out, using a StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc., USA). The differential expression of miR-21 and mir-148a mRNAs estimated as the Ct values and normalized, using U6 as internal controls.

Primers sequences for miR-21 (RT primer: 5′GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGACTCAACA-3′; forward: 5′ CGGCGGTAG CTTATCAGACTGATGT-3′; reverse: 5′-GTGCAGGGTCCGAGGT 3′), miR -148a (RT primer: 5′GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACACAAAGT3′; forward: 5′-AGCTGTTCAGTGCACTACAGA-3′; reverse: 5′-GTGCAGGGTCCGAGGT3 U6 (RT primer: 5′-CGCTTCACGAATTTGCGT GTCAT-3′; forward: 5′-CTCGCTTCGGCAGCACA-3′; reverse: 5′-AACGCTTCA CGAATTTGCGT-3′), were synthesized by the pishgam biotech companies (Iran). The relative expression level was calculated, using the $2^{-\Delta\Delta Ct}$ method. All reactions were carried out in duplicate within the same PCR run.

Statistical Analysis

SPSS 24.0 (Munich, Germany) was used for statistical analysis. Results were representative of two independent experiments. The Kolmogorov–Smirnov normality test was performed to assess of normality and variables were normally distributed. MiRNAs were presented as the mean ± standard error of the mean (SEM). The student *t*-test or Chi-square test was used for analyzing the difference of the mean value between two groups and the relation between two categorical variables, appropriately. One-way analysis of variance (ANOVA) test with Tukey's post hoc was also used for analysis of the differences within more than two groups. In all different analyses, the probability or p value of < 0.05 was considered statistically significant.

Results

The Mutations Frequencies and Distributions of KRAS and Clinicopathological Characteristics of Mutations in Patients with CRC and Polyp

The results of the *KRAS* mutational analysis in the CRC and the adenoma polyps have shown that the majority of somatic mutations in the *KRAS,* affect only one codon, mainly codon 12(p.G12D) with GGT→GAT substitution. All patients, in codon 13 of exon 2 and codon 61 of exon 3, harbored wild-type *KRAS*. Somatic mutations (p.G12D) were found in 2 (13.3%) of the 15 of colorectal adenoma patients with high frequency (100%) in the colon, compared to the rectum (supplementary table). In addition, The *KRAS* p.G12D mutations were found in 9 (36%) of the 25 of CRC patients. The relationship between the *KRAS* mutations and the clinicopathological characteristics of the CRC patients is summarized in Table 1. In general, all patients were divided into two groups according to their *KRAS* mutation status (wild type vs. *KRAS* p.G12D mutations). The frequency of p.G12D mutations, according to TNM stage was 0% in stage I, 11.1% in stage II, 66.7% in stage III, and 22.2% in stage IV. Colorectal carcinomas with p.G12D mutation were positively associated with stage classification mutations, resulting in a trend towards a high frequency (88.9%) of *KRAS* 12 mutation, in advanced stage disease ($p = 0.152$). In addition, our data suggest that the patients with a mutant *KRAS* p.G12D had also a positive correlation with lymph node metastasis ($p = 0.011$). However, no significant association was found between mutant *KRAS,* p.G12D, and age ($p = 0.835$), gender ($p = 0.093$), pathological type ($p = 0.251$), the colonic site of the tumor (55.6 vs. 44.4%; $p = 0.734$), tumor size greater than 30 mm in diameter ($p = 0.973$), vessel and lymphatic invasion ($p = 0.432$), and metastasis ($p = 0.169$) (Table 1).

Table 1 Distributions of clinicopathologic characteristics by *KRAS* codon 12 mutation

Feature	*KRAS* P.G12D mutation	Wild-type *KRAS*	*p* value
N (%)	9 (36)	16 (64)	
Age (years)			
≤ 50	3 (33.3)	6 (37.5)	0.835
> 50	6 (66.7)	10 (62.5)	
Gender			
Male	7 (77.8)	10 (62.5)	0.093
Female	2 (22.2)	6 (37.5)	
Location			
Colon	5 (55.6)	10 (62.5)	0.734
Rectum	4 (44.4)	6 (37.5)	
Tumor size (mm)			
≤ 30 mm	6 (66.7)	6 (37.5)	0.973
> 30 mm	3 (33.3)	10 (62.5)	
Tumor differentiation			
Well	1 (11.1)	6 (37.5)	
Moderate	6 (66.7)	9 (56.3)	
Poor	2 (22.2)	1 (6.3)	0.251
TNM stage			
I	0 (0)	2 (12.5)	0.152
II	1 (11.1)	5 (31.3)	
III	6 (66.7)	8 (50)	
IV	2 (22.2)	1 (6.3)	
Lymph vascular invasion			
Negative	5 (55.6)	9 (56.3)	0.432
Positive	4 (44.4)	7 (43.8)	
Lymph node metastasis			
Negative	2 (22.2)	12 (75)	0.011*
Positive	7 (77.8)	4 (25)	

