Association of lncRNA-p53 regulatory network (lincRNA-p21, lincRNA-ROR and MALAT1) and p53 with the clinicopathological features of colorectal primary lesions and tumors

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Abstract. Colorectal cancer (CRC) is a common intestinal cancer with a high mortality rate. Early detection of this type of cancer is fundamental to the prevention of the disease, which results in improved survival rates. In the human colon tissue, transition from normal epithelium to adenoma is considered to be caused by unknown molecular incidents occurring over 5-10 years. The detection of CRC has proved problematic when in the early stages of disease. In addition, identifying suitable biomarkers for the detection of CRC progress in patients remains one of the most significant challenges. Long non-coding RNAs have been demonstrated to contribute to the promotion of CRC. The aim of the present study was to investigate the clinical and biological significance of long intergenic non-coding (linc)RNA-p21, lincRNA-regulator of reprogramming (ROR) and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in the colon tumor and polyp tissue, and the association that these have with the expression of p53 at the mRNA level. Neoplastic and paired adjacent normal tissue samples were obtained from 72 patients (46 polyps and 26 tumors). Reverse transcription-quantitative PCR was performed to determine the relative fold changes in the expression of lincRNA-p21, lincRNA-RoR, MALAT1 and p53 in the samples. A significant association was observed between the levels of MALAT1 and p53 in neoplasm tissues (R=0.073; P<0.05). The relative expression of the MALAT1 gene revealed a statistically significant difference between the different polyp types and number of polyps (P=0.0028 and 0.022, respectively). Adjuvant therapy in patients with tumors revealed an association between the levels of lincRNA-ROR and lincRNA-p21 expression (P=0.015 and 0.038, respectively). MALAT1 may be selected as an early detection biomarker for CRC. Furthermore, lincRNA-ROR and lincRNA-p21 may serve as prognostic and therapeutic biomarkers in patients with CRC.

Introduction

Colorectal cancer (CRC) is responsible for >1.8 million new colorectal cancer cases and 881,000 cases of CRC-associated mortality worldwide each year (according to GLOBOCAN 2018). It was also the third most commonly diagnosed cancer in both men and women in the United States, in 2017 (1,2). Evidence has suggested that different biological pathways are involved in CRC development, particularly the type that originated from the precursor lesions termed ‘polyps’ (3). Currently, histology is used to classify these lesions as adenomatous or serrated polyps (4). The adenoma subdivisions include villous, tubulovillous or tubuluar, and serrated polyps, which are then subdivided into hyperplastic polyps, sessile serrated polyps or traditional serrated adenomas (4).
Early detection of CRC results in improved prognostic outcomes for patients, and late diagnosis leads to challenges in treatment (5,6). Currently, researchers are striving to identify highly specific novel biological markers for the non-invasive and easy diagnosis of CRC (7-9). Previous studies have indicated that long non-coding RNAs (lncRNAs) are involved in different biological processes of cells, particularly in gene expression via the regulation of transcription and post-transcriptional processing as well as chromatin modification. They can also potentially contribute to tumor progression and metastasis in different types of tissue (10-12). p53 mutations occur in 40-50% of sporadic cases of CRC, resulting in direct deactivation of the affected gene (13). Emerging evidence has suggested that lncRNAs may regulate the p53 gene or p53 targets (14). Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a highly conserved lncRNA and is overexpressed in various types of human cancer (15). It has been revealed that p53 is a downstream mediator of MALAT1 activity, and the decrease in MALAT1 levels leads to the activation of p53 (16). In addition, the 1-kb sequence upstream of regulator of reprogramming (ROR) is a p53-binding site that induces ROR expression. ROR expression suppresses p53 to maintain cellular homeostasis (17). Cyclin-dependent kinase inhibitor 1 (CDKN1A), also known as p21, mediates p53-dependent growth arrest (18). LincRNA-p21 is induced by p53 and is expressed ~15 kb upstream of the CDKN1A (p21) on chromosome 17 (15).

Although there are preliminary data that support the involvement of the aforementioned lncRNAs on p53 regulation, their usage as predictive biomarkers in early detection and management of CRC is not currently well known in screening programs (14,15). Although detection of p53 at the protein level using immunohistochemistry could demonstrate this association, it could not determine the mediation of lncRNAs in this pathway.

Colon tissue homeostasis occurs in connection with the function of lncRNAs and other cellular regulatory systems. Understanding the associations between the p53 regulatory system and colon tissue-specific lincRNAs could aid in identifying the primary lesion and CRC tumor etiology. An increased knowledge of the aberrant expression levels of these lncRNAs and the p53 gene in the colon neoplasm could provide evidence for the potential biomarkers targeting future novel therapeutic approaches. The present study hypothesized that CRC-associated lincRNA-p21, lincRNA-RoR and MALAT1 may serve as prognostic and therapeutic targets in patients with CRC.

Materials and methods

Patients. A total of 125 volunteers with suspected colon polyps and CRC were subjected to colonoscopy. Non-Iranian patients and those who were negative for colon polyps and CRC were excluded (53 volunteers). The final number of patients included in the present study was 72. Finally, this cross-sectional study was performed on a total of 72 patients (46 polyps and 26 tumor tissues), who were referred to the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences (Tehran, Iran) between August, 2014 and 2016, enrolled in the present study. The mean age ± standard deviation of the patients with polyps and tumors was 49.71±17.54 and 59.84±18.87 years, respectively.

The patients were of Iranian descent and provided written informed consent for the present study prior to the sampling procedure. Biopsies were performed during the colonoscopy and diagnoses were confirmed following evaluation by pathologists. Detailed clinicopathological parameters provided the following data on each polyp lesion and invasive colon cancer subject in patients with polyps [including age, body mass index (BMI), sex, smoking status, ethnicity, education, constipation, diarrhea, anemia, weight loss, bleeding from the anus, family history, polyp type, polyp location, number of polyps and polyp size] and patients with tumors (including age, sex, ethnicity, family history, tumor site, grade, tumor stage, tumor size, radiotherapy and adjuvant therapy) from patient records. The tumor stage was determined using Tumor-Node-Metastasis (TNM) classification of the Union for International Cancer Control (UICC) (19). The protocol for the present study conformed to the ethical guidelines of The Declaration of Helsinki (1975) as reflected by the approval from the Ethics Committee of the Gastroenterology and Liver Diseases Research Center, Shahid Beheshti University of Medical Sciences (Date of approval: May 15, 2014, under the ethical code IR.SBMU.RIGLD.1393.Code 765). The non-tumorous tissue samples were provided with a 10 cm margin from the tumor tissue. The tissue samples were frozen in liquid nitrogen immediately following surgical removal and stored at -80°C.

