Austin Journal of Clinical Case Reports

Open Access

Austin Publishing Group

Research Article

Exploring the Diagnostic Potential of miRNAs: A Comprehensive Analysis of miRNA Expression Profiles and Their Correlation with Target Genes in Colorectal Cancer

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Received: February 19, 2024
Accepted: April 01, 2024
Published: April 08, 2024

Abstract

Background: Recently studies reported that miRNAs play vital roles in pathogenesis of many human diseases including cancer, which may serve as diagnostic, prognostic markers and may provide means for cancer treatment. The study aimed to investigate the feasibility of using miRNAs as molecular markers for colorectal cancer detection.

Methods: Paired tumour/normal tissues of 81 CRC patients were investigated to measure the expression level of six miRNAs (miR-20a, 21, 29a, 31, 92a and 224), evaluate mRNA and protein expressions in a further six genes (SMAD4, PTEN, TGFBRII, BCL2, KLF4 and RASA1) genes using RT-qPCR and immunohistochemistry analysis respectively.

Results: In relative to the normal tissue mucosa, statistical analysis revealed a significant increase in the tumour expression level of (miR-20a (p=0.007), miR-21 (p=0.0003), miR-29a (p=0.001) and miR-31 (p=0.0003) with a decrease in the mRNA expression level of TGFBRII. Spearman's rank order correlation demonstrated that high miR-20a expression was inversely correlated with PTEN-mRNA level (r= -0.238, p=0.033) and PTEN-protein level (r= -0.253, p= 0.023). High miR-21 and miR-224 expression were associated with low expression of TGFBRII-mRNA [(r= -0.358, p=0.001), (r= -0.276, p=0.013)] and TGFBRII-protein [(r= -0.328, p= 0.003), (r= -0.319, p=0.004)] respectively. In addition, over expression of both miR-29a and miR-31 inversely correlated with RASA1-mRNA level [(r= -0.217, p=0.014), (r= -0.276, p=0.013)] and RASA1-protein level [(r= -0.222, p=0.046), (r= -0.209, p=0.010)] respectively.

Conclusions: The presence of a high degree of correlation between upregulated miRNAs and downregulation of some of target genes involved in different signalling pathways, indicated that miRNAs may have roles in CRC carcinogenesis. Additionally, upregulation of (miR-20a, 21, 29a and 31) may be suitable to differentiate CRC with a high degree of accuracy from a normal mucosa of CRC patients and can play a critical role on screening CRC in general population.

Keywords: Colorectal cancer; miRNA; mRNA; IHC

Introduction

There is convincing evidence that screening and early detection of CRC has been a pivotal strategy for reducing the incidence and mortality rates of the disease [1]. For example, the 5-year survival rate is as high as 93.2% for TNM stage I as compared to only 8.1% for stage IV [2]. However, existing screening tools such as (1) colonoscopy screening, which is currently the most reliable screening tool, has been hampered because of its invasive nature and high cost, (2) the Faecal Occult Blood Test (FOBT), which has low sensitivity and requires dietary restriction, impedes compliance and use. Additionally, studies have investigated several molecular biomarkers for CRC detection, such as Carcinoembryonic Antigen (CEA), and shown that

Citation: Ham-Karim HA, Ahmad N, Shwan A, Ilyas M. Exploring the Diagnostic Potential of miRNAs: A Comprehensive Analysis of miRNA Expression Profiles and Their Correlation with Target Genes in Colorectal Cancer. Austin J Clin Case Rep. 2024; 11(2): 1317.

high CEA levels are associated with CRC progression. However, its utility in the disease screening is limited due to the serum level of CEA not being elevated after the tumour has entered the serosa membrane [3]. In view of these caveats, there is an urgent need for new specific molecular markers to improve the diagnosis of CRC. In the recent past, researchers have focused on miRNAs due to the roles they play in a variety of cellular processes including development, cell cycle progression, cell differentiation, proliferation and apoptosis [4,5]. Others found aberrant miRNA expression has been associated with several types of cancers [6,7] as these act as either tumour suppressors or oncogenes [8]. Furthermore, miRNAs have been shown to successfully discriminate various types of cancers and predict outcomes in both haematological and solid malignancies [9]. In CRC difference in profiles of miRNA expression between tumour and paired adjacent colorectal normal tissue [10], highlights their potential for early diagnostic and prognostic applications [11, 12]. However, inconsistencies about the diagnostic accuracy of differentially expressed miRNAs still exists.

In the present study, we have chosen a panel of 6 miRNAs including (miR-20a, 21, 29a, 31, 92a and 224) which previous studies have shown to be upregulated in CRC and which could be used as diagnostic and prognostic markers [11,13-18]. The study aimed to investigate whether this panel of miRNAs: (1) have roles in the activation of common singling pathways involved in CRC carcinogenesis, by measuring mRNA and protein expression of some of genes that are targeted by the panel of miRNAs. (2) are suitable to use as screening biomarkers for CRC. miRNA and mRNA were measured using RT-qPCR and immunohistochemistry was used to measure protein expression in a series of 81 CRC samples.

Methods

Selection Criteria

This study utilized paired Formalin Fixed Embedded Paraffin (FFPE) cases, comprising primary Colorectal Cancer (CRC) tissues and corresponding normal mucosa samples, procured from 81 patients. Normal tissue specimens included a combination of margin blocks and tissue immediately adjacent to the tumor, exclusively derived from mucosal tissue. All patients underwent surgery at Queen's Medical Centre (QMC) in Nottingham, UK, between 2012 and 2014. Case selection was based on the availability of comprehensive clinicopathological data and the presence of at least 50% tumor cells in the tumor block, additional file 1. The samples were sourced from the Nottingham Health Science Biobank, and ethical approval was granted by Nottingham Research Ethics Committee (REC reference C02.310). Additionally, it is noteworthy that all tissue samples analyzed in this study originated from adenocarcinomas and were acquired through surgical procedures.

