Evaluation of Radiotherapy on miR-374 Gene Expression in Colorectal Cancer Patient Blood Samples

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Abstract

Background: Current cancer treatments include surgery, radiotherapy, chemotherapy, and immunotherapy. Despite these treatments, a main issue in cancer treatment is early detection. microRNAs (miRNAs) can be used as markers to diagnose and treat cancers. This study investigated the effect of radiotherapy on miR-374 expression, and APC and GSK-3β, two of its target genes, in the WNT pathway, in peripheral blood samples from radiotherapy-treated colorectal cancer (CRC) patients.

Methods: Peripheral blood was collected from 25 patients before and after radiotherapy. RNA was extracted from the blood and cDNA synthesized. miR-374, APC, and GSK-3β expression was determined by real-time polymerase chain reaction (RT-PCR) and the amplicons were sequenced. Finally, the data were statistically evaluated.

Results: Quantitative RT-PCR revealed significant down-regulation of miR-374 (0.63-fold) and up-regulation of APC (1.12-fold) and GSK-3β (1.22-fold) in CRC patients after five weeks of radiotherapy. Sequencing of PCR-produced amplicons confirmed the conservation of mature and precursor sequences encoding miR-374. miR-374 expression changed with time after radiotherapy treatment and related tumor grading. Increased age and tumor grade positively correlated with decreased miR-374 expression.

Conclusions: miR-374 expression, and that of its two target genes, APC and GSK-3β, changed after radiotherapy. These genes can likely be used as diagnostic radiotherapy markers in CRC.

Keywords: Biomarker, Colorectal cancer, Mir-374, Radiotherapy.

Introduction

Colorectal cancer (CRC) is one of the most common high-mortality-rate cancers. Types of known CRCs include adenocarcinoma, which originates from mucus-producing cells, carcinoid tumors, which derive from specific hormone-producing cells, gastrointestinal stromal tumors (GISTs), lymphoma, which originates from immune cells in colorectal tissue, and sarcoma, which originates from blood vessel cells (1, 2). These various CRCs result from 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, tumor suppressor genes such as Rb, and APC, and those involved in metastasis, repair, and apoptosis. The products of these genes are present in various signaling pathways, including the p53, TGFβ, JAK/STAT, PI3K, and WNT pathways (3-8). Several key gene products are present in each pathway. One component in the WNT pathway is beta-catenin, which is normally surrounded and degraded by scaffolds from APC, GSK3β.
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(glycogen synthase kinase-3 beta), and Axin2 gene products. After degradation, beta-catenin enters the nucleus and activates proliferative genes including C-myc and cyclin D. Gene deregulation caused by mutations in the cancer process lead to the survival of beta-catenin and expression of proliferative genes (9-12). One group of regulatory RNA molecules involved in recent oncology topics is miRNAs. Currently, the role of miRNAs as diagnostic biomarkers is being considered by researchers (13). Peter Jo et al. in 2017 showed that miRNAs in the blood plasma of CRC patients can be used as biomarkers for detection of response rate to treatment (12). Currently, biomarkers for CRC tumors used in chemotherapy include KRAS, BRAF, MSI, and SMAD4. Common treatments for CRC include surgery, radiation, chemotherapy, and immunotherapy. Each of these methods has side effects. Despite these treatment options, a major challenge in cancer treatment is early defect diagnosis. This study investigated the effect of radiotherapy on expression of miR-374, and APC, and GSK-3β, two of its target genes, in the WNT pathway in peripheral blood samples from radiotherapy-treated CRC patients.

Materials and Methods

Collection of blood samples
Two ml of peripheral blood were collected from 25 CRC patients referred to Khansari Hospital, Arak, Iran (2018-2020) before, and in the first, third, and fifth weeks after starting radiation therapy. All patients treated with radiotherapy were treated using an Elekta Precise linear accelerator with a daily dose of 1.8 Gy (dose per fraction: 1.8 Gy). The radiation treatment dose in treatment phases I and II were 45 Gy with 5.4 Gy boosts to a total of 50.4 Gy for all patients (Table 1). Radiotherapy was performed as a 3D-conformal protocol with 18 MV photon beams five days per week.

| Phases of treatment | number of sessions | dose per fraction (Gy) | dose in per phase (Gy) |
|---------------------|--------------------|-----------------------|------------------------|
| Phase I             | 25                 | 1.8                   | 25*1.8= 45             |
| Phase II (Boost)    | 3                  | 1.8                   | 3* 1.8= 5.4            |
| Total               | 28                 | 1.8                   | 28 * 1.8= 50.4         |

In addition to radiation therapy, 15 (60%) of the patients in the study also received FOLFOX, an anti-cancer drug containing folinic acid, fluorouracil, and oxaliplatin. The study was approved by the Ethics Committee of Arak University of Medical Sciences with a Code of Ethics (IR.ARAKMU.REC.1397.52).