Reverse transcription-quantitative (RT-q) PCR and PCR analysis. RNA was extracted from 72 tissue samples (46 polyps and 26 tumor tissues) and paired adjacent normal tissues from the patients included in the present study using an RNeasy Mini kit (50) (Qiagen GmbH), according to the manufacturer's protocol. RNA integrity, quantity and quality were determined using 1% standard agarose gel electrophoresis and a NanoDrop™ 2000 system (Fisher Scientific, Inc.). RNA was stored at -70°C prior to use. cDNA was synthesized using 1 μg samples of RNA, and the RNAs Revert Aid RT Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used to amplify the lncRNAs-p21, lincRNA-RoR, MALAT1 and p53 genes using SYBR® Premix Ex Taq™ (Takara Bio, Inc.), according to the manufacturer's protocol. The experimental protocol was performed as follows: i) Thermocycling conditions consisted of an initial activation step for 30 sec at 95°C, 40 cycles at 95°C for 5 sec and 60°C for 35 sec; and ii) melting curve analysis. A duplicate, no template control, consisting of water, cDNA fragments were used as templates to amplify the lncRNA-p21, lincRNA-RoR, MALAT1 and p53 genes using SYBR® Premix Ex Taq™ (Takara Bio, Inc.), according to the manufacturer's protocol. The experimental protocol was performed as follows: i) Thermocycling conditions consisted of an initial activation step for 30 sec at 95°C, 40 cycles at 95°C for 5 sec and 60°C for 35 sec; and ii) melting curve analysis. A duplicate, no template control, consisting of water, was included in every run for each primer pair to test for DNA contamination in buffers as well as solutions and to assess the primer-dimers.

To select the best normalizing gene, the previous study by Kheirelselid et al (20) was reviewed and the variation of Ct in several randomly selected samples using primers targeting B2M, GAPDH and β-actin was also compared. The results of these comparisons confirmed the use of B2M gene as a normalizer endogenous gene.
The primer sequences for lincRNA-p21, lincRNA-ROR, MALAT1, p53 and B2M, were as follows: lincRNA-p21: Forward: 5’-GGGTGGCTCACTCTTCTGGC-3’, and reverse: 5’-TGGCCTTGCCCGGGCTTGTC-3’; lincRNA-ROR: Forward, 5’-CCAGGACAAATGAAACCAC-3’, and reverse: 5’-AGGAGCCAAAGTAACAG-3’; MALAT1: Forward, 5’-GGTAACGGTGTCGAGGTC-3’, and reverse: 5’-CCAGCATTACAGTTCTTAACATG-3’; p53: Forward: 5’-TCTAGAGCCACCGTCACGG-3’, and reverse: 5’-ACGCTAGGATCTGACTGCG-3’; and B2M: Forward: 5’-TGCTGTCTCCATGTTTGATGTATCT-3’ and reverse: 5’-TCTCTGCTCCCCACCTCTAAGT-3’. The 2^-ΔΔCq method was used to determine the expression fold changes (tumor vs. normal) (21). In addition, patients were divided into low-expression (≤median) and high-expression (>median) groups. The median cut-off value of 1.5 fold was used.

Statistical analysis. Data was plotted and statistical analysis was performed using GraphPad Prism (v.5.04; GraphPad Software, Inc.), and the significance was determined using paired t-test and one-way analysis of variance test, with a Tukey’s multiple comparison post-hoc test with a P<0.05 was considered to indicate a statistically significant difference. Differences in clinicopathological outcome between groups were assessed using the SPSS software (v.19.0; IBM Corp.). The association between p53 and lncRNA expression was assessed via linear regression. The receiver operating characteristics (ROC) curve was constructed to describe diagnostic specificity and sensitivity, and was performed using the GenEx program (v.6.1; MultD Analysys AB).

Results

General statistical information. CRC was characterized in 36.1% of the patients (26/72), and polyps were detected in 63.9% (46/72). In those patients with polyps in the evaluations (46 in total), the mean age of the patients was 49.71±17.54 years with a mean BMI of 25.74±3.42, 24 patients (52.2%) were male, 9/46 (25.7%) were smokers, 3/46 (8.6%) consumed alcohol and 9/46 (25.7%) had a positive family history for gastrointestinal polyps. The most common type of polyp was adenomatous polyp in 23 (50.0%) patients, followed by hyperplastic and inflammatory polyps in 13 (28.3%) and 10 (21.7%) patients, respectively. The most common site of the polyps was the recto-sigmoid in 17 (37.0%) patients; followed by the transverse and descending colon, each in 9 (19.6%) patients; ascending colon in 6 (13.0%) and cecum in 3 (6.5%) patients. In the majority of cases [31 (67.4%) patients] the number of polyps was <5 and their size was 5-10 mm. The most common symptom in these patients was constipation (34.8%) followed by bleeding from the anus (21.7%), diarrhea (19.6%) and weight loss (17.4%).

In those patients with colon cancer (26 cases in total), the mean age was 59.84±18.87 years, 18 patients (69.2%) were female, 5 (19.2%) were smokers and 5 (19.2%) had family
history of colon cancer. In 16 patients (61.5%), the site of the
cancer was the colon, whereas in 10 others (38.5%), it was
the rectum. In 12, 10 and 4 patients, the tumor grade was
determined as II, I and III, respectively. The tumor stage was
III, I and II in 12, 10 and 4 cases, respectively. In 18 (69.2%)
of the cases, the size of the tumor was ≥5 cm and 5 (19.2%)
and 6 (23.1%) patients underwent radiotherapy and adjuvant
therapy, respectively. The cancer patients' data were limited
than those of the polyp patients, including BMI, consumed
alcohol, constipation, bleeding from the anus, anemia, diar-
rhea and weight loss.