Note: Chi-square test was used to evaluate how relation between two categorical variables

*p value < 0.05 was considered statistically significant

Distributions of miR-21 Tissue Expression and Its Clinical Implications in CRA, CRC Patients with KRAS Status (Wild Type Versus Mutated), Compared to Adjacent Non-neoplastic Mucosa

To better comprehend the potential biological significance of altered miR-21 expression in CRC progression, we evaluated miR-21 expression in 60 pairs of human CRC tissues and their corresponding non-tumor tissues, using qRT–PCR. Significant upregulation of miR-21expression was detected in 50 CRC cases (83%), compared with the corresponding non-neoplastic mucosa. We also examined miR-21 in 15

colorectal adenoma samples, and compared with its expression in CRC and non-neoplastic colon mucosa samples. It was observed that miR-21 expression was clearly upregulated in colorectal adenocarcinoma, compared to non-neoplastic mucosa ($p < 0.001$), in colorectal adenoma vs non-neoplastic mucosa ($p < 0.001$), and in CRC vs adenoma ($p < 0.001$) (Fig. 1a). A comparison with other clinical parameters showed that clinical parameters, such as age ($p = 0.583$), gender ($p = 0.074$), tumor size ($p = 0.108$), were revealed no significant correlation with miR-21 expression levels, measured as fold change. Nevertheless, there was a significant difference between *KRAS* mutational status ($p = 0.004$), localization ($p = 0.006$), pathological type ($p = 0.003$), and miR-21 expression. Our data revealed a lack of aberrant high-level tumor miR-21 expression in patients with advanced CRC (stage III, $p = 0.021$ and stage IV, $p = 0.049$) with metastases, compared with stage II of CRC (Table2; Fig. 2).

MiR-148a Differential Tissue Expression and Its Clinical Characteristics in CRA, CRC Patients with KRAS Mutation Status (Wild Type Versus Mutated), Compared to Adjacent Non-neoplastic Mucosa

From the 65 cases included in these analyses, the miR-148a expression was significantly downregulated in slightly more than 80% of CRCs, compared to non-neoplastic mucosa ($p < 0.001$, in colorectal adenoma (71%) vs non-neoplastic mucosa

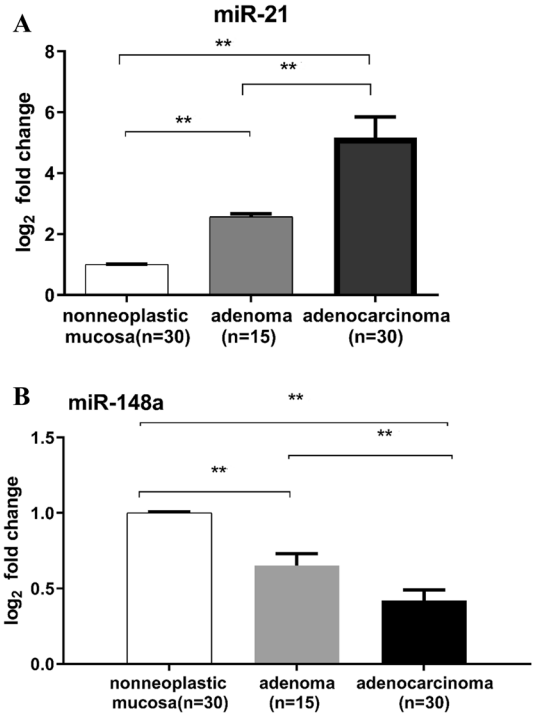

Fig. 1 The differences between miR-21 (**a**) and miR-148a (**b**) expression in non-neoplastic colon mucosa, colorectal adenoma, and CRC tissue. The miRNAs expression levels are normalized to U6 snRNA. One-way analysis of variance (ANOVA) test followed by post hoc analyses with Tukey's test was used for analysis of significant differences between more than two groups. The statistical significance was designated with asterisks (**, $p < 0.001$) and p value less than 0.001 was considered as significant

