Original Article

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miRNA Signature and its Clinicopathological Association in Colorectal Cancer: A Cross-sectional Study

Oncology Section

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ABSTRACT

Introduction: Colorectal Cancer (CRC) is a significant health problem worldwide, with more than 70,000 new cancer cases recorded in India in 2020. A better understanding of CRC prognosis is needed. The substantial intratumoural heterogeneity among tumours of different stages has entailed the research for new biomarkers to more clearly identify tumour biology and behaviour. Micro Ribonucleic Acids (miRNAs) are non coding RNAs comprised of approximately 20-25 nucleotides and play an important role in epigenetic regulations. Studies have demonstrated that miRNAs play a critical role in tumourigenesis, metastasis, and tumour response to treatment. Comprehensive knowledge of miRNAs as potential markers of colon cancer diagnosis, prognosis, and predictive factors is crucial.

Aim: To assess the miRNA signature (miR21, miR31, and miR34a) in colon cancer tumour samples and to evaluate the association of the miRNA signature with the clinicopathological profile and Pathological Tumour/Node/Metastasis (pTNM) stage of CRC patients.

Materials and Methods: This cross-sectional and prospective study was conducted at the Departments of Pathology, Molecular Research and Diagnostics and Surgical Oncology of Sri Shankara Cancer Hospital, Bengaluru, Karnataka, India and was comprised of a total of 69 CRC patients who underwent surgery with a curative intent. The study was conducted over an 18-month period from January 2020 to June 2021. miRNA was extracted from Formalin-fixed Paraffin-embedded (FFPE) samples, and quantitative Polymerase Chain Reaction (qPCR) was done to get corresponding ΔCT (Threshold Cycles) values. The expression of miR21, miR31, and miR34a was evaluated, and their association with different clinicopathological parameters like age, sex, tumour stage, grade, Carcinoembryonic Antigen (CEA) levels, Perineural Invasion (PNI), and Lymphovascular Invasion (LVI) status was studied. The expression of the mentioned miRNAs was assigned low and high values on the basis of median values. Spearman's correlation was done to check for any significant associations.

Results: All three miRNAs (miR21, miR31, miR34a) were found to have lower values in the >60 years age group compared to the <60 years age group. Higher values of miRNA were found in male patients than in females, with a p-value of 0.022 for miR21. However, miRNA expression (miR21, miR31, miR34a) did not show any statistically significant correlation with tumour location (p-values 0.543, 0.255, 0.255), lymph node status (p-values 0.676, 0.153, 0.930), TNM stage (p-values 0.273, 0.509, 0.898), LVI (p-values 0.233, 0.233, 0.733), PNI (p-values 0.686, 0.263, 0.756), and serum CEA level (p-values 0.543, 0.255, 0.255).

Conclusion: The present study showed the possible tumour-suppressive role of miR34a in CRC. Although miR21 acts as an oncogenic miRNA in many cancers, in CRC, its expression differs between males and females, with most tumours in males exhibiting high expression.

Keywords: Colon cancer, Micro ribonucleic acids, Tumour

INTRODUCTION

The miRNAs are endogenous small non coding Ribonucleic Acid of 20-22 nucleotides that participate in numerous intracellular processes. The human genome is estimated to encode as many as 1,000 unique miRNAs. These miRNAs participate in tumourigenesis by functioning as tumour suppressors or oncogenes. They achieve this by targeting the 3' Untranslated Regions (UTRs) of target genes, and mediating cell proliferation, migration, metabolic shifts, epithelial-mesenchymal transition, autophagy, apoptosis, and radiosensitivity. miRNAs can signals suppress or amplify signals and mediate or modulate signals by participating in positive or negative feedback loops, respectively. They are reported to be involved in the occurrence and progression of various cancers, including brain, breast, lung, liver, prostate, and CRC [1]. Recent evidence shows that several miRNAs with potential biological and clinical relevance have been identified and are being explored as diagnostic, prognostic, and predictive biomarkers [2-4].

A 6-miRNA classifier has been shown to distinguish between stage II colon cancer patients with low and high risks of disease progression, resulting in 5-year disease-free survivals of 89% and 60%, respectively [5]. High miR31 expression observed in serrated lesions has been suggested as a diagnostic biomarker and therapeutic target for BRAF-mutated CRCs [6]. Furthermore, a 3-miRNA signature has been shown to discriminate invasive carcinoma from adenoma with high-grade dysplasia in colonoscopic biopsy specimens, which holds promise for early diagnosis [7].

