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Association of microRNA-652 Expression with Radiation Response of Colorectal Cancer: A Study from Rectal Cancer Patients in a Swedish Trial of Preoperative Radiotherapy to Public Data Analysis and in Vitro Investigation

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Abstract

Purpose: Radiotherapy (RT) is a standard adjuvant therapy in progressive rectal cancer patients, but many patients are resistant to RT, leading to poor prognosis. Our study identified microRNA-652 (miR-652) value on RT response and outcome in rectal cancer patients.

Methods: miR-652 expression was determined by RT-PCR in primary rectal cancer from 48 patients with and 53 patients without RT. The relationship of miR-652 with biological factors and prognosis were examined. The biological function of miR-652 was identified through TCGA and GEPIA database search. Two human colon cancer cell lines (HCT116 p53+/+ and p53-/-) were used for *in vitro* study.

Results: miR-652 expression was augmented significantly in cancer than normal mucosa in non-RT patients (P=0.044). High miR-652 expression in non-RT patients was related to more apoptosis (P=0.036), ATM (P=0.010) and DNp73 expression (P=0.009). High miR-652 expression was related to worse disease-free survival of non-RT patients, independent of gender, age, tumor stage and differentiation (P=0.028; HR=7.398, 95% CI 0.217-3.786). The biological functional analysis further identified the prognostic value and potential relationship of miR-652 with the apoptosis in rectal cancer. In RT patients, miR-652 expression was notably decreased in cancers when compared to non-RT cases (P=0.047), and miR-652 expression in cancers was negatively related to WRAP53 expression (P=0.022). After miR-652 inhibition, the estimation of reactive oxygen species, caspase activity and apoptosis in HCT116 p53+/+ cells were significantly increased compared with HCT116 p53-/- cells after radiation.

Conclusions Our findings suggest the potential value of miR-652 expression as a marker for the prediction of radiation response and clinical outcome in rectal cancer patients.

1. Introduction

Today's radiotherapy (RT) strategy is still designed to treat large and heterogenous groups of rectal cancer patients mainly based on tumor stage, but more than 30% of patients are resistant to RT, resulting in poor prognosis. One of the main reasons is that the currently used clinicopathological criteria including tumor stage for designing RT strategy have their limitations for predicting RT response. Thus, it is urgent to find promising biomarkers for refining RT strategy for individual patients.

miRNAs are considered as prominent markers for diagnosis, prognosis, and prediction of treatment response for malignancies of rectal cancer [1]. miRNAs such as miR-200c, miR-125a/b, miR-451, and miR-587 are shown to be involved in chemoresistance in the different types of cancers [2]. There is no study particularly conducted to examine the involvement of miR-652 in radiation response of rectal cancer so far.

In the present study, we determined the relative level of miR-652 in the samples taken from 101 rectal cancer patients obtained from a Swedish trial of preoperative RT in order to identify miR-652 value on RT response and clinical outcome in rectal cancer patients. Moreover, miR-652 expression, its association

with other vital proteins in a protein-protein interaction network, cellular functions, and survival value in rectal cancer was further analyzed and confirmed from the public databases.

2. Materials And Methods

2.1. Patients

The study includes 101 rectal cancer patients from the South-east Swedish Health Care region where the patients participated in a Swedish trial of preoperative RT between 1987 and 1990 (Swedish Rectal Cancer Trial, 1997). Of them, 53 patients underwent surgery alone and 48 underwent RT and then surgery. An RT dose of 25 Gy in 5 fractions over a median of 8 (6-14) days prior to the surgery was administered. Surgery was carried out in a median of 4 days (range: 0-8 days) after RT. Consent was procured from the patients and the approval was given for the study protocol by the Institutional Review Board of Linköping University, Sweden. There was no significant difference between the non-RT and RT patients regarding the characteristics of the patients and tumors (P>0.05; Table 1).

2.2. Cell lines

Human colon cancer cell lines were given as a kind gift from Johns Hopkins University by Dr. Vogelstein. Wild-type p53 (HCT116 p53+/+) and its p53-null counterpart (HCT116 p53-/-) were produced by HCT116, in which both alleles of p53 were removed through homologous recombination. The cell lines were maintained in McCoy's-5A medium (Sigma-Aldrich), which were supplemented with 10% fetal bovine serum (GIBCO, Invitrogen), 1.5 mM L-glutamine (GIBCO), and also 1X PEST (GIBCO) in 5% CO_2 incubator at 37□C.

