Evaluation of the Chemo-Radiotherapy Effect on the Level of Expression of miR-374 Gene Expression in Rectal Cancer Samples

Azam Ahmadi  
Arak University of Medical Sciences  
Mohamd Reza Bayatiani  
Arak University of Medical Sciences  
Fatemeh Seif  
Arak University of Medical Sciences  
Jamshid Ansari  
Arak University of Medical Sciences  
Parisa Rashidi  
Arak University of Medical Sciences  
Mona Moghadasi  
Arak University of Medical Sciences  
Mobarake Etemadi  
Arak University of Medical Sciences

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Abstract

**Background:** There are several methods for treatment of rectal cancer including surgery, radiation therapy and chemotherapy. Despite all the treatment approaches for cancer, still one of the major challenges in the treatment of cancers is defect in early diagnosis. miRNAs can be used as markers of diagnosis and treatment of cancers. This study will be investigated the effect of radiotherapy on the expression level of miR-374 gene and two its target genes in WNT pathway in Samples of rectal cancer treated with radiotherapy.

**Methods:** In previous study, we predicted the miR-374 that target main genes of the WNT signal transduction pathway using bioinformatics analysis. The RNA extraction and cDNA synthesis were performed on 25 patients with rectal cancer in three periods before and after radiotherapy treatment. The expression levels of miR-374, GSK-3β and APC were evaluated by using qRT-PCR. The amplicon products were sent to sequencing by Macrogen Company. Finally, expression data were evaluated with demographic feathers and significantly of data were evaluated using specific software.

**Results:** The results sequencing confirmed properly amplification reaction. Quantitative RT-PCR revealed significant down-regulation of miR-374 (0.63 folds) and up-regulation of APC (1.12 folds) and GSK-3β (1.22 folds), on patients with rectal cancer after radiotherapy treatment compared with before starting radiotherapy interestingly. The alterations of expression levels of miR-374 gene were critically changed with passing a more time after radiotherapy treatment and related to tumor grading.

**Conclusion:** Our results revealed change in the expression of miR-374 and its two target genes, in patients with rectal cancer after radiotherapy. The evaluation of aforementioned genes in present study can be used as a marker for radiotherapy treatment. In other words finding a reliable diagnostic tumor marker would be critical in more effective therapy of cancer.

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide with high percentage of mortality rate. Types of known colorectal cancers based of originating include adenocarcinoma; a type that originates from mucus-producing cells, Carcinoid tumors; a type derived from specific hormone-producing cells, Gastrointestinal stromal tumors (GISTs); Lymphomas originate from immune cells present in the colorectal tissue, and Sarcomas originate from blood vessel cells. Also a type that originates from the intestinal wall-specific cells called interstitial cells of Cajal (1, 2). Genetic causes of this heterogeneous disease are changes in several genes. Generally, genes involved in CRC are classified into several groups, including oncogenes and proto-oncogenes such as transcription factors, growth factors and their receptors and tumor suppressor genes such as Rb, APC and involved genes in metastasis, repair and apoptosis process. The products of each of these genes are present in different signalling pathways, including the p53, TGFβ, JAK / STAT, PI3K and WNT pathways (3–8). There are several key genes in each signalling pathway. One of the important components in the WNT pathway is beta-catenin which is normally surrounded and degraded by scaffolds from APC, GSK3B, Axin2, etc. After degradation was unable to enter the nucleus and activate proliferative genes such as C-myc, cyclinD, and so on. Deregression of genes causing by mutations in the cancer process lead to survival of beta-catenin, and expression of proliferative genes (9–12). One of the regulatory RNA molecules involved in new oncology topics are miRNAs. Today the role of miRNAs as diagnostic biomarkers is considered by researches (13). Peter Jo et al. in 2017 showed that presented miRNAs in the blood plasma of patients with rectal cancers can be used as biomarkers of response to treatment (12). Today, routine biomarkers for rectal cancer tumors used in chemotherapy regimens include KRAS, BRAF, MSI and SMAD4. Common treatments for colorectal cancer include rectal surgery, radiation therapy, chemotherapy and immunotherapy. Each of these methods has its side effects. Despite all the treatment methods for cancer, it is still one of the major challenges in the treatment of cancers is a defect in early diagnosis. This study will be investigated the effect of radiotherapy on the expression level of miR-374 gene and two its target gene in wnt-pathway in Samples of rectal cancer treated with radiotherapy.