Expression of lncRNAs in the samples of patients. In order
to investigate the expression of MALAT1, lincRNA-p21,
lincRNA-ROR and p53 genes in the colon cancer tissues, a
tPCR analysis was performed on 66 neoplastic and adjacent
non-neoplastic tissues (46 with polyps and 20 with tumor
tissue). The results indicated that there were no significant
differences in the expression of MALAT1, lincRNA-p21,
lincRNA-ROR and p53 between the aforementioned
tissues (Fig. 1A-D). The extent of the gene expression of the
lncRNAs was compared between the polyp (adenomatous,
inflammatory and hyperplastic types) and colon cancer tissues.

Table 1. Evaluation between MALAT1, lincRNA-ROR and lincRNA-P21 expression in tumor tissue and polyp tissue.

| Variables     | MALAT1     | LincRNA-ROR | LincRNA-p21 |
|---------------|------------|-------------|-------------|
|               | Low, n (%) | High, n (%) | Low, n (%)  | High, n (%) | Low, n (%)  | High, n (%) |
| Polyp tissue  | 34 (73.9)  | 12 (26.1)   | 37 (80.4)   | 9 (19.6)    | 32 (69.6)   | 14 (30.4)   |
| Tumor tissue  | 15 (57.7)  | 11 (42.3)   | 18 (69.2)   | 8 (30.8)    | 13 (50.0)   | 13 (50.0)   |
| P-value       | 0.156      | 0.282       | 0.1         |

MALAT1, metastasis-associated lung adenocarcinoma transcript 1; lincRNA, long intergenic non-coding RNA; ROR, regulator of reprogramming.

Figure 2. Relative mRNA expression between the different types of polyp (adenomatous polyp, inflammatory polyp and hyperplastic polyp) and colon cancer
tissues. (A) Relative expression of MALAT1 between the different types of polyp and colon cancer tissues (P=0.0028). (B) Relative expression of lincRNA-p21
between the different types of polyp and colon cancer tissues (P=0.4402). (C) Relative expression of lincRNA-ROR between the different types of polyp
and colon cancer tissues (P=0.5809). (D) Relative expression of p53 between the different types of polyp and colon cancer tissues (P=0.5837). MALAT1,
metastasis-associated lung adenocarcinoma transcript 1; lincRNA, long intergenic non-coding RNA; ROR, regulator of reprogramming; p53, tumor protein 53.
Table II. Association between clinicopathological characteristics and MALAT1, lincRNA-ROR and lincRNA-P21 expression in the 46 patients with colon polyps.

| Variables            | MALAT1  | LncRNA-ROR | LncRNA-p21 | p53     |
|----------------------|---------|------------|------------|---------|
|                      | Low, n (%) | High, n (%) | P-value    | Low, n (%) | High, n (%) | P-value    | Low, n (%) | High, n (%) | P-value    |
| Age, years<sup>a</sup> | 0.939   | 0.112      | 0.191      | 0.957    |
| <50                  | 12 (41.4) | 14 (46.7)  |            | 5 (41.7) | 11 (40.7)   |            |
| ≥50                  | 17 (58.6) | 17 (53.1)  | 16 (53.3)  | 7 (77.8) | 7 (58.3) | 16 (59.3) |            |
| BMI<sup>b</sup>      | 0.608   | 0.313      | 0.489      | 0.656    |
| Underweight, ≤18.5   | 1 (5.0)  | 1 (4.8)    | 1 (16.7)   | 1 (16.7) | 1 (5.0)    |            |
| Normal weight, 18.6-24.9 | 6 (30.0) | 6 (26.1)  | 5 (23.8)   | 2 (24.0) | 2 (33.3) | 5 (25.0) |            |
| Overweight, 25-29.9  | 11 (55.0) | 14 (60.9)  | 13 (61.9)  | 3 (50.0) | 1 (60.0)    |            |
| Obese, ≥30           | 2 (10.0) | 2 (8.7)    | 2 (9.5)    |        |            |            | 2 (10.0) |            |
| Sex                  | 0.619   | 0.207      | 0.403      | 0.403    |
| Male                 | 17 (50.0) | 21 (56.8)  | 18 (56.3)  | 6 (42.9) | 18 (56.3)   |            |
| Female               | 17 (50.0) | 16 (43.2)  | 14 (43.8)  | 8 (57.1) | 8 (57.1) | 14 (43.8) |            |
| Smoking<sup>c</sup>  | 0.330   | 0.639      | 0.847      | 0.781    |
| Yes                  | 8 (29.6) | 7 (24.1)   | 7 (25.0)   | 2 (22.2) | 7 (26.9)    |            |
| No                   | 19 (70.4) | 22 (75.9)  | 21 (75.0)  | 7 (77.8) | 19 (73.1)   |            |
| Ethnicity<sup>d</sup> | 0.228   | 0.654      | 0.644      | 0.626    |
| Turkish              | 2 (7.4)  | 3 (10.3)   | 2 (7.1)    | -        | 3 (11.5)   |            |
| Persian              | 22 (81.5) | 22 (75.9)  | 21 (75.0)  | 7 (77.8) | 20 (76.9)   |            |
| Lurs                 | 1 (3.7)  | 2 (6.9)    | 3 (10.7)   | -        | 1 (11.1) | 2 (7.7)    |            |
| Kurd                 | 2 (7.4)  | 2 (6.9)    | 2 (7.1)    | -        | 1 (11.1) | 1 (3.8)    |            |
| Education<sup>e</sup> | 0.865   | 0.817      | 0.865      | 0.492    |
| Academic             | 13 (46.4) | 13 (44.8)  | 13 (46.4)  | 5 (55.6) | 11 (42.3)   |            |
| Non-academic         | 15 (53.6) | 16 (55.2)  | 15 (53.6)  | 4 (44.4) | 15 (57.7)   |            |
| Constipation         | 0.902   | 0.497      | 0.447      | 0.152    |
| Yes                  | 12 (35.3) | 12 (32.4)  | 10 (31.3)  | 7 (50.0) | 9 (28.1)    |            |
| No                   | 22 (64.7) | 25 (67.6)  | 22 (68.8)  | 7 (50.0) | 23 (71.9)   |            |
| Diarrhea             | 0.768   | 0.099      | 0.833      | 0.308    |
| Yes                  | 7 (20.6) | 9 (24.3)   | 6 (18.8)   | 4 (28.6) | 5 (15.6)    |            |
| No                   | 27 (79.4) | 28 (75.7)  | 26 (81.3) | 10 (71.4) | 27 (84.4) |            |
| Anemia               | 0.190   | 0.389      | 0.418      | 0.129    |
| Yes                  | 9 (26.5) | 9 (24.3)   | 8 (25.0)   | 5 (35.7) | 5 (15.6)    |            |
| No                   | 25 (73.5) | 28 (75.7)  | 24 (75.0) | 9 (64.3) | 27 (84.4)   |            |
| Weight loss          | 0.419   | 0.159      | 0.713      | 0.633    |
| Yes                  | 5 (14.7) | 5 (13.5)   | 6 (18.8)   | 3 (21.4) | 5 (15.6)    |            |
| No                   | 29 (85.3) | 32 (86.5)  | 26 (81.3) | 11 (78.6) | 27 (84.4) |            |
Table II. Continued.