MicroRNA-21 is an oncogenic miRNA that regulates the expression of multiple cancer-related target genes and is overexpressed in various human tumours. Additionally, miR21 expression is upregulated in CRC tissues, elevated during tumour progression, and is associated with poor survival and chemotherapeutic response [8].

A first-in-human phase I clinical trial (NCT01829971) has been conducted to investigate the safety, pharmacokinetics, and clinical activity of a liposomal formulation of miR34 mimic (known as MRX34) in patients with advanced solid tumours, including CRC. However, enrollment was halted following multiple immune-related severe adverse events [9].

Since miRNAs play a pivotal role in many cancers and their roles in CRC are still not well elucidated, particularly in the Indian population, there is an utmost need to evaluate the expressions of miRNAs in CRCs. The objective of the study was to assess the miRNA

signature (miR21, miR31, and miR34a) in colon cancer tumour samples and to evaluate the association of the miRNA signature with the clinicopathological profile and pTumour Node Metastasis (pTNM) stage of CRC patients.

MATERIALS AND METHODS

The present study was a cross-sectional and prospective study carried out at a tertiary care Institute in Bengaluru, Karnataka, India over an 18-month period from January 2020 to June 2021 (Ethical Committee approval no.: SSCHRC/IEC9/49). All cases of colorectal carcinomas planned for surgery with curative intent were included in the study.

Inclusion criteria:

1. Patients with no distant metastasis at presentation;
2. Patients with non invasive tumours, recurrent tumours, or incomplete surgical margins;
3. Final histological diagnosis of adenocarcinoma only;
4. Patients with no history of chemotherapy/radiotherapy or surgery received outside;
5. Patients who provided informed consent for the study;
6. Patients more than 18 years of age and non pregnant at the time of presentation.

Exclusion criteria: Metastatic disease at presentation, recurrent tumours, or incomplete surgical margins, and patients who received chemotherapy/radiotherapy.

Study Procedure

All patients underwent a thorough evaluation of history and clinical examination, colonoscopy and tissue biopsy, measurement of serum CEA levels (reference range 0-2.9 ng/mL) at presentation, Contrast-enhanced Computed Tomography (CECT) scan of the abdomen and thorax, Magnetic Resonance Imaging (MRI) of the pelvis, and/or Positron Emission Tomography (PET) scan. Serum CEA levels were analysed with a threshold of >5 ng/mL and <5 ng/mL. Histopathological reports of the final surgical specimen were evaluated after curative surgery. Over two years, out of 148 cases who underwent surgery for CRC, only 69 cases fulfilled the inclusion criteria and were taken for miRNA analysis using qRT-PCR techniques [10].

A meta-analysis of the TCGA (The Cancer Genome Atlas) database and published literature yielded a series of tumour-promoting and suppressing miRNAs, which were ranked according to their expression levels [11]. Markers of higher significance were chosen for measurement and analysis after addressing logistical issues. miRNAs21, 31, and 34a with the following HS ID and assay ID were used in the study [Table/Fig-1].

miRNA	HS ID	Assay ID	Part number
miRNA21	hsa-miR-21	397	4427975
miRNA31	hsa-miR-31	2279	4427975
miRNA34a	hsa-miR-34a	426	4427975

[Table/Fig-1]: HS ID and assay ID.

Extraction of miRNA from FFPE Samples: A 20-micron section was done from FFPE blocks, and two sections were placed in a 1.75 mL microcentrifuge tube. Two tubes were prepared for each specimen, labeled with serial numbers and Histopathology (HPE) numbers. Deparaffinisation and clearing of the solution were performed. The tubes were centrifuged at room temperature for two minutes at 13,300 rpm. The supernatant was discarded, and 1 mL of 100% Ethanol was mixed with the pellet in each tube. After washing, the tubes were rotated in a vortex mixer and centrifuged for two minutes at room temperature to remove any traces of xylene used.

