RESEARCH ARTICLE

Diagnostic and Prognostic Value of miR-205 in Colorectal Cancer

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Abstract

Emerging evidence has shown associations of microRNA-205 (miR-205) with crucial cell processes such as the epithelial-mesenchymal transition (EMT) and aberrant expression with tumorigenesis in many types of human malignancy. This prospective study characterized the contribution of miR-205 to the colorectal cancer (CRC) tumorigenesis. The real-time reverse transcription–polymerase chain reaction was used to examine miR-205 levels prospectively in 36 pairs of samples of CRC tissue and adjacent noncancerous tissue (>2 cm from cancer tissue). In addition, the relationship between miR-205 levels and clinicopathological features was explored. The capability of miR-205 to function as a tumor marker was also examined. miR-205 expression levels did not show significant changes overall. However, miR-205 was significantly downregulated in a group of CRC samples compared with matched noncancerous tissue samples. Moreover, decreased miR-205 correlated significantly with lymphatic metastasis. A receiver operating characteristic (ROC) curve also showed an optimum cut off point of 1.4×10^{-3} to distinguish lymphatic metastatic CRCs from non-metastatic CRCs. Interestingly we found lymphatic metastasis in almost 80% of the depressed samples. This study suggested that miR-205 could be reduced in the majority of metastatic CRCs and the risk of CRC metastasis may be predicted by monitoring miR-205 in patient samples collected at the time of the initial diagnosis. Therefore, targeting miR-205 and its potential environmental activators might be a promising therapeutic option to prevent malignant progression toward metastasis.

Keywords: microRNA - miR-205 - colorectal cancer - metastasis - biomarker

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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer mortality which accounts for 10 percent of cancer deaths (Pourhoseingholi, 2012). CRC has approximately one million new cases every year and affect men and women equally (Siegel et al., 2012). Most patients with early CRC stage are cured by surgery alone whilst additional chemotherapy can provide survival benefits to patients with later stages (Poste et al., 2012; Salimzadeh et al., 2012). Although since the mid-1980's, the death rate for colorectal cancer has been dropping due in part to increased awareness and development in screening approaches, the search for early diagnostic, prognostic and predictive biomarkers is indispensable and would allow to select patients with early stage disease for closer monitoring and possibly systemic treatments (Haghighi et al., 2010; Poste, 2011; Shemirani et al., 2011; Henry and Hayes, 2012).

The involvement of tiny non-coding RNAs known as miRNAs in the development and progression of human cancer is supported by an increasing body of experimental evidence (Iorio and Croce, 2009). Several miRNAs with potential biological and clinical relevance have been identified in colorectal cancer and are being explored (Azimzadeh et al., 2012; Bonfrate et al., 2013; Janbabai, 2013). Each individual miRNA predicted to target multiple genes based on the seed sequence matches in their 3'-UTRs. Therefore, perturbation in miRNAs may result in facilitating tumor initiation and proliferation and/ or inhibiting proliferation and invasion (Ahmed et al., 2009; Bandres et al., 2009).

miR-205 is a highly conserved microRNA and it has been proved that miR-205 shares a similar expression pattern with miR-200 family (Gregory et al., 2008b). miR-205 is an epithelial-specific miRNA and has been shown to orchestrate some cellular processes such as epithelial mesenchymal transition (EMT) and differentiation fate of stem cells in mammary gland (Greene et al., 2010a; 2010b). miR-205 conducts conflicting functions in human cancers, where it may functions either as a tumor suppressor or an oncogene in a single tumor type

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depending on targeted mRNA (Savad et al., 2012; Qin et al., 2013; Zhao et al., 2013; Orang et al., 2014).

As EMT has been reported to be miss-regulated in CRC and miR-205 has been considered to regulate EMT, it is therefore an intriguing possibility that miR-205 serves as a critical molecule in colorectal tumorigenesis and thus is of immediate interest as a candidate in CRC prognosis and treatment (Spaderna et al., 2006; Gregory et al., 2008a). In this paper, we hypnotized that aberrant expression of miR-205 may be an inevitable circumstance in cancer cells. However, expression level of this miRNA has not been yet quantified in colorectal cancer tissue samples and the previous observations raised the question that to what extent the colorectal tissue samples express miR-205 compared to normal tissue samples.