Macrodissection

Due to the fact that stromal cells can confound the interpretation of the tumour gene expression profiles, tumour specimens were macrodissected after haematoxylin–eosin slide evaluation by a pathologist to ensure a minimum of 50% tumour tissue content as recommended by Chretien et al, [19]. Two 20µm-thick serial sections were cut from each paraffin block and placed on glass slides (unstained section). The area containing the region of tumour was identified by trained pathologist after examining Haematoxylin–Eosin (H&E) slides that were used as templates to mark the region on unstained sections. The unstained sections were all prepared in the same orientation as the original H&E slides and the region of tumours and normal stroma were correctly identified. Before starting macrodissection the area of tumour was marked on the underside by using an indelible marked pen. Then the area within the mark settled scraped off with the disposable scalpel and collected in Eppendorf® vials. The same procedure has been done for the normal stroma as well. Total RNA and miRNA isolation was performed using the miRNAeasy FFPE kit (Qiagen, Hilden, Germany).

Reverse Transcriptase-Quantitative Polymerase Chain Reaction

Following the generation of cDNA using the miScript II RT kit and QuantiTec Reverse Transcription Kit (Qiagen, Hilden, Germany) for miRNA and mRNA, respectively, the identified genes underwent quantification employing the miScript SYBR Green PCR kit (Qiagen) on a 7500 Fast Real-Time PCR System (Applied Biosystems). The primer sequences and primer efficiencies, derived from assay optimization, are detailed in Additional file 1. The ΔΔCt method of relative mRNA quantification was used to quantify the relative miRNA and mRNA expression between normal and CRC tissues using RNU6B and HPRT as reference genes for both miRNA and mRNA, respectively [18, 19].

Evaluation of Protein Expression

Immunohistochemistry (IHC)

Tissue microarray: Following the verification of specificity and optimal antibody concentrations, Tissue Microarray (TMA) sections from colorectal cancer tissue underwent protein expression measurement through staining. Prior to Immunohistochemistry (IHC) staining, Western blotting analysis was employed to assess the primary antibodies' specificity, with a detailed procedure provided in Additional file 2. TMAs, a recent advancement in pathology, offer high-throughput evaluation of biomarker expression across numerous tissue samples. These arrays consist of paraffin blocks generated from minute tissue samples (single core/tumor) in an array configuration for multiplex histological analysis. Antibodies used in this study are detailed in additional file 1. TMA sections were prepared at Nottingham Health Science Biobank/QMC/Nottingham/UK, with 4-µm paraffin-embedded CRC TMA sections stained using Novolink Polymer Detection Systems (Leica Microsystems) for anti-SMAD4, anti-KLF4, anti-RASA1, anti-PTEN, anti-TGFBRII, and anti-BCL2 antibodies. Each run included positive and negative controls to validate experimental success. The detailed procedure of the IHC stinging is in the Additional file 3.

Assessment of protein expression: Initially the stained TMA slides were checked with the light microscope to confirm the validity and staining, followed by scanning slides with a Nanozoomer Digital Pathology scanner (Hamamatsu Photonics) at 20x magnification, a semi-quantitative method (H-score) was used to assess protein expression in tumour cells. In the H-scoring method, presence and intensity of immunoreactivity were assessed. Staining intensity of each core was assessed as (0 was negative, 1 was weak, 2 was moderate and 3 was strong staining), then H-scores were calculated by multiplying the percentage of positive tumour cells (minimum 0 and maximum 100) by the staining intensity. [20]. After that to ensure reproducibility, slides were all assessed by a second scorer and intraclass correlation coefficient was applied to assess concordance between both scorers.

Statistical Analysis: Statistical analysis was performed using the SPSS software package (version 22). Categorical data was tested for assessing the statistical significance using a chi-square test and continuous data was tested for assessing the statistical significance of the differences between data sets using Wilcoxon test. Fisher's exact test correlation was used for association between unpaired tumour groups. Spearman's correlation was used for detecting correlation between targets. Multiple corrections testing (Bonferroni step-down (Holm) correction) and for both statistical analyses, P-values of <0.05 were statistically significant.

Results

MiRNA Quantification

MicroRNA quantification by real-time quantitative RT-PCR: The expression levels of miRNAs, including miR-20a, miR-21, miR-29a, miR-31, miR-92a, and miR-224, were assessed in 81 Colorectal Cancer (CRC) samples and their corresponding normal mucosa using RT-qPCR assay, normalized to RNU6B. All assays were conducted in triplicate, and the Ct values for all targets in all samples were below 27 (range 16.1-26.8), with a standard deviation less than 0.5 between replicate Ct values. Statistical analysis, specifically the Wilcoxon test (due to non-normal distribution of data), was employed to identify miRNAs with significantly different expression in CRC samples compared to normal mucosa, considering a fold change greater than 1.5. Among the six miRNAs studied, four exhibited significantly higher expression levels in CRC samples compared to normal mucosa (Figure 1). Specifically, miR-31 showed an average 10.83-fold higher expression in CRC than in adjacent normal colon tissue (0.52-161.69 fold, p=0.0003), miR-29a exhibited an average 8.11-fold higher expression in CRC compared to adjacent normal colon tissue (0.52-108.36 fold, p=0.001), miR-21 demonstrated a 6.42-fold higher expression in CRC than in normal tissue (0.5-63.84 fold, p=0.0003), miR-20a showed a 3.27-fold higher expression in CRC than in normal tissue (0.53-109.16 fold, p=0.007), miR-92a displayed a 2.2-fold higher expression in CRC than in normal tissue (0.37-34.8 fold, p=0.2), and miR-224 exhibited a 2.68-fold higher expression in CRC than in normal tissue (0.51-19.35-fold, p=0.042). However, after applying the Bonferroni correction for multiple testing, miR-224 no longer retained statistical significance (p=0.22), while the significance of the others persisted (miR-20a, p=0.04; miR-21, p=0.001; miR-29a, p=0.006; miR-31, p=0.001) (Table 1).