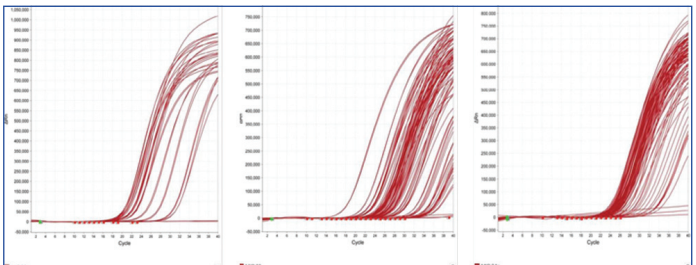
The tissue sections were washed again, followed by drying in a ThermoMixer C at 40°C and 300 rpm for 10 minutes. Confirmation was made by observing the formation of powdery material that should not be attached to any wall upon tapping. A 300-microliter Tris-Ethylenediamine Tetracetic Acid (EDTA) Sodium Dodecyl Sulfate (TE-SDS) solution and 10 microliters of Proteinase K solution were added to each tube and kept for overnight for 12-16 hours at 65°C in the ThermoMixer C. The following day, 1 mL of TRI (Guanidium Isothiocyanate) reagent was added to each 1.75 mL Eppendorf tube, mixed well, and incubated at room temperature for five minutes for RNA precipitation. Then, 200 µL of chloroform was added to each tube, shaken for 20 seconds, and incubated at room temperature for 10 minutes. The tubes were centrifuged at 4°C for 15 minutes at >10000 rpm.

Each tube contained three phases: the upper supernatant phase containing RNA, the middle coloured interphase containing DNA, and the lower phase containing proteins. The upper aqueous phase was collected in a new separate tube using a micropipette, and an equal amount of Isopropanol was added to it for any residual protein precipitation. After mixing, the tubes were incubated at -20°C for 30 minutes. They were then centrifuged at 4°C for 10 minutes at >10000 rpm. The supernatant was discarded, and 1 mL of 75% Ethanol (made with nuclease-free water) was added to the tube, mixed in a vortex, and centrifuged at 4°C for 10 minutes at >10000 rpm. The supernatant was discarded, and the pellet containing RNA was suspended in 25 microliters of nuclease-free water.

Preparation of cDNA from RNA: A master mix was prepared with 1.5 mL of RT Buffer for pH stabilisation, 0.3 mL Deoxynucleotide Triphosphate (dNTP) for base pairing, 0.19 mL RNase inhibitor for RNA protection, 1 mL Nuclease-free water, 1 mL each of Stem loop of RNU44, MiR21, MiR31, and MiR34 for complementary DNA sequence, and 1 mL of Murine Leukemia Virus (MuLV) Reverse Transcriptase enzyme for facilitating the reaction. Seven mL from the RNA stock solution containing approximately 40 ng of RNA was mixed with 7.98 mL of the master mix solution and made into 15 mL in 0.2 mL PCR Tubes. The tubes were then placed in the Veriti (Gradient PCR Machine), Applied Biosynthesis for cDNA synthesis and run for 30 minutes at 16°C, followed by 30 minutes at 42°C, 5 minutes at 85°C, and then maintained at 4°C as long as required. 0.75 mL of this cDNA was then diluted with 3.75 mL of Nuclease-free water and made into 4.5 mL of working cDNA solution.

Quantitative Polymerase Chain Reaction (PCR): An optical multiwall plate was prepared as per pre-determined protocol with a mixture of 5 mL of TAQMANTM MicroRNA and 0.5 mL of miRNA-specific Probe. This was followed by the addition of 4.5 mL of the sample-specific working cDNA solution in two consecutive wells. Samples of each miRNA were set up in duplicate. Similar process was repeated for all samples and all 4 miRNAs - RNU44 (control), MiR21, MiR31, and MiR34a, except for those marked as No Template Control (NTC). Quantitative PCR was then performed on the Quant studio five platform of Applied Biosystems with the following settings: Denaturation at 95°C for two minutes followed by 45 cycles of PCR at 95°C for five seconds and 60°C for 30 seconds.