In line with previous studies, we quantitatively analysed the expression levels of miR-205 in CRC tissues, relative to their non-tumor counterparts. Moreover, the potential relationship between miR-205 levels and clinicopathological and prognostic outcomes in CRC patients has been investigated.

Materials and Methods

Patients and tissue samples

In this prospective study, a total of 40 CRC samples and normal adjacent tissues were collected following colonoscopy and sigmoidoscopy at Imam Reza Hospital (Tabriz, Iran), the first affiliated hospital of Tabriz University of Medical Sciences. The non-tumor counterparts were obtained from a section of the resected specimen at the farthest distance from tumor (>2 cm from tumor). All study participants were Iranian-born individuals. The study was approved by the Research Ethics Committee of Imam Reza Hospital in accordance with institutional protocol and informed consents were obtained from all patients. Resected specimens were routinely processed for histopathological assessment. The clinicopathological factors and histological grades of cancer was classified using the TNM staging system of the American Joint Committee on Cancer (AJCC, 2010) and International Union Against Cancer (UICC), according to the standard of the World Health Organization (WHO). A number of cases were excluded from subsequent statistical analysis for the following reasons: the patients had a previous malignancy and/or had undergone chemotherapy, radiation therapy or immunotherapy, the samples had histology other than adenocarcinomas.

Sample preparation and RNA isolation

All the tissue samples were immediately flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. We applied phenol based RNA extraction using TRIzol reagent (Takara) according to the manufacturer's instructions with some small changes. Briefly 1 ml TRIzol LS reagent was added into homogenized tissue sample, pipetted up and down several times, and incubated for 5 minutes at room temperature. 200µl chloroform was added, shaken vigorously for 15 seconds, and incubated for 2-15 minutes at room temperature. A centrifugation at 12,000 rpm for 15 minutes at 4°C was followed. The aqueous phase was transferred into a new Eppendorf tube and 500µl of 100% isopropanol was added, stored at 20°C overnight followed by a 13,000 rpm centrifuge for 10 min at 4°C, pelleting the nucleic acid. The supernatant was removed, and 1 ml of 75% ethanol was added to wash the RNA pellet. The sample was then centrifuged at 7, 500 rpm for 5 minutes at 4°C. The supernatant was removed and the RNA pellet was air dried for 5-10 minutes. 25 µl RNase-free water was pipetted onto the RNA pellet up and down several times, incubated in a water bath at 55-60°C for 10-15 minutes. The concentration of isolated RNA was quantified by NanoDrop 2000 (Thermo Fisher Scientific, USA). The extracted RNAs were stored at -80°C until cDNA synthesis. To degrade any DNA contamination in extracted RNAs we performed a 10 µl DNase I treatment reaction (Takara).

Reverse transcription and quantitative Real-time PCR

Reverse transcription was carried out on 120 ng of total RNA in a final volume of 10 µl reaction system. The 10 µl RT reaction mixture was incubated at 37°C for 60 minutes, 85°C for 5 seconds, and then held at 4°C using the PrimeScript(R)miRNA cDNA Synthesis Kit (Takara) according to the manufacturers' instructions. Then 90 µl of the RNase-free water was added to dilute the RT product.

Real-time PCR was performed using SYBR® Green Supermix (Takara). 4 µl diluted RT product was added into a 10- µl PCR reaction, which also contained 10 µl SYBR Green supermix, 1 µl primer mix (Parsgengan) and 1 µl RNase-free water. miR-205 and 5S rRNA(as a control RNA) primers are also purchased from Parsgengan. All PCR reactions, including non-template controls, were run in triplicate using iQ5 Real-time PCR Detection System (BIO-RAD, USA) as follows: 1) 1 cycle of 95°C for 30 seconds, 2) 45 cycles of 95°C for 5 seconds, 60°C for 30 seconds and 72°C for 30 seconds, 3) 71 cycles of 60°C for 10 seconds. Finally, the raw data were analyzed with the iQ5 Optical System Software version 2.1. All samples were processed in triplicate. The threshold cycle (CT) was defined as the cycle number at which the fluorescence passed the fixed threshold. A control without a template was included in each experiment. The final products of real-time PCR were confirmed by Polyacrylamide gel electrophoresis (PAGE).