| Variables                              | MALAT1 | LncRNA-ROR | LncRNA-p21 | p53  |
|----------------------------------------|--------|------------|------------|------|
|                                        | Low, n (%) | High, n (%) | P-value    | Low, n (%) | High, n (%) | P-value    | Low, n (%) | High, n (%) | P-value |
| Bleeding from the anus                 | 0.171  | 0.959      | 0.080      | 0.699  |
| Yes                                    | 13 (38.2) | 2 (16.7)   | 12 (32.4)  | 3 (33.3) | 13 (40.6)  | 2 (14.3)  | 4 (28.6)  | 11 (34.4)  |
| No                                     | 21 (61.8) | 10 (83.3)  | 25 (67.6)  | 6 (66.7) | 19 (59.4)  | 12 (85.7) | 10 (71.4) | 21 (65.6)  |
| Family history of colon polyps<sup>a</sup> | 0.958  | 0.639      | 0.246      | 0.136  |
| Yes                                    | 7 (25.9)  | 2 (25.0)   | 7 (24.1)   | 2 (33.3) | 6 (21.4)   | 3 (42.9)  | 4 (44.4)  | 5 (19.2)   |
| No                                     | 20 (74.1) | 6 (75.0)   | 22 (75.9)  | 4 (66.7) | 22 (78.6)  | 4 (57.1)  | 5 (55.6)  | 21 (80.8)  |
| Consumption of vegetables              | 0.126  | 0.976      | 0.509      | 0.509  |
| Low                                    | 12 (46.2) | 1 (14.3)   | 11 (39.3)  | 2 (40.0) | 11 (42.3)  | 2 (28.6)  | 2 (28.6)  | 11 (42.3)  |
| High                                   | 14 (53.8) | 6 (85.7)   | 17 (60.7)  | 3 (60.0) | 15 (57.7)  | 5 (71.4)  | 5 (71.4)  | 15 (57.7)  |
| Consumption of red meat<sup>a</sup>    | 0.943  | 0.743      | 0.943      | 0.943  |
| Low                                    | 4 (15.4)  | 1 (14.3)   | 4 (14.3)   | 1 (20.0) | 4 (15.4)   | 1 (14.3)  | 1 (14.3)  | 4 (15.4)   |
| High                                   | 22 (84.6) | 6 (85.7)   | 24 (85.7)  | 4 (80.0) | 22 (84.6)  | 6 (85.7)  | 6 (85.7)  | 22 (84.6)  |
| Consumption of soda<sup>a</sup>        | 0.641  | 0.516      | 0.641      | 0.390  |
| Low                                    | 13 (50.0) | 4 (57.1)   | 15 (53.6)  | 2 (40.0) | 13 (50.0)  | 4 (57.1)  | 2 (28.6)  | 15 (57.7)  |
| Moderate                               | 3 (11.5)  | -          | 3 (10.7)   | -       | 3 (11.5)   | -          | 1 (14.3)  | 2 (7.7)    |
| High                                   | 10 (38.5) | 3 (42.9)   | 10 (35.7)  | 3 (60.0) | 10 (38.5)  | 3 (42.9)  | 5 (71.4)  | 15 (57.7)  |
| Consumption of fast food<sup>a</sup>   | 0.385  | 0.605      | 0.346      | 0.922  |
| Low                                    | 18 (69.2) | 3 (42.9)   | 17 (60.7)  | 4 (80.0) | 15 (57.7)  | 6 (85.7)  | 4 (57.1)  | 17 (65.4)  |
| Moderate                               | 3 (11.5)  | 1 (14.3)   | 4 (14.3)   | -       | 4 (15.4)   | -          | 1 (14.3)  | 3 (11.5)   |
| High                                   | 5 (19.2)  | 3 (42.9)   | 7 (25.0)   | 1 (20.0) | 7 (26.9)   | 1 (14.3)  | 2 (28.6)  | 6 (23.1)   |
| Exercise<sup>a</sup>                  | 0.279  | 0.976      | 0.833      | 0.126  |
| Yes                                    | 17 (65.4) | 3 (42.9)   | 17 (60.7)  | 3 (60.0) | 16 (61.5)  | 4 (57.1)  | 6 (85.7)  | 14 (53.8)  |
| No                                     | 9 (34.6)  | 4 (57.1)   | 11 (39.3)  | 2 (40.0) | 10 (38.5)  | 3 (42.9)  | 1 (14.3)  | 12 (46.2)  |
| Type of polyp                           | 0.024  | 0.409      | 0.434      | 0.727  |
| Adenomatous                            | 19 (82.6) | 4 (17.4)   | 17 (73.9)  | 6 (26.1) | 14 (60.9)  | 9 (39.1)  | 6 (26.1)  | 17 (73.9)  |
| Hyperplastic                           | 6 (46.2)  | 7 (53.8)   | 12 (92.3)  | 1 (7.7)  | 10 (76.9)  | 3 (23.1)  | 4 (30.8)  | 9 (69.2)   |
| Inflammatory                           | 9 (90.0)  | 1 (10.0)   | 8 (80.0)   | 2 (20.0) | 8 (80.0)   | 2 (20.0)  | 4 (40.0)  | 6 (60.0)   |
| Location of polyp<sup>a</sup>          | 0.542  | 0.170      | 0.098      | 0.439  |
| Cecum                                  | 2 (6.1)   | 1 (9.1)    | 2 (5.7)    | 1 (11.1) | 2 (6.5)    | 1 (7.7)   | 2 (14.3)  | 1 (3.3)    |
| Ascending colon                        | 5 (15.2)  | 1 (9.1)    | 3 (8.6)    | 3 (33.3) | 2 (6.5)    | 4 (30.8)  | 1 (7.1)   | 5 (16.7)   |
| Transverse colon                       | 8 (24.2)  | 1 (9.1)    | 9 (25.7)   | -       | 9 (29.0)   | -          | 2 (14.3)  | 7 (23.3)   |
| Descending colon                       | 5 (15.2)  | 4 (36.4)   | 8 (22.9)   | 1 (11.1) | 6 (19.4)   | 3 (23.1)  | 2 (14.3)  | 7 (23.3)   |
| Recto-sigmoid                          | 13 (39.4) | 4 (36.4)   | 13 (37.1)  | 4 (44.4) | 12 (38.7)  | 5 (38.5)  | 7 (50.0)  | 10 (33.3)  |
The level of expression of the MALAT1 gene revealed a statistically significant difference between the different polyp types and the tumor tissue (P=0.0028; Fig. 2A). However, there was no statistically significant difference identified in the expression of lincRNA-p21, lincRNA-RoR and p53 mRNA between the polyp and colon cancer tissues (P>0.05; Fig. 2B-D). Patients were divided into two groups, consisting of colon cancer tissue and colon polyps, and the association between the MALAT1, lincRNA-ROR and lincRNA-P21 genes was evaluated (Table I).