2.3. Radiation in cells

In 9.5 cm^2 surface area plates, cells were seeded at a density of 1×10^5 cells and further radiated with photon spectra of 6 MV by utilizing a linear accelerator (Clinac 4/100, Varian; PaloAlo, CA) to assess the effect of radiation. Cells were located below 3 cm PMMA and 105 cm from photon source (photon source to PMMA-surface distance: 100 cm). Cells were introduced to 2 Gy or 10 Gy radiations at room temperature. Then, cells were harvested 2 hrs after radiation for analysis of miRNAs.

2.4. Reverse transcription reaction and qRT-PCR

RNA was isolated from the primary tumor and distant normal mucosa from patients, respectively, using mirVana-miRNA Isolation Kit (Ambion) as per manufacturer's instructions. The cDNA synthesis was performed using gene-specific primers according to the protocol of TaqMan MicroRNA Assay. Comparative qRT-PCR was carried out in triplicates and included no-template controls.

2.5. Normalization and data analysis

Threshold cycle values (Ct) were computed by SDS 2.4.1 software (Applied Biosystems) using manual threshold settings (threshold value=0.2). Here, the reference gene selected was RNU6b (Assay No.

001006; Applied Biosystems).

2.6. Assessment of the factors associated with apoptosis, carcinogenesis, and radioresponse

The data for DNp73 (*n*=83) [3], p130 (*n*=71) [4], phosphatase of regenerating liver-3 (PRL-3; *n*=73) [5], endosialin (TEM1; *n*=75) [6], survivin (*n*=47) [7], PPARδ (*n*=67) [8], WRAP53 (*n*=88) [9], AEG-1 (*n*=70) [10], cyclooxygenase-2 (COX-2; *n*=77) [11] and SATB1 (*n*=68) [12] of primary tumors determined by immunohistochemistry, was taken from earlier studies carried out with the same cases at our Lab. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay was carried out to detect apoptosis (*n*=71) with the same cases at our Lab [13].

2.7. Transfection of HCT 116 cells with miR-652 inhibitor

Transfection with miR-652 inhibitor was performed using Lipofectamine 2000. Before transfection, HCT116 p53+/+ as well as HCT116 p53-/- cell lines were seeded into the plates. This transfection was carried out to observe the expression levels of miR-652 in HCT116 p53+/+ and HCT116 p53-/- cell lines.

2.8. Flow cytometry for intracellular reactive oxygen species (ROS) estimation, caspase activity and apoptosis

Cells were cultured and treated according to the experimental setup and incubated with 5μM 2', 7'-dichlorofluorescin diacetate (DCF-Da). In the case of caspase-3 activity and cell death estimation, the cells were cultured and treated similarly as mentioned above without DCF-Da treatment. The cells were then incubated with NucView 488 caspase-3 substrate (Biotium) and Po-Pro dye (Life Technologies Inc.) for 30 mins on ice as per the manufacturer's protocol. Cells were then subjected to flow cytometry (Gallios) using FL1 (DCF-Da and caspase-3) and FL9 filters (Po-Pro) and analyzed using FlowJo software (vX.0.7).

2.9. TCGA data analysis

The data of miR-652 expression for 161 rectal cancer and three paired normal rectal specimens was downloaded from TCGA portal (National Cancer Institute & National Human Genome Research Institute, accessed 1st November 2017). The data collection process complied with all laws and regulations. MiR-652 expression and its prognostic value for rectal cancer based on TCGA were investigated.

2.10. miRNA-gene network construction

The miRNet database provided the miRNA-gene interaction information, and Cytoscape software was used to visualize the networks.

2.11 Biological functional analysis

Gene ontology (GO) annotation and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis were carried out by “ClusterProfiler” package on R language to explore the biological function for miRNAs-related genes on pathway level.

2.12 Statistical analysis

Statistical differences in miR-652 expression between primary cancer and normal mucosa were assessed by Wilcoxon test. Two-tailed Mann-Whitney U-test and Kruskal-Wallis test were also used to assess the association between miR-652 expression and clinicopathological or biological variables of the patients. Kaplan-Meier and Cox regression hazard model were used for examining miR-652 in relation to clinical outcome in univariate and multivariate. *P* values <0.05 were considered as statistical significance. STATISTICA software was used to perform the calculations.