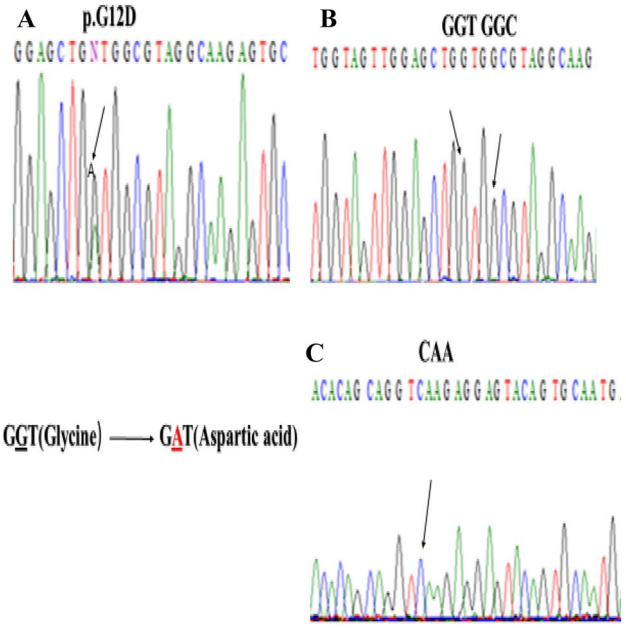

Fig. 2 Direct Sequencing to detect *KRAS* Mutations. **a** Human colorectal cancer and adenoma DNA samples showed the only mutant heterozygote type of p.G12D in *KRAS* codon 12 mutation in tissue **b** Colorectal cancer and adenoma DNA samples of wild-type *KRAS* in other sites' codon 12 and codon 13 **c** Colorectal cancer and adenoma DNA samples of wild-type *KRAS* at codon 61

($p < 0.001$), and in adenoma vs CRC ($p < 0.001$) (Fig. 1b). In addition, no significant association was found between miR-148a expression and clinicopathological features, for instance, age ($p = 0.976$), gender ($p = 0.376$), tumor size ($p = 0.173$), site of the tumor ($p = 0.152$), and pathological type ($p = 0.850$). Taken together, our results indicate that decreased miR-148a expression in CRC was associated with advanced clinical stage, lymph node metastasis, and *KRAS* mutational status. That way miR-148a revealed a significant down-regulation in patients with lymph node metastasis (stage III), compared to patients with no metastasis (stage II) ($p = 0.001$), showing that this microRNA has important roles in CRC progression and distant metastasis (IV) (Table2). However, no significant differences were found between stage III and stage IV ($p = 0.581$).

Discussion

The majority of the mutations in the *KRAS* are point mutations at codons 12 and 13 (exon 2), but less often in codon 61 (exon 3), with nearly 80% happening in codon 12 and 15% in codon 13 (Palmirotta et al. 2011), respectively. In clinical research, the focus is on the most frequent KRAS mutations in codon 12 (p.G12D) (p.G12V), and codon 13 (p.G13D) transversion, frequently occurring in advanced adenomas and CRCs (Lorentzen et al. 2016). In the first section of the current study, we

Table 2 The association of miRNA-21 and miRNA-148a, with clinical and pathological characteristics in CRC patients

Characteristic	*N*	miRNA 21 expression Mean ± SEM	*p* value	miRNA 148a expression Mean ± SEM	*p* value
Age(years)					
≤ 50	2	3.52 ± 1.5	0.583	0.61 ± 0.21	0.979
> 50	28	4.28 ± 0.44		0.35 ± 0.04	
Gender					
Male	21	3.89 ± 0.43	0.074	0.38 ± 0.06	0.376
Female	9	5.02 ± 98		0.36 ± 0.08	
Location					
Colon	22	4.66 ± 0.53	0.006**	0.39 ± 0.05	0.152
Rectum	8	3.04 ± 0.39		0.37 ± 0.15	
Tumor size mm					
≤ 30 mm	2	2.88 ± 0.60	0.108	0.31 ± 0.08	0.173
> 30 mm	28	4.32 ± 0.44		0.39 ± 0.05	
Tumor differentiation					
Well	13	5.38 ± 0.59		0.50 ± 0.09	
Moderate	13	2.62 ± 0.19	0.003**	0.30 ± 0.050	0.095
Poor	4	2.53 ± 0.51		0.26 ± 0.55	
Lymph vascular invasion					
Negative	22	4 ± 0.86	0.678	0.48 ± 0.05	0.003**
Positive	8	4.30 ± 0.49		0.10 ± 0.02	
TNM stage					
I	4	4.49 ± 1.37	0.001**	0.57 ± 0.07	0.003**
II	9	5.83 ± 0.55		0.56 ± 0.11	
III	13	2.63 ± 0.23		0.25 ± 0.04	
IV	4	1.96 ± 0.15		0.19 ± 0.03	
KRAS					
Wild	16	4.92 ± 0.53	0.004**	0.47 ± 0.05	0.001**
Mutation	9	2.61 ± 0.29		0.13 ± 0.02	
Lymph node metastasis					
Negative	22	3.52 ± 0.75	0.961	0.47 ± 0.05	0.037*
Positive	8	2.65 ± 0.88		0.27 ± 0.05	