Data retrieval: After the PCR was completed, raw data [Table/Fig-2] from the machine were compiled and analysed. The mean value of



[Table/Fig-2]: Left: Amplification plot for miRNA 21 after qRT-PCR; Middle: Amplification plot for miRNA 31 after qRT-PCR; Right: Amplification plot for miRNA 34a after qRT-PCR.

the Threshold cycle (Ct) from both wells for each miRNA was taken, and the data were organised along with clinical details for each of the patients and maintained as a data master sheet. Data normalisation was done using the ΔCT (Delta Cycle Threshold) method as demonstrated by Livak KJ and Schmittgen TD [12]. ΔCT values were calculated by subtracting the RNU44 value from the corresponding MiR CT sample value. Relative Normalised Units (RNU) were calculated as $2^{-\Delta\Delta CT}$. All these values obtained were arranged in a Microsoft Excel sheet for analysis with clinicopathological variables. Median values were obtained for each miRNA after sorting the values in order. Median groups - high and low - were divided based on values more or less than the corresponding median values, respectively.

STATISTICAL ANALYSIS

All the RNU values obtained after normalisation were arranged in a Microsoft Excel sheet with the data collected from the proforma. Standard descriptive analysis was then performed using XLSTAT software against histopathological prognostic factors. Spearman's correlation was performed to see any correlation between two variables. The p-value obtained was considered significant only if <0.05.

RESULTS

The age of the patients ranged from 22-81 years (median age 63 years) with an equal male-female ratio (Male=35, Female=34). Total 49 colon and 20 rectal specimens were included in the study [Table/Fig-3].

Variable/Statistic	Categories	Frequency per category	Rel. frequency per category (%)
Age (in years)	≤60 years	30	43.478
	>60 years	39	56.521
Gender	Female	34	49.275
	Male	35	50.725
Hb	<10 g/dL	26	37.68
	>10 g/dL	43	62.31
Albumin	<3 mg/dL	4	5.79
	>3 mg/dL	65	94.20
Tumour site	Colon	49	71.014
	Rectum	20	28.986
Histologic type	Adenocarcinoma	64	92.754
	Mucinous carcinoma	5	7.246
Histologic grade	Well	9	13.043
	Moderately	53	76.812
	Poorly	7	10.145
CEA	CEA high (>5)	30	50.000
	CEA low (<5)	30	50.000
pT	1	2	2.899
	2	17	24.638
	3	43	62.319
	4	7	10.135
pN	Negative (0)	24	34.783
	Positive (1)	45	65.217
TNM stage	1	11	15.942
	2	11	15.942
	3	44	63.769
	4	3	4.348
Lymphovascular Invasion (LVI)	No (0)	23	33.333
	Yes (1)	46	66.667
Perineural Invasion (PNI)	No (0)	60	86.957
	Yes (1)	9	13.043

[Table/Fig-3]: Clinicopathological details.
Hb: Haemaglobin; CEA: Carcinoembryonic antigen; pT: prothrombin time; pN: peripheral neuropathy

Among the 69 cases, 49 (71.01%) had carcinoma of the colon and 20 (28.99%) had rectal carcinoma. The histologic type was adenocarcinoma in 64 (92.75%) and mucinous carcinoma in 4 (7.25%). The histologic grade was well-differentiated (Grade-1) in 9 (13.04%), moderately differentiated (Grade-2) in 53 (76.81%), and poorly differentiated (Grade-3) in 7 (10.15%).

The T1, T2, T3, and T4 diseases were observed in 2 (2.89%), 17 (24.63%), 43 (62.31%), and 7 (10.13%) cases, respectively, in present cohort. Nodal metastasis was seen in 45 (65.22%) cases. Lymphovascular Space Invasion (LVI) was positive in 46 (66.67%) cases, and PNI was positive in 9 (13.04%). High (>5 ng/mL) serum CEA was observed in 50% of cases (9 patients did not have CEA values, so analysis was done with 60 patients).

Median values were obtained for each miRNA after sorting values in order. Median groups - high and low - were divided based on values more or less than corresponding median values, respectively [Table/Fig-4]. All miRNAs were found to have lower values in the >60 years age group compared to the <60 years age group. Fisher's exact test was done between RNU values of miRNAs and age (<60 years and >60 years). The p-value was insignificant (0.281, r=-0.130) for both miRNA21 and miRNA 31, but for miRNA34a, it was 0.039 (r=-0.272) [Table/Fig-5]. Fisher's exact test was done between RNU values of miRNAs and sex (male and female). Higher values of miRNA were found in male patients than female. The p-value=0.022 (r=-0.304) was significant only for miRNA 21. However, miRNA expression did not show any statistically significant association with tumour location, stage, lymph nodal status, TNM stage, LVI, PNI, and serum CEA levels [Table/Fig-6,7].