Statistical analysis

The expression levels of miR-205 in CRC tissues relative to matched non-tumor counterparts were analyzed using the $2^{-\Delta\Delta Ct}$ method. Briefly, the threshold cycle (Ct) of fluorescence for each sample was determined. ΔCt indicated the difference in expression levels with the Ct value between miR-205 and 5s rRNA ($\Delta Ct=Ct_{miR-205}-Ct_{5s}$), and ΔΔCt indicated the difference in the ΔCt value between cancer tissue and the matched control ($\Delta\Delta Ct=\Delta Ct_{cancer}-\Delta Ct_{control}$). The $2^{-\Delta\Delta Ct}$ value (fold value) was also calculated. When the fold value was <1, there was a low expression of miR-205 in the cancer tissues compared to their non tumorous counterparts. The statistical differences in miR-205 expression in cancer tissues relative to the matched adjacent non tumor tissues were analyzed by a paired t-test. Moreover, the association

between miR-205 expression and clinicopathological parameters was analyzed by a non parametric test (Mann Whitney U test between 2 groups and Kruskal Wallis H test for ≥3 groups). Statistical analysis was performed using the GraphPad Prism 6. Receiver operating characteristic (ROC) curve was also constructed to evaluate the specificity and sensitivity of predicting metastatic CRCs and non-metastatic tissues by miR-205 expression levels and the sensitivity/specificity at various cutoff values was calculated using SigmaPlot 12.5. P values less than 0.05 were considered to indicate a statistically significant difference.

Results

Expression levels of miR-205 in CRC tissues

Among 36 patients with CRC, expression levels of miR-205 were detected using real-time PCR in cancer tissues compared to matched non-tumor counterparts. Generally, miR-205 expression levels were found to be low. However, a single aberrant regulation pattern of miR-205 was not found in our experiments, since both miR-205 up-regulation and down-regulation were observed. Interestingly, significant differences were found in miR-205 expression levels of tumor and normal cohorts when separating two dysregulation types. Table 1 shows the values of ΔCt (means±SD) and fold changes. Differences of miR-205 expression levels were illustrated in Figure 1.

Correlation between miR-205 expression levels and clinicopathological characteristics in patients with colorectal cancer

Table 1. Differentially Expressed miR-205 in Human Colorectal Cancer Compared with Normal Tissues Through Two Anti-parallel Dysregulation, Up- and Down-regulation

Dysregulation	Normal miR-205 expression	Tumor miR-205 expression	Mean fold change	p value
De-regulation*	0.07±0.07	0.04±0.07	0.65	NS
Up-regulation	0.07±0.07	0.07±0.07	1.06	NS
Down-regulation	0.07±0.06	$1.00\times10^{-3}\pm6\times10^{-4}$	0.04	2.1×10^{-3}

*Both up- and down-regulation;**Values of ΔCt are presented as mean±SD; NS, not statistically significant (p≥0.05); p values obtained using Paired t-test

In total, 36 patients (22 male, 14 female) were involved in the study. The non-parametric test between the relative expression of miR-205 in CRC samples and its clinicopathological characteristics have been done to investigate the associations with clinicopathological variables. However, no significant relations were detected between expression levels of miR-205 and clinicopathological characteristics, including age, gender, tumor stage, differentiation, localization, and smoking status.

Nevertheless, decreased expression levels of miR-205 in patients with CRC tended to be associated with lymphatic metastasis as shown by non-parametric tests (p<0.01; Mann-Whitney U test). Patient characteristics with respect to miR-205 expression are shown in Table 2.

Table 2. Relationships between miR-205 Expression Levels in Cancer Tissue Samples from Patients with CRC and Clinicopathological Features

Feature	N	miR-205, ΔCt	Statistical significance	p value
Gender				
Male	22	0.04±0.07	NS	0.77
Female	14	0.04±0.06		
Age (years)				
<65	12	0.09±0.11	NS	0.22
≥65	24	0.02±0.02		
Smoking status				
Never	11	0.07±0.01	NS	0.15
Current or ex-smoker	25	0.03±0.05		
Tumor location				
Colon	21	0.03±0.05	NS	0.13
Rectum	15	0.07±0.09		
Tumor size (cm)				
<5	16	0.06±0.09	NS	0.43
≥5	20	0.02±0.02		
Histological grade				
Well differentiated	17	0.06±0.09	NS	0.64
Moderately differentiated	13	0.03±0.03		
Poorly differentiated	6	0.02±0.03		
pTNM stage				
I+II	22	0.04±0.08	NS	0.45
III+IV	14	0.04±0.04		
Invasion into lymphatic vessels				
Positive	13	0.01±0.01	**	8.3×10^{-4}
Negative	23	0.03±0.03		