3. Results

3.1. miR-652 expression in non-RT patients

We first examined the value of miR-652 expression in patients without RT. miR-652 expression was significantly increased in primary cancer compared with normal mucosa (*P*=0.044). The correlation between miR-652 expression and clinicopathological features was analyzed, and there was no significant association between them (Table 2). Furthermore, the relationship of miR-652 expression with biological factors and prognosis was analyzed. A high level of miR-652 expression was related to more apoptosis (*P*=0.036), ATM expression (*P*=0.010) and DNp73 expression (*P*=0.009) (Table 3).

3.2. miR-652 expression in RT patients

We then examined the value of miR-652 expression in patients received RT. miR-652 expression was significantly decreased in primary cancer compared with normal mucosa (*P*=0.047). No significant association was present between miR-652 expression and clinicopathological features (*P*>0.05, Table 2). The level of miR-652 expression in RT cases was negatively correlated to WRAP53 expression (*P*=0.022, Table 3). Univariate analysis showed that a high level of miR-652 expression correlated with worse DFS (*P*=0.044, Figure 1). The significance even remained after adjusting for gender, age, tumor stage and differentiation in a multivariate analysis (*P*=0.028; HR=7.398, 95% CI 0.217-3.786, Table 4).

3.3. miR-652 expression in TCGA database

To validate the role of miR-652 expression in rectal cancer, we used the TCGA database and obtained the expression for a panel of rectal cancers (*n*=161) and the paired normal rectal specimens (*n*=3). The level of miR-652 expression in rectal cancers was significantly higher than that in normal rectal tissue (*P*=0.013). Then, we analyzed the association between miR-652 expression in rectal cancer and prognosis. High miR-652 expression was related to worse survival OS (*P*=0.025, Figure 2).

3.4. ROS estimation, caspase activity and apoptosis in vitro

To further explore the underlying mechanism by which miR-652 responded to radiation, the ROS estimation, caspase activity, and apoptosis of cells inhibited by miR-652 inhibitor were determined by flow cytometry after radiation (2 Gy and 10 Gy). There was no significant difference of ROS estimation, caspase activity and apoptosis between HCT116 p53+/+ and HCT116 p53-/- cell lines in miR-652 inhibition when no radiation was given (P>0.05). After miR-652 inhibition, however, the ROS estimation (Figure 3a), apoptosis (Figure 3b) and caspase activity (Figure 3c) were significantly increased in HCT116 p53+/+ cells when 2 Gy and 10 Gy of radiation were given (P<0.05).

3.5. miR-652 biological functional analysis

The miRNA-gene network for miR-652 was shown (Figure 4a). Several significant genes were showed including *LLGL1*, *INO80D*, *MTFP1*, and *ZFAND5* (Figure 4b). GO annotation showed that the miRNA catabolic process and RNA catabolic process were highly related to miR-652 regulated genes (Figure 4c). The ribosome pathway is the only significantly related pathway for miR-652 related genes from KEGG pathway analysis (Figure 4d).

4. Discussion

miRNAs are intricated in several biological processes and play a noteworthy role in pathological approaches. In several types of cancers, miRNAs are mainly responsible for tumorigenesis, cancer progression, cell invasion and metastasis [14]. When compared with colon cancer, the existing studies pertinent to rectal cancer are relatively less. While there are some previous approaches to identify the role of miR-652 in cirrhosis of liver, focusing on their control in liver tissue has not been characterized in humans or rodents [15]. In the present study, we examined whether selected candidate miRNAs could serve as a marker for rectal cancer by analyzing the relative level of miR-652 in samples from 101 rectal cancer patients who participated in a Swedish trial of preoperative RT.

In plasma of gastric cancer patients, a miRNA-microarray platform was used to screen the differentially expressed miRNAs and upregulated miR-652 was further selected for validation [16]. However, expression levels of miR-652 were found to be downregulated in primary squamous cell lung carcinoma [17].

In the present study, miR-652 expression was increased significantly in primary cancer than normal mucosa in non-RT patients. Further, high miR-652 expression in non-RT cases was positively associated with apoptosis, ATM expression and DNp73 expression. A previous study exhibited that ATM expression level was significantly increased in colorectal cancer [18]. Likewise, the increased expression of DNp73 has been observed in several types of tumors and cell lines, and it is linked to pro-tumor activities [19]. Furthermore, we found that the high level of miR-652 expression was independently related to worse DFS non-RT patients.