Association of the expression of biomarkers and clinicopathological variables: We have defined normal and high level of the selected miRNAs level (using <0.5 fold as showing down-regulation and >1.5 fold as showing up-regulation). Pearson Chi-square test was applied to identify association between miRNAs and clinicopathological features, and the results showed that normal miRNA92a expression was associated with grade two (X2=7.037, d.f.=2, p=0.03). High miRNA21 expression was associated with Duke`s B stage (X2=6.115, d.f.=2, 0.04). However, following multiple correction testing using the Bonferroni correction, they both failed to retain significance (p=0.28 and p=0.36, respectively) (Table 2).

mRNA Quantification by Real-Time Quantitative RT-PCR

The mRNAs exhibiting significant differences in expression between CRC samples and normal mucosa were identified through rigorous statistical analysis, specifically a paired t-test. Downregulation was defined as <0.6 fold, while up-regulation

Table 1: Expression profiles of candidate microRNAs.

Genes	Fold of change	p value	Bonferroni correction test
miRNA20a	3.27	0.007	0.04
miRNA21	6.42	0.003	0.001
miRNA29a	8.11	0.001	0.006
miRNA31	10.83	0.003	0.001
miRNA92a	2.2	0.29	0.87
miRNA224	2.68	0.042	0.22

Table 2: Association between miRNAs expression and clinic-pathological variables.

Variables		Normal	High	P Value	Correction
miR-20a					
Tumour Grade	well	1 (2.6%)	1 (2.3%)	0.8	0.2
	good	34 (89.5%)	40 (93.0%)		
	poor	3 (7.9%)	2 (4.7%)		
Nodal State	pN 0	21 (55.3%)	29 (67.4%)	0.3	0.7
	pN I	12 (31.6%)	12 (27.9%)		
	pN II	5 (13.2%)	2 (4.7%)		
Duke's Stage	A	5 (13.2%)	7 (16.3%)	0.5	0.9
	B	16 (42.1%)	22 (51.2%)		
	C	17 (44.7%)	14 (32.6%)		
EMVI	0	15 (39.5%)	26 (60.5%)	0.1	0.3
	1	22 (57.9%)	16 (37.2%)		
	2	1 (2.6%)	1 (2.3%)		
miR-21					
Tumour Grade	well	2 (6.3%)	0 (0.0%)	0.1	0.3
	good	29 (90.6%)	45 (91.8%)		
	poor	1 (3.1%)	4 (8.2%)		
Nodal State	pN 0	15 (46.9%)	35 (71.4%)	0.08	0.2
	pN I	13 (40.6%)	11 (22.4%)		
	pN II	4 (12.5%)	3 (6.1%)		
Duke's Stage	A	2 (6.3%)	10 (20.4%)	0.04	0.1
	B	13 (40.6%)	25 (51.0%)		
	C	17 (53.1%)	14 (28.6%)		
EMVI	0	12 (37.5%)	29 (59.2%)	0.05	0.1
	1	20 (62.5%)	18 (36.7%)		
	2	0 (0.0%)	2 (4.1%)		
miR-29a					
Tumour Grade	well	1 (2.8%)	1 (2.2%)	0.9	0.9
	good	33 (91.7%)	41 (91.1%)		
	poor	2 (5.6%)	3 (6.7%)		
Nodal State	pN 0	22 (61.1%)	28 (62.2%)	0.6	0.9
	pN I	12 (33.3%)	12 (26.7%)		
	pN II	2 (5.6%)	5 (11.1%)		
Duke's Stage	A	4 (11.1%)	8 (17.8%)	0.6	0.9
	B	18 (50.0%)	20 (44.4%)		
	C	14 (38.9%)	17 (37.8%)		
EMVI	0	19 (52.8%)	22 (48.9%)	0.4	0.8
	1	17 (47.2%)	21 (46.7%)		
	2	0 (0.0%)	2 (4.4%)		
miR-31					
Tumour Grade	well	1 (2.9%)	1 (2.1%)	0.1	0.3
	good	29 (85.3%)	45 (95.7%)		
	poor	4 (11.8%)	1 (2.1%)		
Nodal State	pN 0	22 (64.7%)	28 (59.6%)	0.1	0.3
	pN I	7 (20.6%)	17 (36.2%)		
	pN II	5 (14.7%	2 (4.3%)		
Duke's Stage	A	2 (5.9%)	10 (21.3%)	0.1	0.3
	B	19 (55.9%)	19 (40.4%)		
	C	13 (38.2%)	18 (38.3%)		
EMVI	0	14 (41.2%)	27 (57.4%)	0.3	0.7
	1	19 (55.9%)	19 (40.4%)		
	2	1 (2.9%)	1 (2.1%)		
miR-92a					
Tumour Grade	well	1 (1.8%)	1 (4.2%)	0.03	0.2
	good	55 (96.5%)	19 (79.2%)		
	poor	1 (1.8%)	4 (16.7%)		
Nodal State	pN 0	38 (66.7%)	12 (50.0%)	0.2	0.6
	pN I	14 (24.6%)	10 (41.7%)		
	pN II	5 (8.8%)	2 (8.3%)		
Duke's Stage	A	9 (15.8%)	3 (12.5%)	0.3	0.7
	B	29 (50.9%)	9 (37.5%)		
	C	19 (33.3%)	12 (50.0%)		
EMVI	0	31 (54.4%)	10 (41.7%)	0.3	0.7
	1	24 (42.1%)	14 (58.3%)		
	2	2 (3.5%)	0 (0.0%)		
miR-224					
Tumour Grade	well	1 (2.3%)	1 (2.7%)	0.4	0.8
	good	39 (88.6%)	35 (94.6%)		
	poor	4 (9.1%)	1 (2.7%)		
Nodal State	pN 0	29 (65.9%)	21 (56.8%)	0.6	0.9
	pN I	11 (25.0%)	13 (35.1%)		
	pN II	4 (9.1%)	3 (8.1%)		
Duke's Stage	A	6 (13.6%)	6 (16.2%)	0.5	0.9
	B	23 (52.3%)	15 (40.5%)		
	C	15 (34.1%)	16 (43.2%)		
EMVI	0	23 (52.3%)	18 (48.6%)	0.9	0.9
	1	20 (45.5%)	18 (48.6%)		
	2	1 (2.3%)	1 (2.7%)		