Materials And Methods

Collecting of cancer samples

In this study, 2 ml blood samples from patients with rectal cancer referred to Khansari Hospital, Arak, Iran (2018–2020) were collected the day before radiotherapy and in the first, third and fifth weeks after starting radiation therapy. All patients were treated with radiation as part of curative treatment using a linear accelerator (Elekta, precise) with a daily dose of 1.8 Gy, (dose per fraction: 1.8 Gy). The radiation treatment dose was 45 Gy with 5.4 Gy boost (to a total of 50.4 Gy) for all patients. Radiotherapy was done as a 3D-conformal protocol with 18 MV photon beams (5 days per week).

The number of samples studied was estimated to be 25 cases. These patients also received Folfox. This study was approved by the Ethics Committee of Arak University of Medical Sciences with Code of Ethics (IR.ARAKMU.REC.1397.52).

Evaluation of coding nucleotide sequence of miR-374 in tumoral samples

To ensure conservation of the miR-374 coding region and confirmation of amplicons, its coding sequence was examined in tumor and non-tumor samples using sequencing method.

Performance of amplification and sequencing reactions

Genomic material was extracted using an extraction kit (PZP Cat No. s120796, Iran) from collected blood samples of tumor and non-tumor as control. Non tumor sample (used as control) is related to blood sample from non-cancerous patient. Specific primers (forward: 5’TTTATAATACAACCTGATAGAGCAGGGTCCGAGG 3’ and reverse: 5’AGAGCGGGGTCCGGAGGT 3’) were mixed in an appropriate concentration of 20 pmol with Red mastermix (Amplicon, UK) in a thermocycler (Eppendorf, Germany) at an Annealing temperature of 62.5. The samples were loaded on 1.3% agarose gel (Sinagen, Iran) and their concentration was evaluated. A no
Assessment of gene expression in samples using real-time PCR

The expression levels of miRNAs and their target genes were evaluated by quantitative real-time PCR. On blood samples were performed RNA extraction using RNX kit (Sinaclone, Iran) and then cDNA synthesis using mmulv enzyme (YTA, Iran), random hexamer and stem-loop primers from previous study (data not published). cDNAs were used as templates in a real-time PCR reaction with Master Mix SYBR green (YTA, Iran) and 10 pmol of forward and reverse primers in protocols with annealing temperature 54 degrees centigrade in Roche PCR. Also GAPDH and SNORD47 (U47) used as internal control.

Analysis of statistical results

Expression analysis data in this study were performed using Excel 2007 and GraphPad 7.0 software and the differences between the groups were considered statistically significant at less than 0.05. Sequencing results were also analyzed with Bioedit alignment tool and Chrome software.

Results

Demographic data from the collected samples are shown in the following table.

Table 1: Demographic data of the studied samples

| Characteristic       | Gender | Median age, range | BMI | History of cancer | Grade of tumor | Differentiation | Anatomical distribution | D (%) |
|----------------------|--------|-------------------|-----|-------------------|----------------|-------------------|------------------------|-------|
| Detail               | Male   | ≤ 50              | > 50| < 27              | > 27           | No               | Poorly                | 10.06 |
|                      | Female | ≤ 50              | > 50| < 27              | > 27           | Yes              | Moderately            | 10.06 |
|                      |        |                   |     |                   |                | T2 & T3          | Well                   | 10.06 |
|                      |        |                   |     |                   |                | T1 & unknown     | Sigmoid               | 10.06 |
|                      |        |                   |     |                   |                |                  | Colon                 | 10.06 |
|                      |        |                   |     |                   |                |                  | Rectum                | 10.06 |
|                      |        |                   |     |                   |                |                  | Sigmoid               | 10.06 |
| patients             | 64     | 36                | 28  | 72                | 56             | 85                | 15                     | 68    |
|                      | 36     |                   | 72  |                   | 56             | 15                | 85                     | 68    |

hsa-miR-374 predicted as miRNA target of key WNT signalling pathway receptor genes in a previous study using bioinformatics (data not published).