To validate the role of miR-652 expression, we used the TCGA database and identified a high significant expression in rectal cancer tissue. Prognostic analysis showed that expression of miR-652 was independently related to unfavorable survival of patients. The results from the TCGA database were in

accordance with those in our samples. To further determine the biological function of miR-652, MiRNet was used for network creation, and analysis and a lot of related genes were shown. GO annotation showed that miRNA catabolic process and RNA catabolic process were found to be highly related to miR-652 regulated genes. The ribosome pathway was the only significant related pathway for miR-652 related genes from KEGG pathway analysis. In the miRNA-gene network, several genes, including *LLGL1*, *INO80D*, *MTFP1*, and *ZFAND5* were significantly related to miR-652. A previous study showed that downregulation of *LLGL1* was associated with the progression of colorectal cancer (CRC) [20]. *INO80D* is a subunit of the human INO80 chromatin-remodeling complex and is intricated in the regulation of transcription, DNA replication as well as repair mechanism [21]. For *MTFP1*, the loss of function instigates the cytochrome c release, which activates caspase cascade and further leads to apoptosis [22–23]. *ZFAND5* plays an important role in controlling NF-kappa-B activation and apoptosis [24].

In RT patients, miR-652 expression was significantly decreased in cancers when compared to non-RT cases. However, the level of miR-652 expression was negatively related to WRAP53 expression. Previous studies showed that COX-2 was instigated by p53-mediated activation of RAS or RAF or ERK cascade [25], and WRAP53 encoded a regulatory RNA required for the function of p53 upon DNA damage [26].

To further explore the underlying mechanism by which miR-652 response to radiation, the ROS estimation, caspase activity, and apoptosis were determined by flow cytometry after radiation and miR-652 inhibition. It showed that the ROS estimation, caspase activity and apoptosis in HCT116 p53+/+ cells rather than HCT116 p53-/- cells were significantly increased after radiation. Further studies are needed to determine whether miR-652 was involved in p53-dependent or -independent apoptosis induced by radiation.

5. Conclusions

In this study, miR-652 expression in primary cancer was significantly higher than that in normal mucosa in non-RT patients, which was related to increased apoptosis, ATM expression and DNp73 expression, as well as worse disease-free survival, indicating an aggressive tumor phenotype of miR-652. Moreover, biological functional analysis of miR-652 further identified its prognostic value and potential relationship with the apoptosis in rectal cancer. In RT patients, miR-652 expression in cancer was negatively related to WRAP53 expression, *in vitro* study in HCT116 cell lines identified the apoptosis-related mechanism of miR-652 in radiation, suggesting that the selected candidate miR-652 serves as a predictive prognostic biomarker for radioresponse of rectal cancer. However, there are few limitations in this study where relatively small sample sizes in patient population were not enough to verify the value of miR-652 expression in response to the radiation in rectal cancer. Secondly, the target genes and signaling pathways revealed by bioinformatical analysis should be further analyzed.

Declarations

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Ethical approval: Institutional Review Board of Linköping University, Sweden approved the study protocol.

Availability of data and material: Yes

Author Contributions: Conceptualization: Surajit Pathak, Hong Zhang and Xiao Feng Sun; Methodology and data analysis: Surajit Pathak, Wen-Jian Meng, Jaganmohan Reddy Jangamreddy and Gunnar Adell; Bioinformatic and statistical analysis: Xueli Zhang and Hong Zhang, Wen-Jian Meng and Ganesan Jothimani; Writing original draft: Surajit Pathak and Sushmitha Sriramulu; Revision and editing: Surajit Pathak, Wen-Jian Meng, Antara Banerjee, Alexander Sun Zhang, Hong Zhang, and Xiao-Feng Sun. The content of the submitted manuscript was approved by all authors.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1. Characteristics of the rectal cancer patients without and with preoperative radiotherapy (RT).

Characteristics	Non-RT (%)	RT (%)	*P* value
Gender			
Male	31(50.0)	31(50.0)	0.671
Female	22(56.4)	17(43.6)	
Age (year)			
≤ 70	29(48.3)	31(51.7)	0.420
> 70	24(58.5)	17(41.5)	
TNM stage			
I	18(51.4)	17(48.6)	0.933
II	13(52.0)	12(48.0)	
III	19(55.9)	15(44.1)	
IV	3(25.0)	4(75.0)	
Differentiation			
Well + moderate	42(53.2)	37(47.8)	0.793
Poor + mucinous	11(50.0)	11 (50.0)	
Surgical type			
Anterior resection	25(56.8)	19(43.2)	0.572
Abdominoperineal	28(49.1)	29(50.9)	

Table 2. Correlation between miR-652 expression and clinicopathological features in rectal patients without and with preoperative radiotherapy (RT).