Table 3: Expression profiles of candidate target genes mRNA.

Genes	Fold of change from tumour to normal	*p* value	Bonferroni multiple correction
SMAD4	0.98	0.12	
PTEN	0.97	0.22	
BCL2	3.72	0.05	0.2
TGFB-RII	0.46	0.001	0.006
KLF4	1.18	0.8	
RASA1	0.66	0.002	0.01

was set at >1.8 fold. All assays were meticulously conducted in triplicate, and replicates with a Ct standard deviation greater than 0.5 were excluded from further analysis to ensure data integrity. Among the six targets examined, two mRNAs from distinct genes, namely RASA1 (p=0.002, with a fold change of 0.66 ± SD 0.74, 95% CI 0.5-0.82) and TGFBRII (p=0.0001, with a fold change of 0.46 ± SD 0.78, 95% CI 0.29-0.63), demonstrated significantly lower expression levels in tumor samples compared to normal tissues. Conversely, BCL2 exhibited a significantly higher expression level (p=0.05, with a fold change of 3.72 ± SD 8.2, 95% CI 1.93-5.53). For the remaining genes, including SMAD4 (p= 0.12, with a fold change of 0.98 ± SD 0.92, 95% CI 0.78-1.18), PTEN (p=0.22, with a fold change of 0.97 ± SD 0.91, 95% CI 0.77-1.17), and KLF4 (p=0.8, with a fold change of 1.16 ± SD 1.6, 95% CI 0.81-1.51), the mRNA expression levels were relatively similar in tumor and normal tissues. However, subsequent to meticulous multiple correction testing using the Bonferroni correction, RASA1 and TGFBRII retained their significance (p=0.01 and p=0.006, respectively), underscoring their robust association with CRC. On the other hand, BCL2 failed to maintain statistical significance (p=0.2), (Table 3).

Cut-off point to detect mRNAs: Prior to analyzing samples to discern mRNA expression levels, we aimed to establish a cut-off point to delineate high and low expression levels. To accomplish this, RNA was extracted from 20 individual pure normal colon tissues and pooled with equal volumes. Subsequently, the expression levels of all mRNAs were assessed in each normal colon tissue sample in comparison to the pooled sample. On average, the minimum fold of expression for all mRNAs in normal colon tissues was 0.6, while the maximum was 1.8. Downregulation was defined as <0.6 fold, and up-regulation was characterized as >1.8 fold. This approach allowed us to set a robust benchmark for differentiating between high and low mRNA expression in subsequent analyses.

Protein Evaluation

Optimisation of primary antibodies for IHC: Prior to Immunohistochemical (IHC) staining of the proteins of interest in Colorectal Cancer (CRC) tissues, the specificity of antibodies against SMAD4, TGFBR2, RASA1, and KLF4 was validated through Western blotting. These primary antibodies target proteins with molecular weights of 65 KDa (SMAD4), 75 KDa (TGFBR2), 140 KDa (RASA1), and 55 KDa (KLF4). In Figure 2A, the anti-SMAD4 antibody successfully detected the expected band (65 KDa) in the SW480 cell lysate but not in other human colorectal cell lines (HCT116, HT29, RKO, and Lovo). For RASA1 (Figure 2B), the Western blotting displayed the anticipated band (140 KDa) in HT29 and Lovo. Meanwhile, the anti-KLF4 antibody (Figure 2C) exhibited the predicted bands (55 KDa) in the HT29 cell lysate but not in other human colorectal cell lines. Finally, as illustrated in Figure 2D, the TGFBR2 antibody detected the expected band at approximately 75 KDa in HT29 and SW480. These Western blotting results served to confirm the specificity of the antibodies intended for use in staining target proteins within CRC Tissue Microarrays (TMAs) through IHC. Other antibodies employed in our project were sourced from our group and the histopathological department at Nottingham Queens Medical Centre, with prior confirmation of their specificity. Furthermore, the concentration of each antibody utilized in IHC underwent additional optimization.

Different concentrations of each antibody were employed to achieve an optimal concentration. In Figure 3, anti-SMAD4 at 1:100 exhibited a heterogeneous staining pattern with no background staining, while 1:50 was excessively high, and 1:200 was too low for expression detection. TGFBR2 at 1:400 showed heterogeneous staining but was too high at 1:200 and 1:300. Anti-RASA1 at 1:40 resulted in a heterogeneous staining pattern with no background staining, whereas 1:50 and 1:100 gave weak staining. The optimal concentration for anti-KLF4 was determined to be 1:100, as 1:50 presented background staining, and 1:200 yielded weak staining. For BCL2 and PTEN, staining was performed by the Histopathology department at Queens Medical Centre.