Evaluation of miR-374 precursor coding sequence was performed in tumor and non-tumor samples after DNA genomic extraction and amplification. The size of bands of products (291 bp) in electrophoresis showed the correctness of the amplification reaction.

The expression of miR-374, APC and GSK3β genes after the first week, third week and fifth week after radiotherapy in the studied samples are shown as column form in Fig. 3 and Fig. 4 respectively using two-ways Anova analysis.

Table 2: Quantification of expression changes of the studied genes at different times after radiotherapy than before

| Fold of | APC expression | GSK-3β expression | miR-374 expression |
|---------|----------------|-------------------|--------------------|
| First week after starting radiation therapy | 1.02 | 1.11 | 0.76 |
| Third week after starting radiation therapy | 1.10 | 1.21 | 0.66 |
| Fifth week after starting radiation therapy | 1.12 | 1.22 | 0.63 |

The analysis of the changes in expression of the studied genes and some demographic features of the affected individuals (age, grade and differentiation) showed a positive correlation (pearson r, p = 0.0005, r = 0.08) between increase in BMI, age and increase in tumor grade (decrease in differentiation) with the fold-change of expression of miR-374.

Discussion
According to statistics from the US Centers for Disease Control and Prevention, CRC is the most common cause of death after lung cancer (1, 2, 14). In the UK, there are about 40,000 diagnoses and 10,000 deaths annually. Stages of CRC include Stage A where the cancer is confined to the mucous membrane, Stage B where cancer spreads to the intestinal wall but the lymph nodes are not infected, Stage C, where the lymph nodes also become cancerous, and finally Stage D. The onset is metastasis. Therefore, early diagnosis is necessary. Existing screening methods are not capable of detecting this cancer early. Therefore, the need for new biomarkers for early detection of this cancer is urgent, and this study investigated this issue. The molecular studies of signalling pathways involved in CRC on cancer samples have revealed that multiple pathways, including the WNT pathway, are altered (15). The components of the signaling pathways in the body can be controlled by miRNAs.

The most previous studies showed that miRNAs have highly conserved gene sequences and over two decades of their identification, there is still much unknown information on their roles and target genes (16–19). In the production process of these molecules, initial processing leads to the production of a nucleotide sequence of 10–160 nucleotides, which is transferred into the cytoplasm. In this study, we amplified miR-374 precursor and the conservation of miR-374 precursor coding sequence in tumoral samples were demonstrated using sequencing (Fig. 2).

Numerous studies have reported altered expression of multiple miRNAs in CRC for example miR-145 inhibits the targeting of hypoxia-inducible factor in many cancers, including neuroblastoma cells, and its down-regulation in carcinoma has been shown to inhibit tumor growth. Numerous evidence suggests that miRNA expression can be a predictive marker for some cancers. In 2009, EKO et al. showed expression alteration of plasma level of miR-92 on 90 patients with CRC, 50 plasma samples of healthy subjects, 20 plasma samples of patients with abdominal pain, and 20 plasma samples of gastric cancer patients. The plasma of patients with CRC was significantly more expressed than healthy controls. This observation is important because the plasma sample is superior to procedures such as biopsy and non-invasive surgery (6). Cai et al. showed in 2015 that miR-144 can reduce ROCK1 migration and proliferation of rectal tumor cells by reducing ROCK1 levels. miRNA and protein expression of ROCK1 and ROCK2 proteins were analyzed using RT-qPCR and Western blot analysis (9). In a 2014 study, they conducted a study to investigate the relationship between miRNA and tumor suppression. In their study, miR-30b was shown to target the rectal tumor via targeting the oncogenes KRAS, PIK3CD and BCL2 (10). In addition to being a diagnostic marker, miRNAs can also be used as markers for the treatment process. One study identified the role of miR-196b in radiation resistance of CRC. A study in 2017 showed that miRNAs can be used as biomarkers to predict treatment response. Based on the results of their study, miR-125b-1, miR-1183, miR-130a, miR-375 had a distinct order of treatment in the sample and control groups before treatment. According to the results of aforesaid study, these miRNAs target key genes such as ATM and CHEK1 that are involved in chemo-radiation.