*TNM, tumor–node–metastasis; Data presented as mean±SD; NS, not statistically significant (p≥0.05); p values obtained using Mann Whitney U test and Kruskal Wallis H test

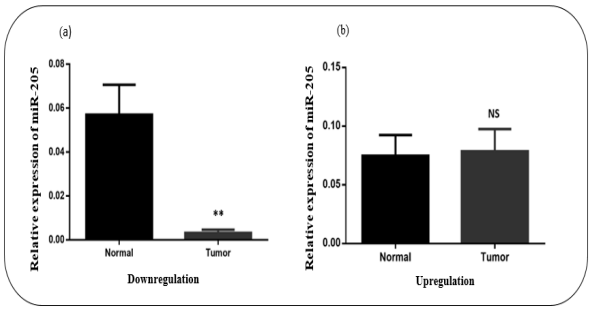

Figure 1. Differential Expression of miR-205. miR-205 relative expression in tumor samples showed a significant decrease compared to normal cohorts in some samples (a). However, in other samples, although miR-205 had undergone upregulation during tumorigenesis process, the p value was not significant (b). p values were **p<0.01 and p>0.05 respectively

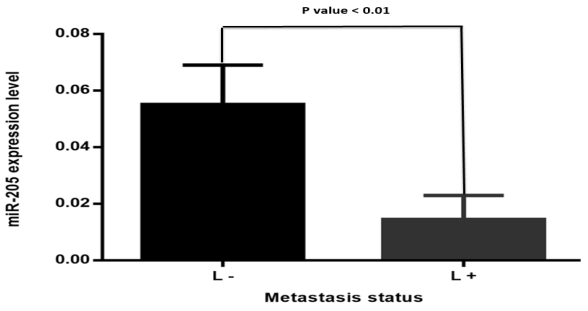

Figure 2. Statistically Different Expression of miR-205 in Two Different Metastasis Statuses. The Mann-Whitney U test of expression levels of miR-205 in cancerous tissues revealed that miR-205 is significantly downregulated in positive lymphatic metastatic samples (L+) compared to non-metastatic tissues (L-) (**p value<0.01)

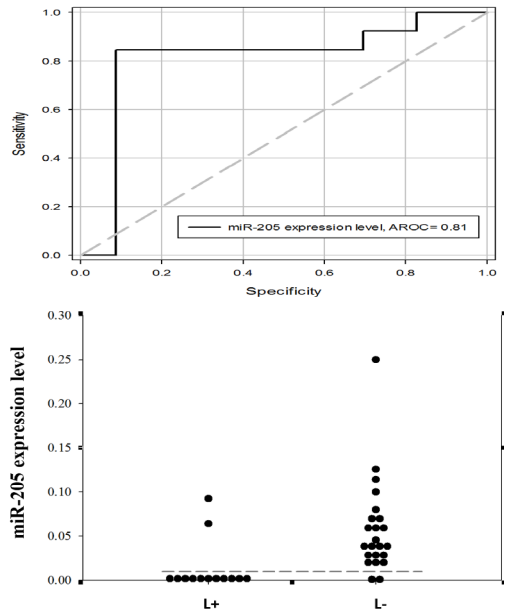

Figure 3. Receiver Operating Characteristic (ROC) in Detection of Lymphatic Metastatic CRCs. The ROC curve was automatically generated from 36 points of cutoff values set by the software SigmaPlot. The area under the ROC curve (AROC) is 0.81 out of 1 (a). The dot histogram shows the optimum 1.4×10^{-3} cutoff point (gray dashed line) where specificity and sensitivity is 91% and 85% respectively (b)

Correlation between miR-205 expression levels and lymphatic metastasis status of samples

Studying the association between miR-205 expression levels and clinicopathological characteristics of patients indicated that miR-205 levels decrease during metastasis (Figure 2). The mean miR-205 expression in metastatic positive tissues (0.01±0.01) was extremely decreased compared with that in non-cancerous tissues (0.03±0.03) ($p<1\times10^{-3}$). Interestingly positive metastatic CRC samples were found to be among 76.9% of samples (11 of 13) in which miR-205 was downregulated.