Quantitative RT-PCR
The expression of miR-374, APC, and GSK-3β was evaluated by quantitative RT-PCR. RNA was extracted from the blood samples using an RNX kit (Sinaclone, Iran), and cDNA was synthesized using M-MuLV enzyme (YTA, Iran), random hexamers, and stem-loop primers. cDNAs were used as templates in RT-PCRs using master mix, SYBR green (YTA, Iran), and 10 pmol of forward and reverse primers with an annealing temperature of 54 °C in a thermocycler (Roche). GAPDH and SNORD47 (U47) were used as internal controls.

Evaluation of miR-374 sequences
miR-374 amplicons were produced by real-time PCR using two series primers (14, and Fig. 1) PCR Products were sequenced on an ABI-Biosystem x100 (Macrogen, South Korea). Data were analyzed with BioEdit and Chromas software.

Measurement of the number of eosinophils, neutrophils, lymphocytes
Three observers calculated eosinophil,
neutrophil, and lymphocyte on BAL fluid with a blind test using a light microscope with a magnification of 400x, then averaged the results.

Fig. 1. Schematic overview of primers used to amplify the miR-374 mature and precursor sequences. RT primers F and R (A) and precursor primers F and R (B).

**Statistical analysis**

Expression data was analyzed using Excel 2007 and two-way ANOVA in GraphPad Prism 7.0 software. Differences between the groups were considered statistically significant at P less than 0.05. Sequence alignments were performed using MEGA4 and BioEdit alignment tools. Sequencing results were analyzed with Blast, BioEdit, and Chromas Lite version 2.01.

**Results**

*miR-374, APC, and GSK-3β expression in samples from radiotherapy-treated CRC patients*

Peripheral blood was collected from each of the 25 radiotherapy-treated CRC patients before, and one, three, and five weeks after the start of therapy. Patient demographic data are shown in Table 2.

| Characteristic                  | Detail         | Patients (%) |
|--------------------------------|----------------|--------------|
| Gender                         | Male           | 64           |
|                                | Female         | 36           |
| Median age range               |                |              |
| ≤ 50                           |                | 28           |
| > 50                           |                | 72           |
| BMI                            |                |              |
| < 27                           |                | 56           |
| > 27                           |                | 44           |
| History of cancer              |                |              |
| No                             |                | 85           |
| Yes                            |                | 15           |
| Tumor grade                    |                |              |
| T2 & T3                        |                | 68           |
| T1 & unknown                   |                | 32           |
| Differentiation                |                |              |
| poorly                         |                | 33           |
| moderately                     |                | 40           |
| well                           |                | 27           |
| Anatomical distribution        |                |              |
| colon                          |                | 54           |
| rectum                         |                | 54           |
| sigmoid                        |                | 27           |
| Taking drugs                   |                |              |
| FOLFOX                         |                | 60           |
| other                          |                | 40           |

Table 2. Patient demographics and tumor sample characteristics.
miR-374 expression was significantly down-regulated, while GSK-3β and APC expression were significantly up-regulated, one, three, and five weeks after the start of radiotherapy (Fig. 2 and Table 3).

**Fig. 2.** Expression of miR-374, GSK-3β, and APC before, and 1, 3, and 5 weeks after radiotherapy. GraphPad prism, Two-way ANOVA method.

**Table 3.** Fold-change of APC, GSK-3β, and miR-374 expression one, three, and five weeks after radiation therapy.

| Gene Expression Changes          | Fold-change of APC expression | Fold-change of GSK-3β expression | Fold-change of miR-374 expression |
|---------------------------------|-------------------------------|---------------------------------|----------------------------------|
| First week after starting radiation therapy | 1.02↑                         | 1.11↑                           | 0.76↓                            |
| Third week after starting radiation therapy | 1.10↑                         | 1.81↑                           | 0.66↓                            |
| Fifth week after starting radiation therapy      | 1.12↑                         | 1.62↑                           | 0.63↓                            |
Conservation of the miR-374 precursor coding sequence

RT-PCR with mature and precursor miR-374 primers resulted in 66- and 291-bp amplicons (Figs. 3A and 3B, respectively). Comparisons of the PCR products and Nucleotide Blasts of the amplicon sequences confirmed their identities (Fig. 3C).

Relationship between demographic characteristics and gene expression changes

Increased BMI and age were associated with increased tumor grade and decreased differentiation. In addition, with increased age in cases where the tumor grade increased and differentiation decreased, the expression of GSK3B and APC genes is not significant as the number of radiotherapy sessions increased. Although with increased age and grade and decreased differentiation, miR-374 expression was significantly down-regulated. The correlation showed the miR-374 expression decrease over treatment time positively correlated with increased patient age, tumor grade, and BMI (Pearson r, p= 0.0005, r= 0.08).