Characteristics	*n*	Non-RT		*P* value	*n*	RT		*P* value
		Low (%)	High (%)			Low (%)	High (%)	
Gender								
Male	31	13(41.9)	18(58.1)	0.146	31	7(22.6)	24(77.4)	0.328
Female	22	5(22.7)	17(72.3)		17	9(52.9)	8(47.1)	
Age (year)								
≤ 70	29	7(24.1)	22(75.9)	0.097	31	10(32.3)	21(67.7)	0.831
> 70	24	11(45.8)	13(54.2)		17	6(35.3)	11(64.7)	
TNM stage								
I	18	6(33.3)	12(66.7)	0.741	17	8(47.1)	9(52.9)	0.234
II	13	3(23.1)	10(76.9)		12	3(25.0)	9(75.0)	
III	19	8(42.1)	11(57.9)		15	3(20.0)	12(80.0)	
IV	3	1(33.3)	2(66.7)		4	2(50.0)	2(50.0)	
Differentiation								
Well + moderate	42	14(33.3)	28(66.7)	1.000	37	12(32.4)	25(67.6)	1.000
Poor + mucinous	11	4(36.4)	7(63.6)		11	4(36.4)	7(63.6)	
Necrosis								
Negative	28	9(32.1)	19(67.9)	0.788	24	10(41.7)	14(58.3)	0.306
Positive	21	6(54.5)	15(45.5)		22	6(27.3)	16(72.7)	
Fibrosis								
Weak	22	7(31.8)	15(68.2)	0.120	15	9(60)	6(40.0)	0.087
Moderate	12	6(50.0)	6(50.0)		13	2(15.4)	11(84.6)	
Strong	15	2(13.3)	13(86.7)		8	5(62.5)	3(37.5)	

Table 3. Correlation between miR-652 expression and biological factors in rectal patients without and with preoperative radiotherapy (RT).

Characteristics	*n*	Non-RT		*P* value	*n*	RT		*P* value
		Low (%)	High (%)			Low (%)	High (%)	
Apoptosis								
Negative	19	10(52.6)	9(47.4)	***0.036***	10	2(20.0)	8(80.0)	0.696
Positive	30	7(23.3)	23(76.7)		33	11(33.3)	22(66.7)	
ATM								
Negative	13	9(69.2)	4(31.8)	***0.010***	11	7(63.6)	4(36.4)	0.198
Positive	16	3(28.7)	13(81.3)		10	3(30.0)	7(70.0)	
p130								
Negative	42	13(31.0)	29(69.0)	0.791	30	9(30.0)	21(70.0)	0.842
Positive	9	3(33.3)	6(66.7)		10	4(40.0)	6(60.0)	
WRAP53								
Negative	32	11(34.4)	21(65.6)	0.742	28	6(21.4)	22(78.6)	***0.022***
Positive	16	4(25.0)	12(75.0)		12	7(58.3)	5(41.7)	
DNp73								
Negative	3	3(100)	0	***0.009***	4	2(50.0)	2(50.0)	0.482
Positive	44	12(27.3)	32(72.7)		37	12(32.4)	25(67.6)	

Table 4. Multivariate analysis of miR-652 expression with disease-free survival in rectal cancer patients with preoperative radiotherapy (RT).

Characteristics	Beta	Standard	t-value	Exponent	Wald	*P* value
miR-652 expression						
Low vs High	2.001	0.910	2.198	7.398	4.832	***0.028***
Gender						
Male vs Female	-3.482	1.235	-2.819	0.031	7.950	***0.005***
Age (year)						
≤ 70 >70	-2.408	0.764	-3.151	0.090	9.930	***0.002***
TNM stage						
I vs II vs III	4.033	1.169	3.451	56.422	11.908	***0.001***
Grade						
Well+moderately vs Poorly + mucinous	-2.444	0.893	-2.736	0.0868	7.484	***0.006***