Associations between the expression of lncRNAs and clinical characteristics. In order to further evaluate the role of MALAT1, lincRNA-p21, lincRNA-RoR and p53 in colon cancer and polyps, the associations between the transcript levels of the genes and several clinicopathological features were also investigated (Tables II and III).

The MALAT-1 relative expression groups demonstrated a statistically significant difference between the polyp type and polyp number (Table II). The statistical analyses between these two groups revealed a significant association between the p53 transcript levels and family history (P=0.018). LincRNA-ROR and lincRNA-p21 expression was significantly associated with adjuvant therapy (P=0.015 and 0.038, respectively; Table III). No significant associations were identified between the transcript level groups and other clinicopathological variables (Tables II and III).

Relative expression of lncRNAs and p53 in individual samples. In order to determine whether any association was present between the expression of the LncRNAs and the p53 gene, the relative expression of these genes was compared in each set of the samples. A significant association was observed between the levels of MALAT1 and p53 in neoplastic tissues (R=0.073; P=0.034; Fig. 3A), but there was no significant association between the levels of lincRNA-ROR and p53 (R=0.006; P=0.56; Fig. 3B), or lincRNA-p21 and p53 (R=0.015; P=0.32; Fig. 3C).

Evaluation of MALAT-1, lincRNA-p21, lincRNA-ROR and p53 in neoplastic tissue as predictive CRC-associated biomarkers. To investigate the characteristics of MALAT-1, lincRNA-p21, lincRNA-ROR and p53 as potential biomarkers for CRC, the ROC curves and the area under the ROC curves (AUC) were generated and calculated for 66 samples from patients with CRC and healthy adjacent tissues. The ROC curves indicated a strong separation between the patients with CRC and the healthy adjacent tissue group, with an AUC of 0.603 [95% confidence interval (CI), 0.501-0.706; P<0.05] for MALAT1, 0.685 (95% CI, 0.595-0.775; P<0.001) for lincRNA-p21, 0.796 (95% CI, 0.723-0.868; P<0.001) for lincRNA-ROR and 0.643 (95% CI, 0.542-0.744; P<0.05) for p53 (Fig. 4A-D; Table IV).

Discussion

Colonoscopy is the gold standard method for the diagnosis of colon cancer, but the technique is invasive and expensive (22). Early detection of colorectal neoplasms is important since it decreases mortality and increases the survival rate in patients with CRC (23). Identifying a biomarker to detect cancer in...
### Table III. Association between clinicopathological characteristics and MALAT1, lincRNA-ROR and lincRNA-P21 expression in 26 patients with colon cancer tissue.