Discussion

Colorectal cancer is the second-leading cause of death by cancer after only lung cancer (1, 2, 15). Colorectal cancer stages include stage A, in which the cancer is confined to the mucous membrane, stage B, in which the cancer spreads to the intestinal wall but the lymph nodes are not involved, stage C, in which the lymph nodes also become cancerous, and stage D, the metastatic form. Existing screening methods cannot detect CRC in the early stages; however, early detection and diagnosis is necessary for effective treatment, and the need for new biomarkers for early detection is urgent. The molecular studies of signaling pathways involved in CRC have revealed that multiple pathways, including the WNT pathway, are altered (16). Components of the signaling pathways in humans can be controlled by miRNAs. Two decades after miRNAs were first identified much remains unknown regarding their roles in cancer (17-20). The nucleotide sequences of the genes encoding miRNAs may be conserved. In our study, conservation of the miR-374 mature and precursor coding sequences in blood samples from CRC patients was demonstrated (Fig. 3). We also investigated miR-374, APC, and GSK-3β expression in blood samples from radiotherapy-treated CRC patients.
Numerous studies have reported altered expression of various miRNAs in CRC. miR-144 was shown to reduce migration and proliferation of colorectal tumor cells by reducing ROCK1 levels (21). In another study, miR-30b was shown to suppress colorectal tumors via targeting the oncogenes KRAS, PIK3CD, and BCL2 (22). In addition to being diagnostic markers, miRNAs can also be used as markers for CRC treatment and its radiation resistance. A study in 2017 showed that miRNAs can be used as biomarkers to predict treatment responses. Based on the results of that study, miR-125b-1, miR-1183, miR-130a, and miR-375 had different expression levels before and after treatment, and these miRNAs target key genes including ATM and CHEK1, which are involved in chemo-radiation of rectal cancer (23). Peter Jo et al. showed in 2017 that miRNAs in the blood plasma of patients with CRC can be used as biomarkers of treatment response. Their study indicated that miR-30c and miR-31 may be differentiated between cancerous and non-cancerous individuals, and could be used as CRC biomarkers (12). De-regulation of miR-374 in lung, pancreatic, and gastric cancers, melanoma, and atherosclerosis have been demonstrated in other studies (24-26). In gastric cancer, up-regulation of this molecule was responsible for the proliferative phenotype in a mouse model of gastritis transformation to gastric cancer (27). In 2019, Li et al. showed that miR-374 was down-regulated in pancreatic cells. They reported this transcript by targeting JAM-2, which leads to growth suppression in pancreatic tumors (28). Baek SJ et al. (2016) using the microarray technique, evaluated 1265 microRNAs and found that four molecules, including miR-374, were down-regulated in carbon beam-resistant cells, and this molecule is considered as a possible biomarker for optimal cancer treatment (29). Our results, in which miR-374 expression was reduced in CRC patients after radiotherapy, were consistent with his study.

In previous work from our laboratory, bioinformatics, and in vitro studies of miR-374, APC, and GSK-3β expression changes were identified in formalin-fixed paraffin-embedded (FFPE) CRC specimens when compared to non-tumor specimens. Increased miR-374 reduced expression of its target genes in cancer cells, leading to activation of proliferative genes (14).

Our present study found decreased miR-374 expression and increased expression of its two target genes following radiotherapy, contrary to what occurs in tumor cells in CRC. Our results also showed increased expression of the target genes with increasing radiotherapy over time. It should also be noted that 60% of our patients also received FOLFOX, a chemotherapy drug containing folic acid, fluorouracil, and oxaliplatin, often used to treat CRC. Therefore, these genes’ altered expression might be used as a marker to predict the effects of various therapeutic approaches.

CRC risk factors included aging, diet, obesity, smoking, and alcohol. Our data agreed with other studies that cite age and obesity as CRC risk factors. In our samples, increased BMI and age were associated with increased tumor grade and decreased differentiation. Also, with increased age, in cases where the tumor grade increased and the degree of differentiation decreased, miR-374 expression decreased significantly, while the GSK-3β and APC expression might indicate a need for more chemotherapy sessions. It is possible that expression of these genes might be a marker to follow to determine the effectiveness of chemotherapy. We believe more studies with larger sample sizes are needed to verify this hypothesis.

Given the side effects of CRC treatments, predicting their effects is valuable. Considering their effects on expression of miR-374 and its target genes, APC and GSK-3β, two major components of the WNT pathway in CRC, these genes may be useful as predictive markers of patient outcomes.

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