Peter Jo et al. showed in 2017 that miRNAs in the blood plasma of patients with rectal cancers can be used as biomarkers of response to treatment. One of the results of their study were miR-30c and miR-31 may be differentiated between cancer and non-cancer patients in plasma and used as biomarkers in rectal cancer (12). In our study, we evaluated the expression of key genes of the wnt pathway, including APC, GSK3b, and miR-374 before and after chemoradiotherapy treatment. In a previous study, bioinformatics and in vitro studies of expression changes of these three genes, overexpression of miR-374, and downregulation of its two target genes, APC and GSK3b, were identified in colorectal tumor specimens compared to non-tumor specimens. miR-374 increases its expression and decreases expression of its target genes in cancer cells, leading to activation of proliferative genes in colorectal tumor cells and tissues relative to non-tumor or tumor margins (data not published). The data of our study confirmed the results of the previous research. The study also demonstrated the positive effect of chemoradiotherapy on increasing the expression of the two target genes and decreasing the expression of the miR-374 gene, contrary to what occurs in a tumor cell in rectal cancer (Figs. 3 & 4). It should be noted that 60% of the studied samples received Folfox, used drug in the treatment of bowel cancer during chemotherapy and consist of folinic acid and fluorouracil (Table 2). Therfore, the types of prescribed drug can be effect on gene expression probably. In present study, the results of Quantative RT-PCR showed a decrease in miR-374 expression and an increase in expression of two APC and GSK-3β target genes in CRC patients before and after chemo-radiotherapy. This change in expression increased with increasing radiotherapy sessions compared to before, the start of the treatment process (Figs. 3). The altered expression of these genes can be used as a marker to predict the complications of various therapeutic approaches.

Risk factors for CRC included aging, diet, obesity, smoking, alcohol, FAP familial syndromes, Lynch Syndrome, and Juvenile Polyposis. In our study, we also examined the relationship between changes in the expression of these genes and some of demographic features. The results indicated that the increase in BMI and age are directly related to these changes significantly. Our demographic data suggest that in the studied samples, an increase in BMI and age was associated with an increase in tumor grade and a decrease in differentiation. In addition, with increasing age, in cases where the tumor grade increases and the degree of differentiation decreases, the change in expression of GSK3β and APC genes, along with the increase in the number of chemotherapy sessions, is not very noticeable. However, with increasing age in cases where the tumor grade increases and the rate of differentiation decreases, the expression of the miR-374 gene expression is significant. These data are concordance with other studies that cite factors such as weight and obesity as risk factors for CRC. The results of the present study show that considering the change in the expression of the studied genes due to chemotherapy, it is possible that the rate of this change in expression can be a marker and to follow the effectiveness of the treatment process of chemotherapy. However, it is recommended that more samples of cancer before and after radiotherapy and in diverse populations of geographical distribution be considered.

Conclusion

Given the side-effects of any of the CRC treatment approaches, predicting the impact of the treatment process is valuable, and possibly altering the expression of main components of WNT pathway, as one of key pathways of rectal cancer, and miRNA affecting them, miR-374, could be used as a predictive marker probably.

Declarations

Ethics approval and consent to participate: This manuscript was approved by Arak university ethic commitee. (Ethical Code: IR.ARAKMU.REC.1397.52).
Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors’ contributions: Bayatiani designed research; Moghadasi and Etemadi searched articles and wrote the proposal; Rashidi and Ansari collected the samples; Ahmadi and Seif done the experiments and wrote the paper; Bayatiani revised the paper. All authors read and approved the final manuscript.

Corresponding author: Dr.Fatemeh Seif

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Figures
Figure 1

up: Schematic overview of primers to amplify the miRNA precursor region. down: 291 base-pairs of products on 1.3% agarose gel

Figure 2

Results of sequencing products sequenced in the studied samples using software Bioedit. The sequences are shown in comparison with precursor sequence.

Figure 3

Evaluation of expression of miR-374 genes before and after radiotherapy, Two-way Anova, P<0.05
Figure 4

Evaluation of expression of studied genes before and after radiotherapy, Two-way Anova, P<0.05