| Variables                             | MALAT1 | LncRNA-ROR | LncRNA-p21 | p53 |
|---------------------------------------|--------|------------|------------|-----|
|                                       | Low, n (%) | High, n (%) | Low, n (%) | High, n (%) | Low, n (%) | High, n (%) | Low, n (%) | High, n (%) | P-value | Low, n (%) | High, n (%) | P-value | Low, n (%) | High, n (%) | P-value |
| Age, years                            | 0.664  | 0.877      | 0.352      | 0.940       |
| <50                                   | 3 (20.0) 3 (27.3) | 4 (22.2) 2 (25.0) | 2 (15.4) 4 (30.8) | 2 (22.2) 4 (23.5) | 
| ≥50                                   | 12 (80.0) 8 (72.7) | 14 (77.8) 6 (75.0) | 11 (84.6) 9 (69.2) | 7 (77.8) 13 (76.5) | 
| Sex                                   | 0.597  | 0.620      | 1          | 0.492       |
| Male                                  | 4 (26.7) 4 (36.4) | 5 (27.8) 3 (37.5) | 4 (30.8) 4 (30.8) | 2 (22.2) 6 (35.3) | 
| Female                                | 11 (73.3) 7 (63.6) | 13 (72.2) 5 (62.5) | 9 (69.2) 9 (69.2) | 7 (77.8) 11 (64.7) | 
| Ethnicitya                            | 0.592  | 0.086      | 0.042      | 0.293       |
| Turkish                               | 3 (27.3) 3 (37.5) | 2 (16.7) 4 (57.1) | 2 (25.0) 4 (36.4) | 2 (28.6) 4 (33.3) | 
| Persian                               | 6 (54.5) 3 (37.5) | 8 (66.7) 1 (14.3) | 4 (50.0) 5 (45.5) | 5 (71.4) 4 (33.3) | 
| Lurs                                  | 2 (18.2) 1 (12.5) | 1 (8.3) 2 (28.6) | 1 (12.5) 2 (18.2) | - 3 (25.0) | 
| Kurd                                  | - 1 (12.5) | 1 (8.3) - | 1 (12.5) - | - 1 (8.3) | 
| Family history of colon cancer        | 0.261  | 0.619      | 0.135      | 0.018       |
| Yes                                   | 4 (26.7) 1 (9.1) | 3 (16.7) 2 (25.0) | 1 (7.7) 4 (30.8) | 4 (44.4) 1 (5.9) | 
| No                                    | 11 (73.3) 10 (90.9) | 15 (85.3) 6 (75.0) | 12 (92.3) 9 (69.2) | 5 (55.6) 16 (94.1) | 
| Tumor site                            | 0.851  | 0.420      | 1          | 0.648       |
| Colon                                 | 9 (60.0) 7 (63.6) | 12 (66.7) 4 (50.0) | 8 (61.5) 8 (61.5) | 5 (55.6) 11 (64.7) | 
| Rectum                                | 6 (40.0) 4 (36.4) | 6 (33.3) 4 (50.0) | 5 (38.5) 5 (38.5) | 4 (44.4) 6 (35.3) | 
| Grade                                 | 0.121  | 0.528      | 0.255      | 0.771       |
| I                                     | 6 (40.0) 4 (36.4) | 8 (44.4) 2 (25.0) | 6 (46.2) 4 (30.8) | 3 (33.3) 7 (41.2) | 
| II                                    | 5 (33.3) 7 (63.6) | 7 (38.9) 5 (62.5) | 4 (30.8) 8 (61.5) | 5 (55.6) 7 (41.2) | 
| III                                   | 4 (26.7) - | 3 (16.7) 1 (12.5) | 3 (23.1) 1 (7.7) | 1 (11.1) 3 (17.6) | 
| Stage                                 | 0.338  | 0.057      | 0.231      | 0.771       |
| I                                     | 6 (40.0) 4 (36.4) | 9 (50.0) 1 (12.5) | 7 (53.8) 3 (23.1) | 3 (33.3) 7 (41.2) | 
| II                                    | 1 (6.7) 3 (27.3) | 1 (5.6) 3 (37.5) | 2 (15.4) 2 (15.4) | 1 (11.1) 3 (17.6) | 
| III                                   | 8 (53.3) 4 (36.4) | 8 (44.4) 4 (50.0) | 4 (30.8) 8 (61.5) | 5 (55.6) 7 (41.2) | 
| Size of tumor                         | 0.165  | 0.671      | 0.395      | 0.492       |
| <5 cm                                 | 3 (20.0) 5 (45.5) | 6 (33.3) 2 (25.0) | 5 (38.5) 3 (23.1) | 2 (22.2) 6 (35.3) | 
| ≥5 cm                                 | 12 (80.0) 6 (54.5) | 12 (66.7) 6 (75.0) | 8 (61.5) 10 (76.9) | 7 (77.8) 11 (64.7) | 
| Radiotherapy                          | 0.058  | 0.619      | 0.619      | 0.778       |
| Yes                                   | 1 (6.7) 4 (36.4) | 3 (16.7) 2 (25.0) | 2 (15.4) 3 (23.1) | 2 (22.2) 3 (17.6) | 
| No                                    | 14 (93.3) 7 (63.6) | 15 (83.3) 6 (75.0) | 11 (84.6) 10 (76.9) | 7 (77.8) 14 (82.4) |
the early stages is, therefore, a major objective in this field. Although 70% of the human genome is transcribed into RNA, only a limited amount of RNAs encode proteins (24). The majority of lncRNAs have conserved sequences that lead to the concept that suggests lncRNA networks may possess a significant function in biological processes (25). For example, lncRNA regulation is involved in CRC progression, proliferation, apoptosis, differentiation, invasion and metastasis (26). The lncRNA function and expression patterns have been reported to be different in different cell types (27). Therefore, it is important to investigate lncRNA in the colon polyp and tumor to identify the potential biomarkers that can detect the disease in the early stages. More importantly, the molecular function and the biology of p53-regulated lncRNAs should be determined in the colorectal neoplastic tissue. In the present study, the expression of MALAT1, lincRNA-p21 and lincRNA-ROR was determined from the network of p53-regulated LncRNAs, as well as the clinicopathological features of colon polyps and tumor tissues. The results indicated that lincRNA-ROR expression did not reveal any association with radiotherapy sensitivity, but that a significant association did exist between the higher expression of lincRNA-ROR in patients receiving adjuvant therapy. According to a previous study by Yang et al (28) the knockdown of lincRNA-ROR improved sensitivity to radiotherapy in patients with CRC by preventing cell viability and promoting apoptosis. Further studies should be performed on the potential post-transcriptional regulatory mechanisms, with treatments other than radiotherapy. Indeed, previous studies have indicated that lincRNA-ROR did not regulate p53 protein levels in unstressed cells (from intracellular or extracellular stresses, e.g., DNA damage) and increased the level of lincRNA-ROR-suppressed p53; on the other hand, p53 activation can influence lincRNA-ROR expression by an autoregulatory negative feedback loop, which maintains cellular homeostasis (14,15). According to the results of the present study, there was no significant association between the expression of p53 and lincRNA-ROR in the colorectal neoplasm tissue; therefore, the present study hypothesized that this inconsistency could be due to the pathological diversity of the tissues of the patients. Gene expression analysis revealed no significant alteration of MALAT1 in the colon neoplastic tissue in comparison with the adjacent normal tissue. However, the present results demonstrate that the MALAT1 level was higher in patients with hyperplastic polyps when compared with those with other types of polyps and tumors. In addition, a association between MALAT1 expression and number of polyps was observed in the present study. MALAT1 is a functional lncRNA that was first reported in the invasive non-small cell carcinoma and overexpressed in a number of other types of cancer tissue, indicating that MALAT1 was associated with hyperproliferation, invasion and metastasis (15,29-31). To the best of our knowledge, however, the present study is the first to investigate the association between the MALAT1 RNA level and the different types of colon polyp and clinicopathological features.