S. No.	miRNA	RNU maximum value	RNU minimum value	RNU median value
1.	miRNA 21	48.511	0.009	1.495
2.	miRNA 31	11.030	0.000	0.041
3.	miRNA 34a	9.969	0.000	0.062

[Table/Fig-4]: Maximium, minimum and median values of miRNAs after normalisation.

	Age group			Gender		
Median Group	>60 y	<60 y	p-value	Female	Male	p-value
RNU.mir21_high	17	17	0.281	12	22	0.022
RNU.mir21_low	22	13		22	13	
RNU.mir31_high	17	17	0.281	15	19	0.398
RNU.mir31_low	22	13		19	16	
RNU.mir34a_high	15	19	0.039	13	21	0.071
RNU.mir34a_low	24	11		21	14	

[Table/Fig-5]: Contingency table of RNU values of miRNAs with age groups and sex.

With standard coefficients obtained from logistic regression analysis, it was found that miRNAs 21 and miRNA 31 had an inverse relationship with miRNA 34a for lymph node positivity [Table/Fig-8]. In other words, miRNAs 21 and 31 are upregulated while miRNA 34a is downregulated in CRC.

The Cancer Genome Atlas (TCGA) Program

The TCGA has information on 736 CRC patients with miRNA data available for 296 patients. miRNA values were downloaded as log2 normalised reads per million from Colorectal adenocarcinomas (COADREAD) TCGA samples as well as UCSC Xena, and the analysis was done using the Wilcoxon 'r' test through R software package version 4.1.2 for comparison with this cohort. The salient features of the TCGA analysis were (Graphs represented for significant correlations only).

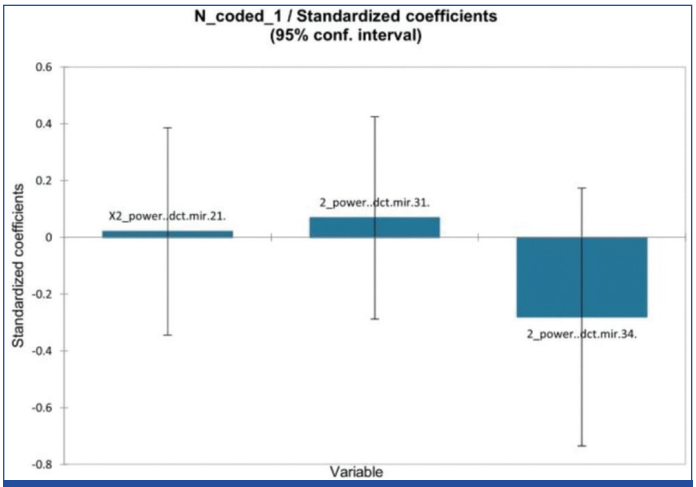
Higher values of miRNAs 21 and 31 were found in colon specimens than rectal specimens, while the opposite was true for miRNA 34a. The p-value was 0.049 for miRNA 21, which is significant

Median group	Tumour pathologic stage					Tumour site			Nodal status		
	T1	T2	T3	T4	p-value	Colon	Rectum	p-value	LN0	LN1	p-value
RNU.mir21_high	1	8	21	4	0.844 (r=0.077)	23	11	0.543 (r=0.055)	11	23	0.676 (r=-0.047)
RNU.mir21_low	1	9	22	3		26	9		13	22	
RNU.mir31_high	0	10	19	5	0.115 (r=0.150)	22	12	0.255 (r=0.119)	9	25	0.153 (r=-0.020)
RNU.mir31_low	2	7	24	2		27	8		15	20	
RNU.mir34a_high	1	10	21	2	0.472 (r=-0.066)	22	12	0.255 (r=0.100)	12	22	0.93 (r=0.065)
RNU.mir34a_low	1	7	22	5		27	8		12	23	