Protein expression of target genes: To delve deeper into the expression of miRNA target genes, namely SMAD4, PTEN, TGFBRII, BCL2, KLF4, and RASA1, an evaluation was conducted in a cohort of primary operable invasive Colorectal Cancer (CRC) patients. The staining patterns for all markers exhibited heterogeneity both between and within certain tumor cores, ranging from weak to intense, as illustrated in Figure 4. Three distinct cores were stained per case, and the average scores of the three cores were used for subsequent analysis. The H-score, representing staining intensity, was determined for the six markers as follows: SMAD4 had a median H-score of 85, ranging from 0 to 300; PTEN had a median H-score of 95, ranging from 0 to 300; TGFBRII had a median H-score of 70, ranging from 0 to 225; BCL2 had a median H-score of 125, ranging from 0 to 300; KLF4 had a median H-score of 90, ranging from 0 to 300; and RASA1 had a median H-score of 65, ranging from 0 to 200.

As previously mentioned, the single measure Intra-class Correlation Coefficients (ICC) between scorers were 0.78, 0.81, 0.73, 0.77, 0.71, and 0.82 for SMAD4, PTEN, TGFBRII, BCL2, KLF4, and RASA1, respectively, indicating excellent concordance between scorers. The mean scores were used to categorize specimens into low and high expression groups. Specifically, for SMAD4, 51 (63%) CRC cases exhibited low expression, while 30 (37%) cases displayed high expression compared to normal mucosa. For PTEN, 67 (83%) CRC cases demonstrated low expression, with 14 (17%) cases characterized by high expression relative to normal mucosa. Similarly, for TGFBRII, 65 (80%) CRC cases were categorized as low expression, and 16 (20%) cases showed high expression compared to normal mucosa. For BCL2, 47 (58%) CRC specimens had low expression, whereas 34 (42%) were characterized by high expression compared to nor-

Table 4: Association between miRNAs and target genes (mRNA and protein).

Variables	TGFBRII-mRNA	TGFBRII-protein	RASA1-mRNA	RASA1-protein
miR-21 CC	-0.358	-0.328		
p-value	0.001	0.003		
miR-29a CC			-0.217	-0.222
p-value			0.01	0.004
miR-31 CC			-0.276	-0.209
p-value			0.01	0.01
miR-224 CC	-0.276	-0.319		
p-value	0.01	0.004		

Table 5: Association between miRNAs.

Variables	miR-29a	miR-31	miR-92a	miR-224
miR-20a CC	0.380	0.403		
p-value	0.0001	0.0001		
iR-21 CC	0.526	0.285		
p-value	0.0001	0.0001		
miR-29a CC		0.275	0.324	
p-value		0.01	0.003	
miR-31 CC				0.328
p-value				0.003
miR-92a CC				0.382
p-value				0.001

CC= Correlation Coefficient

mal mucosa. In the case of KLF4, 36 (44%) CRC cases exhibited low expression, while 45 (56%) cases displayed high expression compared to normal mucosa. Lastly, for RASA1, 61 (75%) CRC cases demonstrated a low H-score, and 20 (25%) cases exhibited high expression compared to normal mucosa. A chi-square test was conducted to examine the correlation between mRNA and protein levels of the markers. The results indicated a significant correlation between mRNA and protein levels in SMAD4 (r=0.466, p<0.0001), TGFBRII (r=0.708, p<0.0001), BCL2 (r=0.623, p<0.0001), and RASA1 (r=0.728, p<0.0001). However, no correlation was observed between mRNA and protein levels in PTEN (r= -0.085, p= 0.450) and KLF4 (r=0.114, p=0.313).

Association the Expression of Biomarkers (target genes) and miRNAs

Spearman's rank-order correlation was employed to evaluate the associations between the markers investigated in this study. Elevated expressions of miR-21 and miR-224 were found to be linked with decreased TGFBRII-mRNA levels [(r= -0.358, p=0.001), (r= -0.276, p=0.01)] and TGFBRII-protein levels [(r= -0.328, p= 0.003), (r= -0.319, p=0.004)], respectively. Furthermore, overexpression of both miR-29a and miR-31 demonstrated an inverse correlation with RASA1-mRNA levels [(r= -0.217, p=0.01), (r= -0.276, p=0.01)] and RASA1-protein levels [(r= -0.222, p=0.004), (r= -0.209, p=0.01)], (Table 4).

A significant correlation was identified between miR-20a and miR-29 (r= 0.380, p=0.0001) and miR-31 (r= 0.403, p=0.0001). Likewise, a noteworthy correlation was observed between miR-21 and miR-29a (r= 0.526, p=0.0001) and miR-31 (r= 0.285, p=0.01). Additionally, a high correlation was noted between miR-29a and miR-31 (r= 0.275, p=0.01) and miR-92a (r= 0.324, p=0.003). Moreover, a significant correlation was established between miR-31 and miR-224 (r= 0.328, p=0.003) and between miR-92a and miR-224 (r= 0.382, p=0.0001), (Table 5).

Discussion

It is undeniable to say that a supreme screening method must have both high sensitivity and high specificity. Biomarkers with high false positive or negative rates will be considered ineffective and cannot be used [22,23]. Then, an improved, reliable, accurate and non-invasive biomarker is still a need to improve the detection of CRC, mainly at early disease stages before the cancer metastasizes and becomes incurable [24]. Now researchers and clinicians are focused on miRNAs as biomarkers for cancer screening because recently different studies reported that miRNAs play a vital role in the development and progression of CRC [25,26]. In this part of our study we investigated the expression of a panel of six oncogenic miRNAs to see whether these miRNAs could be suitable to be used as a marker for CRC detection.

The accurate quantification of mRNA of the targeted genes is reliant upon the selection of a good endogenous control for normalizing quantitative qPCR data. The HPRT was tested and results showed that expression is almost similar between tumour and normal corresponding tissue samples. This result is in line with the study identified HPRT as the best reference gene that could be used as an accurate endogenous control for the measurement of multiple housekeeping genes [27]. Next, the efficiency of the primers was evaluated and results demonstrated that all primers used to estimate expression of targeted mRNAs are more reliable as R2 linear regression ranged between 90-99% [28].