Values were calculated by the student t-test for comparisons between both experimental groups and one-way analysis of variance (ANOVA) test with post hoc Tukey for analysis of significant differences between experiments with more than two groups and one variable. "*" and "**" marks were considered to distinguish between statistically significant ($p < 0.05$) and statistically very significant ($p < 0.001$). TNM system; describes Tumor, node, metastases of cancer

investigated the role of *KRAS* mutation subtypes within the adenomatous polyp and adenocarcinoma CRC to determine adenoma transformation from benignancy to malignancy. In our cases, only mutant heterozygote types were p.G12D, accounting for 100% of all mutations. In this study, low frequency of *KRAS* mutation in

codon12 (13.3%) and no acquired mutations in codon13 were detected in sporadic colorectal adenomas, in contradiction with other reports that have recorded the highest rate of *KRAS* mutations in codons 12 and codon 13, as most common recurring molecular events in colorectal adenoma (Lorentzen et al. 2016). However, our data are consistent with the study of Zauber et al. (2013), reporting that adenomas occurring in the colon were closely related to the *KRAS* mutation at codon 12. The incidence rate of *KRAS* mutations in CRC was approximately 36%, consistent with other studies (Tan and Du 2012), and no mutation was identified at codon 13 in contradiction to other studies (Payandeh et al. 2017). Our data are also consistent with a recent study, showing that *KRAS* mutations are mainly localized in codon12, but not in codon 13 (Li et al. 2015). Frequency of *KRAS* codon 61 mutations in colorectal adenomas is mostly unknown and these mutations were detected only in 2% of CRC (Lorentzen et al. 2016). No mutation was found in this codon in adenoma and CRC cases. To date, four independent cohorts, using Chinese patients with CRC, have reported that *KRAS* mutations were associated with patient gender (Ye et al. 2015); while ours and some studies have not found (Palomba et al. 2016) a link between KRAS mutations and gender ($p=0.093$) and age ($p=0.835$). In addition, in accordance with previous reports, in our cases, the tumors in patients with advanced stage (88.9%, $p=0.152$), lymph node metastasis (77.8%, $p=0.011$) tend to harbor mutations at codon 12 (Li et al. 2015). These data are in parallel with experimental evidence of the study of Li et al. (Li et al.2015), showing that *KRAS* codon 12 mutations confer a more aggressive tumor phenotype than codon 13 mutations. In another study, including 966 CRCs cases, Ye et al. (Ye et al. 2015) indicated that *KRAS* mutations were associated with well-differentiated tumor. While in our study, the *KRAS* codon 12 mutation was associated positively with invasive compare to diffuse type of colorectal adenocarcinoma (7 (77.8%) vs. 2 (22.2%), $p=0.049$, data not shown) and was occurred frequently in moderately and poorly differentiated tumors (66.7% and 22.2%, $p=0.251$, Table 1), but the difference was not statistically significant. Additionally, we found that no apparent significant differences were between the *KRAS* codon 12 mutations and other histopathological criteria, such as tumor size (66.7%, $p=0.973$), lymph vascular invasion (44.4%, $p=0.432$), a finding that is consistent with the study of Liu et al. (2011).

It has been reported that stable overexpression of miR-21 occurred during the progression of colorectal tumor (Wu et al. 2017). In the second part of our study, we determined miR-21 expression alterations, using quantitative RT-PCR method for mature microRNA expression analysis, in colorectal adenoma and carcinoma samples. Healthy colonic tissue samples were also evaluated from the same patients. In the literatures, there is evidence that miR-21 expression levels in patients with advanced adenoma and CRC were higher than those detected in healthy controls consistent with our findings. In a seminal study, Uratani et al., also observed that miR-21 are frequently expressed in premalignant lesions, such as colonic adenomas, as the target lesions of CRC screening (Uratani et al. 2016). However, the level of expression in the adenomatous tissue sample was reported less than that of the cancerous tissue. Recent studies have discovered circRNAs that exhibited a promotive function as microRNA sponges in cancer cells (Han et al. 2017). Notably, miRNA sponging function has been demonstrated, as a miR-21 sponge actively

causes post-transcriptional repression of miR-21 and cell proliferation and increases the expression of miR-21 target genes including the cancer protein DAXX in gastric cancer. Actually, synthetic circRNA sponges targeting oncomiRs could be considered, a simple, effective, convenient strategy to achieve targeted loss of miRNA function in vitro, with promising future for therapeutic application in human patients (Liu et al. 2018).