Figures

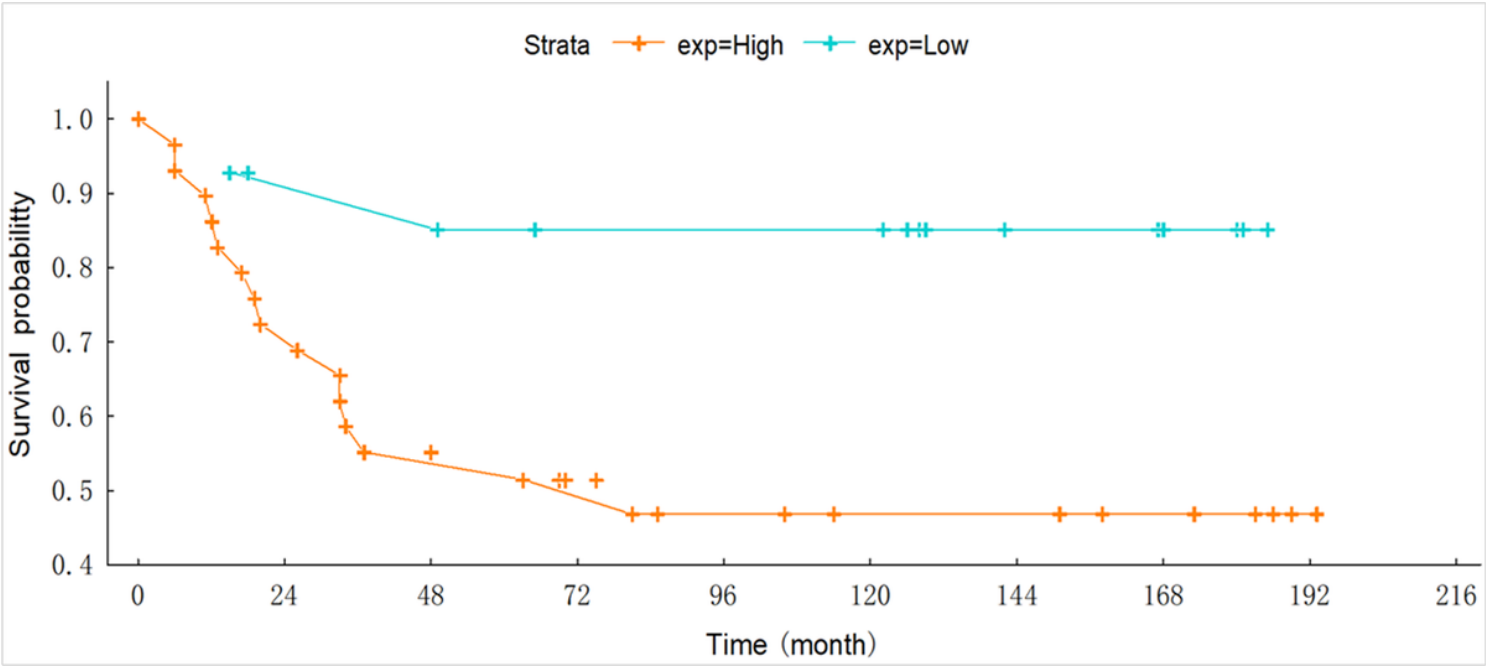

Figure 1

Survival graph for miR-652 expression in rectal cancer patients with preoperative RT showed high level of miR-652 expression correlated with worse disease-free survival (P=0.044)