The present study evaluated the expression level of six miRNAs which are potential diagnostic biomarkers and prognostic factors in cancers. For example, For example, Chai et al, [29] reported that miR-20a has an oncogenic effect in CRC tissue samples and overexpression contributed to the resistance of colorectal adenocarcinoma to chemotherapeutics. Other studies highlighted that overexpression of miR-21 is associated with poor survival and response to chemotherapy in CRC [15,16,30,31]. Moreover, others found that miR-21 expression correlates with clinical stage and could be a potential diagnostic marker, a prognostic marker and it could predict the pathological tumour response to chemotherapy [32,33-35]. Furthermore, Tang et al, [36] found that high expression of miR-29a has a great role in cancer metastasis in CRC tumorigenesis through upregulation of MMP2 and downregulation of E-cad via targeting.

The study profiled 81 paired tumours and matched normal mucosa samples and statistical analysis identified significantly elevated levels of MiR- miR-20a (p=0.04), miR-21 (p=0.001), miR-29a (p=0.006) and miR-31 (p=0.001), this result is in line with other data reported before and detecting that the above miRNAs expression increases dramatically and are diagnostic and prognostic markers in CRC tissue [17,38,39-41]. However, Ahmed et al, [42] and Tao-Wei et al, [43] showed down regulation of miR29a and miR224 in CRC. On the other hand, the study also found a non-significant increase in the expression of, miR-92a and miR-224 in CRCs when compared to normal matched mucosa. This result is not consistent with other studies conducted before and demonstrated an increased level of expression of miR92a and miR224 in CRCs [11,12,41,44]. Differential expression of selected miRNAs makes it possible to use them as diagnostic biomarkers for CRC.

Although the sample size in this study is limited, clinical associations were also analysed and results highlighted no significant correlation between miRNAs and clinic-pathological features. Whereas other studies reported contrary results, for example, Expression level of miR-21 was clearly discernible, with significantly higher levels in stage IV patients compared with stage I or II patients [45]. Schepeler et al, [46] found that miR-31 is significantly increased in stage IV tumours compared to stage II CRC tumours and Zhou et al, [47] showed that high expression of miR-92a correlated with advanced clinical stage, lymph node metastases, and distant metastases.

As many studies, have demonstrated that miRNAs, which have been chosen in this study, are overexpressed and promote colon cancer formation by either up-regulating oncogenic or down-regulating tumour suppressor genes. For example, the miR-20a regulated tumour suppressor gene PTEN, which has a vital role in the inhibition of progression of survival pathways including PI3K/Akt and mitogen activated protein kinase path-

ways [48]. Thus, the study analysed mRNA and protein levels of the targeted genes to see is there any correlation between target genes and selected miRNAs, if yes then find whether miRNAs suppress or activate targeted genes at mRNA or protein levels. Despite finding high correlation between mRNA and protein expression in SMAD4, TGFBRII, BCL2 and RASA1, only down regulation of RASA1 and TGFBRII were significantly associated with the miRNAs upregulation as follow; High miR-21 and miR-224 expression were associated with low expression of TGFBRII, and over expression of both miR-29a and miR-31 inversely correlated with RASA1. This result is in line with previous data reported before [49-51]. However, into our knowledge no one reported any correlation between miR 224 and miR 29a with TGFBRII and RASA1 expression respectively. Moreover, downregulation of RASA1 and TGFBRII in CRC samples is in line with other data reported before and detecting that the RASA1 and TGFBRII decreases dramatically in CRC tissue [49,52]. Additionally, finding no correlation between mRNA and protein levels of PTEN in one hand and high correlation between upregulated miR-20a and low protein expression of PTEN not mRNA in the other hand indicated that miR-20a suppress PTEN at protein level. Despite the present study did not do any functional invitro study, the high correlation between miRNAs and target genes indicated that selected miRNAs may have roles in CRC carcinogenesis as they control some genes which have role in different signalling pathways involved in the development of CRC. For example, upregulation of miR-31 has the potential to drive tumour progression by down regulating RASA1, which acts as a suppressor of RAS function [53]. Suppressing RASA1 leads to the activation of the Ras protein, thereby leading to aberrant intracellular signalling through the RAS-Raf-MAPK and RAS-PI3K-AKT pathways, causing an increase in cell proliferation, anti-apoptosis pro-survival signals and induce cell malignant transformation [54]. The design of the current study has some strengths. For example, tissues of CRC tumours and matched normal mucosa were used and by including the miR expression profiles of adjacent normal tissue, the influence of non-tumorous miRs on the tumours miRs expression was further minimized. However, this study has several limitations. First, the number of samples and the amount of measured miRs were relatively small. Second, the data was not validated by other methods. Third we did not do screening of mutations to find what the correlation is between them.

In conclusion, the study highlights the potential of miRNAs as diagnostic and prognostic markers for CRC. Despite some limitations, the research contributes valuable information about the expression patterns of specific miRNAs and their potential impact on CRC carcinogenesis. Further studies with larger sample sizes and validation methods are recommended for a more comprehensive understanding of the role of miRNAs in CRC.

Author Statements

Authors Contributions

Experimental design (Hersh Ham-Karim, Mohammad Ilyas), experimentation/data acquisition (Hersh Ham-Karim, Narmeen Ahmad, Alan Shwan), data analyses and re-analyses (Hersh Ham-Karim, Alan Shwan), manuscript drafting (Hersh Ham-Karim, Mohammad Ilyas), manuscript review (Mohammad Ilyas), approval of final manuscript draft (all authors)

Data Statement

All datasets on which the conclusions of this paper rely have been presented in the main manuscript and in the additional supporting files. Additionally, raw data can be accessed on request.