CRC cases frequently progress from non-cancerous growths, called polyps, to malignant adenocarcinomas and distant metastases; therefore, CRC is known as a ‘silent’ disease, whose early diagnosis may aid the initiation of early
The results of the present study provide further evidence of the importance of MALAT1 as an early CRC detector and as a prognostic marker. In neoplastic tissues, the analysis revealed that there was an association between the expression of MALAT1 and the p53 gene. Several studies support the potential role of MALAT1 in regulating cell proliferation, invasion and tumor formation in different types of tumor (33,34). According to a previous study by Tripathi et al (16), the destruction of MALAT1 led to the activation of p53 and its targets. Nevertheless, the study of Tripathi et al (16) indicated that when MALAT1 was depleted in the HeLa, U2OS and WI-38-VA13 tumorigenic cell lines, possessing poor functional of p53, tumor suppressor p16 and retinoblastoma protein, the cells did not undergo G1 or G1/S arrest, and exhibited normal S-phase progression. Their data indicate cell cycle defects and the downregulation of the E2F target genes on MALAT1 depletion occurs only in specific cell lines (e.g., human dermal fibroblasts) (16). With regard to the function of MALAT1 in specific cells, the present study

![Figure 3](image-url)
hypothesized that an interaction between MALAT1 and p53 exists in colorectal neoplasms.

Previous studies have assessed the expression of lincRNA-p21 with various types of cancer (18,35,36). However, few studies report the influence of lincRNA-p21 on CRC. In addition, further studies revealed that lincRNA-p21 was associated with malignancy progression, and contributed to the treatment and prognosis of the tumor (18,37,38). The results of the present study indicated that the relative expression of lincRNA-p21 was significantly associated with adjuvant therapy in the CRC tissue. A recent independent study by Wang et al (39), revealed that the increase in lincRNA-p21 inhibited the stability and/or translation of β-catenin. The inhibition of β-catenin leads to the suppression of the Wnt signaling pathway, which promotes cell apoptosis and increases the radiosensitivity for CRC (39). Another study by Zhao et al (40) indicated that lincRNA-p21 levels were significantly increased following surgical treatment in comparison with the time prior to surgery. Despite these results, further studies with a higher sample size are required to investigate the mechanism of this association between treatment models and the aberrant expression of lincRNA-P21.

In conclusion, the results of the present study suggest an interaction between lincRNA-ROR, MALAT1, lncRNA-p21 and certain clinicopathological features, which appear to serve an important role in tumorigenesis, development and influencing the response of cancer cells in treatments.

The results of the present study identified the association between the aberrant expression of lincRNA-p21 and lincRNA-ROR and adjuvant therapy in the CRC tissue. In addition, a association was observed between the MALAT1 level and the type of colon polyp, as well as the number of polyps in the patients. The results of the present study provide further evidence towards the importance of MALAT1 as both an early CRC detector and as a prognostic marker. Low sample size in this cross-sectional study affected the association, and thus, further studies on the same groups of patients are required in order to prove increased lncRNA levels in association with the increased risk of CRC development.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