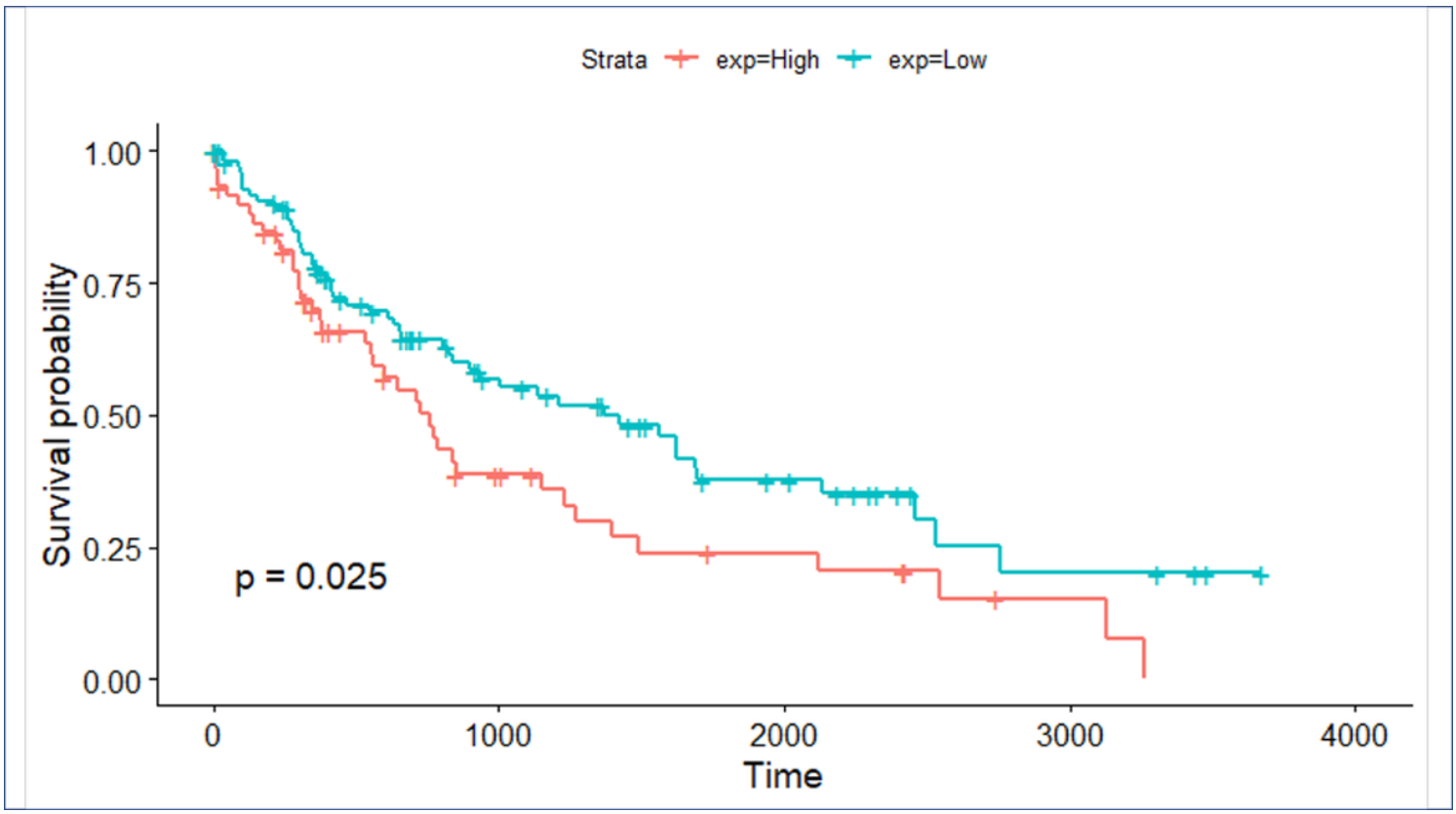

Figure 2

Survival graph for miR-652 expression in rectal cancer patients without preoperative RT showed High expression of miR-652 revealed worse overall survival than that with low miR-652 in rectal cancer patients from TCGA database (P=0.025)

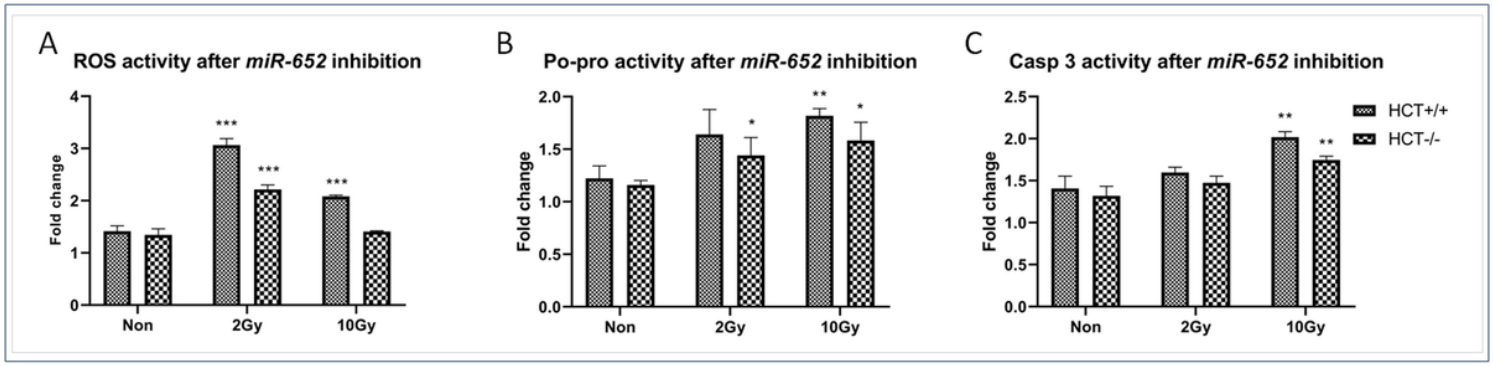

Figure 3

(A) Flow cytometry for intracellular reactive oxygen species (ROS) estimation; (B) apoptosis (Po-Pro) after exposure to various doses of the radiation and miR-652 inhibition in HCT116 p53+/+ cells (solid) and HCT116 p53-/- cells (empty); (C) and caspase 3 (Casp 3) activity