Funding

This work was funded by Universities of Nottingham for (MI).

Ethics Approval and Consent to Participate

Access to tissues and ethics approval were granted by Nottingham Health Sciences Biobank, which has approval as an IRB from North West—Greater Manchester Central Research Ethics Committee (REC reference: 15/NW/0685).

Consent for Publication

No patient consent was needed.

Declaration of Conflict of Interests

The authors declare no conflict of interest.

References

1. Anwar R. Screening for colorectal cancer in the UK. Digestive and liver disease. 2006; 38: 279–82.
2. O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. Journal of the National Cancer Institute. 2004; 96: 1420–5.
3. Huerta S. Recent advances in the molecular diagnosis and prognosis of colorectal cancer. Expert review of molecular diagnostics. 2008; 8: 277–88.
4. Herranz H, Cohen SM. MicroRNAs and gene regulatory networks: managing the impact of noise in biological systems. Genes & development. 2010; 24: 1339–44.
5. Zhou X, Xu X, Wang J, Lin J, Chen W. Identifying miRNA/mRNA negative regulation pairs in colorectal cancer. Scientific reports. 2015; 5.
6. Hannafon BN, Sebastiani P, de las Morenas A, Li J, Rosenberg CL. Expression of microRNA and their gene targets are dysregulated in preinvasive breast cancer. Breast cancer research. 2011; 13: R24.
7. Miko E, Czimmerer Z, Csanky E, Boros G, Buslig J, Dezso B, Scholtz B. Differentially expressed microRNAs in small cell lung cancer. Experimental lung research. 2009; 35: 646–64.
8. Schetter AJ, Okayama H, Harris CC. The role of microRNAs in colorectal cancer. Cancer journal (Sudbury. 2012; 18: 244.
9. Duttagupta R, Jones KW. The curious case of miRNAs in circulation: potential diagnostic biomarkers? Wiley Interdisciplinary Reviews: RNA. 2013; 4: 129–38.
10. Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. Jama. 2008; 299: 425–36.
11. Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. International journal of cancer. 2010; 127: 118–26.
12. Ayaz L, Gorur A, Yaroglu HY, Ozcan C, Tamar L. Differential expression of microRNAs in plasma of patients with laryngeal squamous cell carcinoma: potential early-detection markers for laryngeal squamous cell carcinoma. Journal of cancer research and clinical oncology. 2013; 139: 1499–506.

13. Bandrés E, Cubedo E, Agirre X, Malumbres R, Zarate R, Ramirez N, et al. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. Molecular cancer. 2006; 5: 29.

14. Sung JJ, Chong WS, Jin HC, Lam EKY, Shin VY, Yu J, et al. Differential expression of microRNAs in plasma of colorectal cancer patients: a potential marker for colorectal cancer screening. Gut. 2009.

15. Slaby O, Svoboda M, Fabian P, Smerdova T, Knoflickova D, Bednarikova M, et al. Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. Oncology. 2008; 72: 397–402.

16. Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. Jama. 2008; 299: 425–36.

17. Wang CJ, Zhou ZG, Wang L, Yang L, Zhou B, Gu J, et al. Clinicopathological significance of microRNA-31,-143 and-145 expression in colorectal cancer. Disease markers. 2009; 26: 27–34.

18. Diaz R, Silva J, Garcia JM, Lorenzo Y, Garcia V, Pena C, et al. Deregulated expression of miR-106a predicts survival in human colon cancer patients. Genes, Chromosomes and Cancer. 2008; 47: 794–802.

19. Chretien AS, Harle A, Meyer-Lefebvre M, Rouyer M, Husson M, Ramacci C, et al. Optimization of routine KRAS mutation PCR-based testing procedure for rational individualized first-line-targeted therapy selection in metastatic colorectal cancer. Cancer medicine. 2013; 2: 11–20.

20. Ng EK, Chong WWS, Jin H, Lam EKY, Shin VY, Yu J, et al. Differential expression of microRNAs in plasma of colorectal cancer patients: a potential marker for colorectal cancer screening. Gut. 2009; 58: 1375–81.

21. Ham-Karim H, Negm O, Ahmad N. Investigating genomic, proteomic, and post-transcriptional regulation profiles in colorectal cancer: a comparative study between primary tumors and associated metastases. Cancer Cell Int. 2023; 23: 192.

22. Abd El-Rehim DM, Ball G, Pinder SE, Rakha E, Paish C, Robertson JFR, et al. High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. Int J Cancer. 2005; 116: 340–50.

23. Søreide K. Receiver-operating characteristic curve analysis in diagnostic, prognostic and predictive biomarker research. Journal of clinical pathology. 2009; 62: 1–5.

24. Park SH, Goo JM, Jo C-H. Receiver operating characteristic (ROC) curve: practical review for radiologists. Korean Journal of Radiology. 2004; 5: 11–8.

25. Aslam MI. MicroRNAs are Novel Biomarkers for Detection of Colorectal Cancer. INTECH Open Access Publisher. 2012.

26. Corté H. MicroRNA and colorectal cancer. Digestive and Liver Disease. 2012; 44: 195–200.

27. Ma X, Choudhary SN, Hua X, Dai Z, Li Y. Interaction of the oncogenic miR-21 microRNA and the p53 tumor suppressor pathway. Carcinogenesis. 2013; 34: 1216-1223.

28. Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, et al. Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Laboratory investigation. 2005; 85: 154–9.

29. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomolecular detection and quantification. 2015; 3: 9–16.

30. Chai H, et al. miR-20a targets BNIP2 and contributes chemotherapeutic resistance in colorectal adenocarcinoma SW480 and SW620 cell lines. Acta biochimica et biophysica Sinica. 2011; 125.