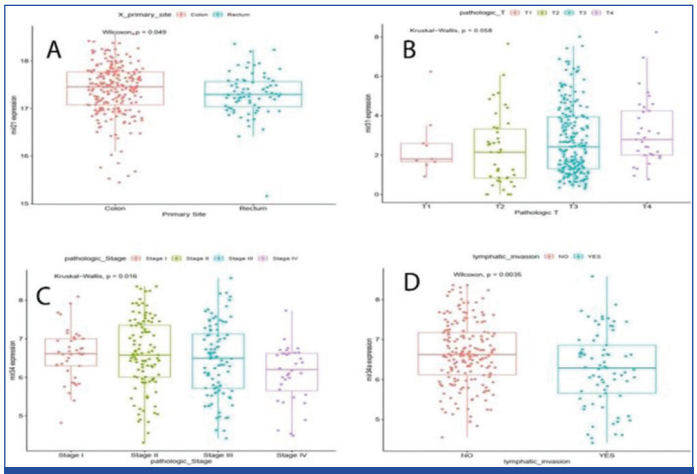
[Table/Fig-6]: Contingency table of RNU values of miRNAs with T stage- T1, T2, T3 and T4, tumour site and lymph node status.

Median group	Histologic grade				Lymphovascular Invasion (LVI)			Perineural Invasion (PNI)		
	Well Diff.	Moderately Diff.	Poorly Diff.	p-value	LVI Absent	LVI Present	p-value	PNI Absent	PNI Present	p-value
RNU.mir.21_high	5	23	6	0.101 (r=0.057)	9	25	0.233	29	5	0.686
RNU.mir.21_low	4	30	1		14	21		31	4	
RNU.mir.31_high	5	24	5	0.396 (r=0.059)	9	25	0.233	28	6	0.263
RNU.mir.31_low	4	29	2		14	21		32	3	
RNU.mir.34a_high	6	22	6	0.086 (r=0.063)	12	22	0.733	30	4	0.756
RNU.mir.34a_low	3	31	1		11	24		30	5	

[Table/Fig-7]: Contingency table of RNU values of miRNAs with histologic grade, Lymphovascular Invasion (LVI) and Perineural invasion (PNI).



[Table/Fig-8]: Probability graph obtained after logistic regression analysis to show relationship between miRNA expression and lymph node positivity.



[Table/Fig-9]: a) Box plot between miRNA 21 expression and primary site- Colon and Rectum after Wilcoxon r test (TCGA data analysis); (b) Box plot between miRNA 31 expression and 'T' stage of tumour-T1, T2, T3 and T4 after Wilcoxon r test (TCGA data analysis); (c) Box plot between miRNA 34a expression and TNM stage of tumour- Stage I, II, III and IV after Wilcoxon r test (TCGA data analysis); (d) Box plot between miRNA 34a expression and LVI- present or absent after Wilcoxon r test (TCGA data analysis).

[Table/Fig-9a]. miRNA31 and 34a had p-values of 0.61 and 0.99, respectively. Only miRNA31 had a significant positive association with the T stage of the tumour with a p-value of 0.058 [Table/Fig-9b], while miRNA21 (p-value=0.55) and 34a (p-value 0.46) had no association.

Association or trend in association was not found to be significant between the N stage of the tumour and miRNA21 (p-value=0.34), 31 (p-value=0.46), and 34a (p-value=0.16). Similarly, no significant association or trend in association was found between the TNM stage of the tumour and miRNA21 (p-value=0.34) and 31 (p-value=0.12); however, an inverse relationship with the TNM stage was observed for miRNA34a with a p-value of 0.016 [Table/Fig-9c]. Additionally, no significant association or trend in association was found between LVI and miRNA21 (p-value=0.98) and 31 (p-value=0.85); while an inverse relationship with LVI was observed for miRNA34a with a p-value of 0.035 [Table/Fig-9d].

DISCUSSION

The CRC is a major health issue globally, with more than 70,000 new cases recorded in India in 2020 [13]. Despite advancements in early detection and treatment, CRC remains one of the leading causes of cancer-related deaths worldwide. To reduce the incidence and mortality of CRC, a comprehensive understanding of the disease at the molecular and cellular levels is crucial. Treatment decisions for CRC are primarily based on disease extent at diagnosis and histopathological staging in CRC, and the search for better biomarkers will always be an unmet need. Considering epigenetic alterations important in the progression of CRC, several miRNAs are being explored as potential biomarkers for CRC. This prospective observational study examines a 3-miRNA signature by comparing their expression values to clinical and pathological factors of CRC.