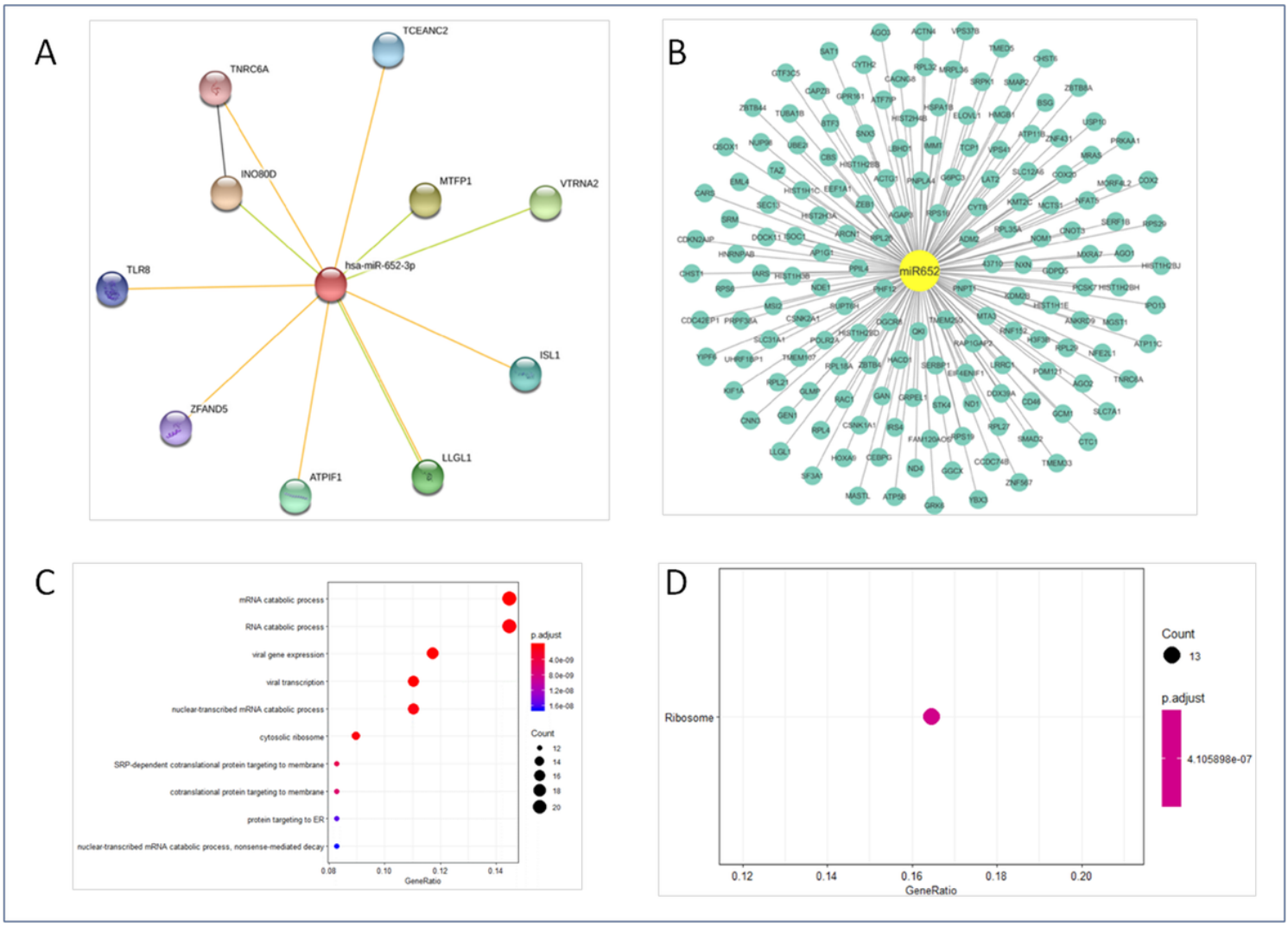

Figure 4

(A) The interactions of miR-652 with miRNA-gene network; (B) The most commonly connected genes with miR-652; (C) miR-652 regulates mainly the mRNA catabolic process and RNA catabolic process in cells; (D) Ribosome pathway is the only significantly related pathway for miR-652 related genes from KEGG pathway analysis