HAA, SI and VC conceived and designed the study. MA, VC and RM performed the experiments. VC, RM, SI, HAA, and MA contributed to the interpretation of the data. VC, and HAA wrote the original draft of the manuscript. SI, MA and HAA revised the manuscript. HAA acquired funding. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of the Gastroenterology and Liver Diseases Research Center, Shahid Beheshti University of Medical Science, Tehran, Iran. The protocol conforms with the ethical guidelines of The Declaration of Helsinki (1975).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A and Jemal A: Colorectal cancer statistics, 2017. CA Cancer J Clin 67: 177-193, 2017.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
3. Tannapfel A, Neid M, Aust D and Baretton G: The origins of colorectal carcinoma: Specific nomenclature for different types of primary lesion/tumor. Int J Mol Med 36: 927-946, 2007.
4. Printz C: Colorectal cancer incidence increasing in young adults. Cancer 121: 1912-1913, 2015.
5. Krishnamurthy A, Kankan S, Wei X, Nanni S, Biagi JJ and Tannapfel A: Chemotherapy delivery for resected colorectal cancer in clinical practice. Eur J Surg Oncol 43: 364-371, 2017.
6. Yates LR and Campbell PF: Evolution of the cancer genome. Nat Rev Genet 13: 795-806, 2012.
7. Neymeyer M, Live D, Gatz G: Advances in understanding cancer genomes through second-generation sequencing. Nat Rev Genet 11: 685-696, 2010.
8. Charaleshi, V, Haghith, MM, Savabkar S, Zali N, Vahedi M, Khanyagama M, Jaijadi A, Assada H and Zali MR: Correlation between the EGF gene intronic polymorphism, rs2298979, and colorectal cancer. Oncol Lett 6: 1079-1083, 2013.
9. Li Y, Egnerov SD, Yang L and Lin C: Molecular mechanisms of long non-coding RNAs-mediated cancer metastasis. Genes Chromosomes Cancer 58: 200-207, 2019.
10. Li XL, Zhou J, Chen ZR and Chng WJ: P53 mutations in colorectal cancer - molecular pathogenesis and pharmacological reactivation. World J Gastroenterol 21: 84-93, 2015.
11. Zhang J, Xu M and Mo YY: Role of the lncRNA-p53 regulatory network in cancer. J Mol Cell Biol 6: 181-191, 2014.
12. Chaudhary R and Lai A: Long noncoding RNAs in the p53 network. Wiley Interdiscip Rev RNA 8: e1410, 2017.
13. Tripathi V, Shen Z, Chakroborty A, Giri S, Freier SM, Wu X, Zhang Y, Gorospe M, Prasanth SG, Lai A and Prasanth KV: Long noncoding RNA MALAT1 controls cell cycle progression by regulating the expression of oncogenic transcription factor B-MYB. PLoS Genet 9: e1003368, 2013.
14. Zhang A, Zhou N, Huang J, Liu Q, Fukuda K, Ma D, Lu Z, Bai C, Watabe K and Mo YY: The human long non-coding RNA-RoR is a p53 repressor in response to DNA damage. Cell Res 23: 340-350, 2013.
15. Chen S, Liang H, Yang H, Zhou K, Xu L, Liu J, Lai B, Song L, Luo H, Peng J, et al.: LincRNA-p21: Function and mechanism in cancer. Med Oncol 34: 98, 2017.
16. Tripathi V, Shen Z, Chakroborty A, Giri S, Freier SM, Wu X, Zhang Y, Gorospe M, Prasanth SG, Lai A and Prasanth KV: Long noncoding RNA MALAT1 controls cell cycle progression by regulating the expression of oncogenic transcription factor B-MYB. PLoS Genet 9: e1003368, 2013.
17. Zhang A, Zhou N, Huang J, Liu Q, Fukuda K, Ma D, Lu Z, Bai C, Watabe K and Mo YY: The human long non-coding RNA-RoR is a p53 repressor in response to DNA damage. Cell Res 23: 340-350, 2013.
18. Chen S, Liang H, Yang H, Zhou K, Xu L, Liu J, Lai B, Song L, Luo H, Peng J, et al: LincRNA-p21: Function and mechanism in cancer. Med Oncol 34: 98, 2017.
19. Somin LH, Gospodarowicz MK and Wittekind C (eds): TNM Classification of Malignant Tumours. 7th edition. Wiley-Blackwell, New Jersey, NY, 2011.
20. Kheirelseid EA, Chang KH, Newell J, Kerin MJ and Miller N: Identification of endogenous control genes for normalisation of real-time quantitative PCR data in colorectal cancer. BMC Mol Biol 11: 12, 2010.
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25: 402-408, 2001.
22. Vatandoost N, Ghanbari J, Mojaver M, Avan A, Ghayour-Mobarhan M, Nedaemin R and Salehi R: Early detection of colorectal cancer: From conventional methods to novel biomarkers. J Cancer Res Clin Oncol 142: 341-351, 2016.
23. Edwards BK, Ward E, Kohler BA, Eheman C, Zauber AG, Anderson RN, Jemal A, Schymura MJ, Lunders-Pogelar I, Sief A, C, et al.: Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. Cancer 116: 544-573, 2010.
24. Xie X, Tang B, Xiao Y-F, Xie R, Li BS, Dong H, Zhou JY and Yang SM: Long non-coding RNAs in colorectal cancer. Onco Targets 7: 5226-5239, 2016.
25. Wilusz JE, Sunwoo H and Spector DL: Long noncoding RNAs: Functional surprises from the RNA world. Genes Dev 23: 1494-1504, 2009.
26. Luo J, Qu J, Wu D-K, Lu Z-L, Sun Y-S and Qu L: Long non-coding RNAs: A rising biotarget in colorectal cancer. Oncotarget 8: 22187-22202, 2017.
27. Yamada A, Yu P, Lin W, Okugawa Y, Boland CR and Goel A: A RNA-sequencing approach for the identification of novel long non-coding RNA biomarkers in colorectal cancer. Sci Rep 5: 875, 2015.
28. Xie X, Tang B, Xiao Y-F, Xie R, Li BS, Dong H, Zhou JY and Yang SM: Long non-coding RNAs in colorectal cancer. Oncotarget 7: 5226-5239, 2016.
29. Wang W, Wang H, Jiang S and Xin Y: MALAT-1, a novel noncoding RNA, and thymosin β4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene 22: 8031-8041, 2003.
30. Sun D, Li X, He Y, Li W, Wang Y, Wang H, Jiang S and Xin Y: YAP1 enhances cell proliferation, migration, and invasion of gastric cancer in vitro and in vivo. Oncotarget 7: 81062-81076, 2016.
31. Pan Y, Brant D, Buerger H, Bulk E, et al: MALAT-1, a novel noncoding RNA, and thymosin β4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene 22: 8031-8041, 2003.
32. Chen X, Liu J, Wang W, Zhan H and Xia D: Prognostic value of long non-coding RNA MALAT1 in colorectal cancer. Oncotarget 7: 63813-63824, 2016.
36. Wang X, Xu Y, Wang X, Jiang C, Han S, Dong K, Shen M and Xu D: LincRNA-p21 suppresses development of human prostate cancer through inhibition of PKM 2. Cell Prolif 50: e12395, 2017.
37. Tang SS, Zheng BY and Xiong XD: LincRNA-p21: Implications in human diseases. Int J Mol Sci 16: 18732-18740, 2015.
38. De Paepe B, Lefever S and Mestdagh P: How long noncoding RNAs enforce their will on mitochondrial activity: Regulation of mitochondrial respiration, reactive oxygen species production, apoptosis, and metabolic reprogramming in cancer. Curr Genet 64: 163-172, 2018.
39. Wang G, Li Z, Zhao Q, Zhu Y, Zhao C, Li X, Ma Z, Li X and Zhang Y: LincRNA-p21 enhances the sensitivity of radiotherapy for human colorectal cancer by targeting the Wnt/β-catenin signaling pathway. Oncol Rep 31: 1839-1845, 2014.
40. Zhao W, Song M, Zhang J, Kuerban M and Wang H: Combined identification of long non-coding RNA CCAT1 and HOTAIR in serum as an effective screening for colorectal carcinoma. Int J Clin Exp Pathol 8: 14131-14140, 2015.

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