All miRNAs were found to have lower values in the >60 years age group compared to the <60 years age group. The p-value was insignificant at -0.281 for both miR21 and miR31, but for miR34a, it was 0.039 after Fisher's exact test. Previous studies have shown no association with age for miR21 by Nielsen BS et al., (p-value 0.62), Liu K et al., (p-value NS), Wu Y et al., (p-value 0.834), and You C et al., (p-value 0.535) [14-17]. Similarly, no association was found with age for miR31 by Nosho K et al., (p-value 0.34)), Yang MH et al., (p-value=0.169) and Ito M et al., [6,18,19]. For miR34a, Bader AG reported no association with age (p-value 0.668), and Hasakova K et al., also found no association. However, Zhang L et al., reported a significant positive association with age (p-value 0.020), contrasting with the results of this study [20-22].

Young Indian CRC patients with high levels of miRNA could be an indicator of aggressive disease prevalent in that cohort. Patil PS et al., from Tata Memorial Hospital have shown that CRC is more prevalent in younger patients in India with advanced stages at

presentation [23]. Although for miR21, no association was found with sex for miR21 in many previous studies [15-17,24], the current study revealed a statistically significant higher expression in males, similar to the studies by Nielsen BS et al., (p-value 0.034), Kang WK et al., and Dehghan F et al., (p-value 0.002) [14,25,26]. For miR34a, no association was found with sex by Bader AG (p-value 0.669) and Zhang L et al., (p-value 0.182), however, Hasakova K et al., reported significant downregulation in male patients (p-value <0.05) [20-22]. A possible explanation for this finding could be the preventive role of female sex hormones in the tumourigenesis of CRC, as reported by Williams C et al., [27].

Forty-nine colon specimens (71.014%) and 20 rectal specimens (28.986%) were included in the statistical analysis. All miRNAs had lower values in colon specimens compared to rectal specimens, although statistically insignificant, with p-values of 0.543 for miRNA21 and 0.255 for miRNA31 and 34a, respectively. Nielsen BS et al., (p-value 0.86), Wu Y et al., (p-value 0.210)) and Kang WK et al., did not find any association between tumour location and miRNA21 [14,16,25]. For miRNA31, no association was found with tumour location by Yang MH et al., (p-value 0.369), while Schee K et al., found higher values in colonic tumours (p-value 0.02), contrasting with this study [18,28]. Wang et al., found miRNA34a upregulated in rectal specimens with higher values (p-value 0.006), while in the colon, the relationship was statistically insignificant (p-value >0.05) [7].

An inverse relationship was observed with miR34a between well and moderately differentiated tumours, with a p-value of 0.048, meaning well-differentiated tumours have a higher percentage of high RNU values of miR34a than moderately differentiated tumours. A decrease in values of miR34a with a decline in the grade of tumour grade indicates its down-regulatory and tumour-suppressive actions. Although not statistically significant, there is an increasing trend in the values of miRNA21 with progression in the 'T' stage of the tumour, while a decreasing trend is noted with miRNA34a. Although a positive association was found with miR31 and the T stage of the tumour by Yang MH et al., [18] (p-value 0.045) and Schee K et al., [28] (p-value 0.004), Slaby O et al., [29] had an insignificant result with a p-value of 0.761, similar to this study [18,28,29].

No association can be found between miRNA values and lymph node positivity after Fisher's exact test, as the p-values are insignificant - 0.676, 0.153, and 0.93 for miRNA21, miRNA31, and miRNA34a, respectively. With standard coefficients obtained from logistic regression analysis, it was found that miRNAs 21 and 31 had an inverse relationship with miRNA 34a for lymph node positivity, meaning miRNAs 21 and 31 are upregulated while miRNA34a is downregulated in CRC for present cohort. You C et al., (p-value 0.002), Slaby O et al., (p-value 0.025), and Wu Y et al., (p-value 0.01) found higher values of miRNA21 with lymph node-positive disease [17,29,16], while Schee K et al., with a p-value of 0.82 found no association [28]. Similarly, Yang MH et al., had a positive association of miRNA31 with lymph node-positive disease with a p-value of 0.001, while Slaby O et al., (p-value 0.965) and Schee K et al., (p-value 0.31) had insignificant results [28]. Lymph node-positive disease had a significant association with higher values of miRNA34a in a study by Gao J et al., [30] (p-value 0.004) and Hasakova K et al., (p-value 0.008), while Zhang L et al., (p-value 0.338), Fawzy MS et al., (p-value 0.974), and Wang M et al., (p-value >0.05) failed to show any association [21,22,31,32].