Capability of miR-205 to function as a CRC tumor marker for lymphatic metastatic CRC diagnosis

Receiver operating characteristic (ROC) curves was constructed and the area under the curve (AROC) was calculated to evaluate the specificity and sensitivity of predicting metastatic CRCs and non-metastatic tissues by miR-205 expression levels (Figure 3). Based on the analysis of ROC curves, miR-205 showed a ROC area (AROC) of 81% (Figure 3a; p value<0.01). The plot shows sensitivity and specificity at different cut-off points. To calculate the optimal cut-off value we performed a post-test from pre-test probability of 0.5 and cost ratio of 1.00. The optimal cut-off point was 1.4×10^{-3} with the highest specificity and sensitivity (Figure 3b).

Discussion

The decision to shed light on the role of miR-205 in CRCs is supported by the evidence that miR-205 is a very critical member of miRNAs. However, its thorough functional role and regulation in all human cancers have not been clearly elucidated yet. Recent findings revealed a converse dual function for miR-205, acting either as an oncogene via facilitating tumor initiation and proliferation, or in some cases as a tumor suppressor through inhibiting proliferation and invasion (Orang et al., 2014).

Moreover an accumulating body of evidence suggests that cells that undergone EMT during tumorigenesis, are most likely to detach from primary tumor, invade through the basement membrane (BM) into circulation and form metastasis at a secondary tumor site (Thiery and Sleeman, 2006; Lim and Thiery, 2012). It is now well-established that miR-205 regulates EMT in adult and embryonic development (Darnell et al., 2006; Tellez et al., 2011). The BM is generally thought to be an important hallmark for tumor migration (Sordat et al., 2000). Many of the differentiated tumor expressing BMs had distant metastasis (Barsky et al., 1983). In line with this, Spaderna et al. (2006) reported that a selective loss of BM correlates with a dedifferentiation/EMT of the CRC cells (Spaderna et al., 2006).

These observations have intensified the study and elucidation of miR-205 levels in CRC specimens compared to normal cohorts. We hypnotized that miR-205 may regulate CRC tumorigenesis and metastasis by regulating BM through EMT pathway. Our results were in consistence with previous findings, indicating that miR-205 has no significant deregulation in CRC samples compared to normal counterparts. Nevertheless, in a group of CRCs that had undergone lymphatic metastasis, miR-205 has significantly decreased (p value of 2.1×10^{-3}). On the other hand, in other samples that miR-205 has been upregulated was just missed the statistical significance. Furthermore, we analyzed the associations between miR-205 expression levels and clinicopathological characteristics of patients with CRC. However, there were no significant relations between expression levels of miR-205 and clinicopathological characteristics, including age, gender, tumor stage, differentiation, localization, and smoking status. Though, miR-205 was found to be significantly downregulated in lymph node metastatic samples compared to non-metastatic CRCs. We also assessed the capability of miR-205 expression level to function as a tumor marker to distinguish metastatic CRCs from non-metastatic samples and we found an optimum cut-off point of 1.4×10^{-3} with 0.91 sensitivity and 0.85 specificity for analyzing CRC metastasis status. In addition, almost all metastatic samples were in downregulated group of miR-205s indicating that the most probable reason for miR-205 downregulation is metastasis.

In conclusion, from these results, we speculate that loss of miR-205 may be a late stage event in CRC progression, occurring in a proportion of the tumor cells that become de-differentiated and have a greater tendency to metastasise via an EMT.

Our data at least suggests that miR-205 could be reduced in the majority of metastatic CRCs and the risk of CRC metastasis may be predicted by monitoring miR-205 in CRC samples collected at the time of the initial diagnosis. In addition, miR-205 is capable of distinguishing lymph node metastatic CRCs from non-metastatic CRCs. Meanwhile, it is unclear whether the

low expression of miR-205 is the cause of metastasis or whether metastasis causes the depression of miR-205 expression. Therefore, targeting miR-205 and its potential environmental activators might be a promising therapeutic option to prevent malignant progression toward metastasis. In either way, it seems clear that the roles of miR-205 in CRC tumorigenesis and metastasis are still undetermined, emphasizing the need for further investigations to clarify its potential clinical diagnostic and therapeutic role.

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