Liu et al. demonstrated that miR-21 upregulation in CRC patients was positively associated with the age, gender, and tumor size, which is inconsistent with our current findings (Liu et al. 2011). Here, we reported that high levels of miR-21 in CRC patients were correlated with early stage (stage II) (5.83 ± 0.55, $p < 0.001$), colonic site of the tumor ($p = 0.006$), and well differentiation (5.38 ± 0.59, $p < 0.001$). Interestingly, our results contradicted previous miR-21 expression studies, reporting that an overexpression of miR21 has been linked to more advanced TNM stages (Schee et al. 2012) and poor differentiation (Wu et al. 2017). In fact, mir-21 upregulation has been found in stage II colon cancer, compared with the advanced stages, in agreement with the previous study by Nielsen et al. (2011) and in another study that high miR-21 levels were determined only in a subgroup of patients with stage II (Nielsen et al. 2011). Lymph vascular invasion has been determined as an indication of lymph node metastases (Yuan et al. 2017), which often constitutes the step prior to increasing tumor malignancy, such as distant metastatic spread (Ulintz et al. 2018). In this research study, no significant relation was found between the miR-21 expression level, in patients presenting with advanced CRC and lymphatic invasion ($p = 0.678$), and positive lymph node metastasis ($p = 0.961$). It is noteworthy that the gradual increase of miR-21 expression, in the oncogenic process of colon cancer, supports the important role of miR-21 as dynamic biomarker for early stages of colon cancer development.

In agreement with Lee et al. studies (2016), our findings demonstrate that the lack of aberrant high-level tumor miR-21 expression in patients with advanced CRC, compared to stage II of CRC, suggests that the invasion and metastasis are tightly controlled by other regulatory pathways. Further studies are required to prove independent biomarkers other than miR-21 that can be used to control further invasion and metastasis. Therefore, because miR-21 expression was heterogeneous in advanced CRC, there is a strong need to use other quantitative measures, such as sensitive, non-invasive biomarkers that could facilitate the detection of the development and progression of cancer in advanced stages. For this reason, in the second phase of this study, we also evaluated the miR-148a expression level.

Our studies show that miR-148a expression is significantly downregulated in CRC mucosa and colorectal adenoma, compared to non-neoplastic, and the expression in term of fold change was less than the cancerous tissue, across the adenoma tissue ($p < 0.001$), referring to the importance of miR-148a in CRC initiation (Hibino et al. 2015). Here, no significant correlation between miR-148a expression and clinicopathological features, for instance, age, gender, tumor size, site of the tumor, and pathological type has been found. Furthermore, data acquired from clinicopathological analysis revealed that down-regulation of miR-148a expression was significantly associated with advanced clinical stage, lymph node invasion, and lymph node metastasis in the CRC patients. Significant down-regulation of miR-148a in tumors

with lymph node invasion (stage III), compared to tumors with no invasion (II), suggests that this microRNA has important roles in CRC progression and distant metastasis (IV). However, no significant differences between stage III and stage IV were found, unlike previous studies. Takahashi et al. also found a significant negative correlation between miR-148a expression and clinical stage of CRC tissues, showing that miR-148a expression was most significantly downregulated, in advanced CRC tissues (stage III and stage IV) (Takahashi et al. 2012). In this clinical research, also the correlation between the expression of miR-21 and miR-148a and the pathologic parameters have been analyzed, including *KRAS* mutation status, showing significant differences between miR-21, miR-148a expression, and wild and mutant adenoma ($p < 0.001$). In addition, it turned out that *KRAS* mutation was associated with high miR-21 expression ($p = 0.004$), and low miR-148a expression ($p = 0.001$). Further studies with larger sample size are required to confirm if these candidate biomarkers and histologic parameters can be used as a screening tool for triaging cases, in early and advance CRC developmental testing.

Conclusions

These results do support the hypothesis that the relation between tissue-specific alteration of miR-21 and miR-148a expression in *KRAS* variants, during progression in a stepwise manner throughout the normal-adenoma-adenocarcinoma sequence, will help us to understand their functional role as biomarkers, which together are useful, along the whole spectrum of CRC development.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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