There is an increasing trend in the values of miRNA21 and 31 with progression in the stage of the tumour, while a decreasing trend is noted with miRNA34a except for Stage IV. The corresponding p-values for miRNA 21, 31, and 34a were 0.273, 0.509, and 0.898, respectively, after Fisher's exact test, which were insignificant owing to the small sample size. TNM stage is the parameter that is highly correlated with miR21, as shown by Liu K et al., (p-value 0.001), Wu Y et al., (p-value 0.01), You C et al., (p-value 0.0001), Schetter AJ et al., (p-value 0.002), and Slaby O et al., (p-value 0.032), while Almeida AL et al., with a p-value of 0.32 found no association [15-17,24,29,33]. For miRNA31, Nosho K et al., (p-value 0.0001) found a positive association with the TNM stage, while Nielson BS et al, Ito M et al., and Slaby O et al., (p-value 0.329) did not [14,19,29]. Bader AG (p-value 0.001) had a significant inverse relationship of miRNA34a with TNM staging [20], while studies by Zhang L et al., [22], (p-value 0.469), Almeida AL et al. [33], (p-value=0.34), and Wang S et al., (p-value >0.05) did not show any significance [7].

No association can be found between miRNA values and LVI after Fisher's exact test, as the p-values are insignificant - 0.349, 0.233, and 0.733 for miRNA21, miRNA31, and miRNA34a, respectively. With standard coefficients obtained from logistic regression analysis, it was found that miRNAs 21 and 31 had an inverse relationship with miRNA34a for LVI, same as it was elaborated with lymph nodal status in present study. Schee K et al., compared both miRNA21 and 31 with LVI in their study and did not find any significant association, with p-values of 0.3 and 0.23, respectively [28]. For miR34a, Gao J et al., had a significant association with LVI with a p-value of 0.02, while Fawzy MS et al., (p-value 0.974) found no association [30,31].

No association can be found between miRNA values and PNI after Fisher's exact test, as the p-values are insignificant - 0.686, 0.263, and 0.756 for miRNA21, miRNA31, and miRNA34a, respectively. Schee K et al., compared both miR21 and miR31 with PNI in their study and did not find any significant association, with p-values of 0.42 and 0.43, respectively [28]. No other study could be found comparing miR34a with PNI in the literature.

Similar to the study by Wu Y et al., this study also found a positive association between higher values of miRNA21 and higher values of CEA (18 of 31 high miR21 cases), although the p-value (0.196) was not significant [16]. Slaby O et al., (p-value 0.101) and Almeida AL et al., (p-value >0.05) did not find any association. Like most of the previous studies [29,32,33], the current study also did not reveal any statistically significant association between CEA and miR31 and miR34a, while Bader AG with a p-value of 0.001 showed an inverse relationship between values of miR34a and CEA [20].

Limitation(s)

Because of the low sample size due to prospective observational study, and owing to the Coronavirus Disease-2019 (COVID-19) pandemic along with the short duration of the study period, any type of survival analysis could not be done in present study. miRNA analysis with a mutational profile or tissue proteomics was also not done due to financial constraints. miRNA analysis with CRC patients receiving Neoadjuvant therapy was not done because of logistic reasons, as tissue samples had to be taken before initiation of therapy. Furthermore, subset analysis with TNM staging could not be done due to the exceedingly small sample size upon division.

CONCLUSION(S)

The present study of 69 CRC patients showed a lower value of miRNA for patients older than 60 years and a positive association with miR34a. This proves the tumour suppressor role of miR34 in CRC patients. Male patients showed a positive association with higher miR21. Heterogeneity observed in this study from those quoted can be explained based on racial differences in the cohort used, selection bias for the sample population, the proportion of metastatic CRC cases included in other studies, and variability in uniform histopathological reporting. There is a need to analyse population-based cohort studies to understand elaborately how the present generation is subjected to epigenetic modifications. miRNAs are one of the ways to study epigenetic alterations that depend on ethnic, racial, and environmental modifications.

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