31. Shibuya H, Linuma H, Shimada R, Horiuchi A, Watanabe T. Clinicopathological and prognostic value of microRNA-21 and microRNA-155 in colorectal cancer. Oncology. 2011; 79: 313–20.

32. Chang K, Miller N, Khierelseid EAH, Ingoldsby H, Hennessy E, Curran CE, et al. MicroRNA-21 and PDCD4 expression in colorectal cancer. European Journal of Surgical Oncology (EJSO. 2011; 37: 597–603.

33. Xiong B, Cheng Y, Ma L, Zhang C. MiR-21 regulates biological behavior through the PTEN/PI-3 K/Akt signaling pathway in human colorectal cancer cells. International journal of oncology. 2013; 42: 219–28.

34. Bullock M, Pickard KM, Nielsen BS, Sayan AE, Jenei V, Mellone M, et al. Pleiotropic actions of miR-21 highlight the critical role of deregulated stromal microRNAs during colorectal cancer progression. Cell death & disease. 2013; 4: 684.

35. Zhang J, Xiao Z, Lai D, Sun J, He C, Chu Z, et al. miR-21, miR-17 and miR-19a induced by phosphatase of regenerating liver-3 promote the proliferation and metastasis of colon cancer. British journal of cancer. 2012; 107: 352–9.

36. Faltejskova P, Svoboda M, Srutova K, Mlcochova J, Besse A, Nekvindova J, et al. Identification and functional screening of microRNAs highly deregulated in colorectal cancer. Journal of cellular and molecular medicine. 2012; 16: 2655–66.

37. Tang W, Zhu Y, Gao J, Fu J, Liu C, Liu Y, et al. MicroRNA-29a promotes colorectal cancer metastasis by regulating matrix metalloproteinase 2 and E-cadherin via KLF4. British journal of cancer. 2014; 110: 450–8.

38. Xia X. Prognostic role of microRNA-21 in colorectal cancer: a meta-analysis. PloS one. 2013; 8: e80426.

39. Reid JF, Sokolova V, Zoni E, Lampis A, Pizzamigilio S, Bertan C, et al. miRNA profiling in colorectal cancer highlights miR-1 involvement in MET-dependent proliferation. Molecular Cancer Research. 2012; 10: 504–15.

40. Schee K, Lorenz S, Worren MM, Gunther CC, Holden M, Hovig E, et al. Deep sequencing the microRNA transcriptome in colorectal cancer. PloS one. 2013; 8: e66165.

41. Earle JS, Luthra R, Romans A, Abraham R, Ensor J, Yao H, et al. Association of microRNA expression with microsatellite instability status in colorectal adenocarcinoma. The Journal of Molecular Diagnostics. 2010; 12: 433–40.

42. Ahmed FE, Amed NC, Vos PW, Bonnerup C, Atkins JN, Casey M, et al. Diagnostic microRNA markers to screen for sporadic human colon cancer in blood. Cancer Genomics-Proteomics. 2012; 9: 179–92.

43. Ke TW, Hsu HL, Wu YH, Chen WTL, Cheng YW, Cheng CW. MicroRNA-224 suppresses colorectal cancer cell migration by targeting Cdc42. Disease markers. 2014; 2014; 617150.

44. Diosdado B, van de wiel MA, Silve Droste JST, Mongera S, Postman C, Meijerink WJHJ, et al. MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression. British journal of cancer. 2009; 101: 707–14.

45. Toiyama Y, Takahashi M, Hur K, Nagasaka T, Tanaka K, Inoue Y, et al. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. Journal of the National Cancer Institute. 2013; 105: 849–59.

46. Schepeler T, Reinert JT, Ostenfeld MS, Christensen LL, Silahtaroglu AN, Dyrskjot L, et al. Diagnostic and prognostic microRNAs in stage II colon cancer. Cancer research. 2008; 68: 6416–24.

47. Zhou T, Zhang G, Liu Z, Xia S, Tian H. Overexpression of miR-92a correlates with tumor metastasis and poor prognosis in patients with colorectal cancer. International journal of colorectal disease. 2013; 28: 19–24.

48. Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. Journal of Clinical Oncology. 2004; 22: 2954–63.

49. Sun D, Yu F, Ma Y, Zhao R, Chen X, Zhu J, et al. MicroRNA-31 activates the RAS pathway and functions as an oncogenic MicroRNA in human colorectal cancer by repressing RAS p21 GTPase activating protein 1 (RASA1. Journal of Biological Chemistry. 2013; 288: 9508–18.

50. Cekaite L, Rantala JK, Bruun J, Guriby M, Agesen TH, et al. MiR-9,-31, and-182 deregulation promote proliferation and tumor cell survival in colon cancer. Neoplasia. 2012; 14: 868–21.

51. Chang KH, Miller N, Kheirelseid EAH, Lemetre C, Ball GR, Smith MJ, et al. MicroRNA signature analysis in colorectal cancer: identification of expression profiles in stage II tumors associated with aggressive disease. International journal of colorectal disease. 2011; 26: 1415–22.

52. Mishra S, Deng JJ, Gowda PS, Rao MK, Lin CL, Chen CL, et al. Androgen receptor and microRNA-21 axis downregulates transforming growth factor beta receptor II (TGFBR2) expression in prostate cancer. Oncogene. 2014; 33: 4097–106.

53. Donovan S, Shannon KM, Bollag G. GTPase activating proteins: critical regulators of intracellular signaling. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2002; 1602: 23–45.

54. Gong B, Liu WW, Nie WJ, Li DF, Xie ZJ, Liu C, et al. MiR-21/RASA1 axis affects malignancy of colon cancer cells via RAS pathways. World journal of gastroenterology. 2015; 21: 1488–97.