A Panel of Three MicroRNA Signatures as a Potential Biomarker for CRC Screening Based on Stages and Functional Prediction Using Bioinformatic Analysis

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Abstract: (1) Background: MicroRNA (miRNA) has been linked to colorectal cancer (CRC) tumorigenesis due to its post-transcriptional mechanism in targeting cancer-associated genes. Although miRNAs appear to be promising screening biomarkers, functional prediction analysis is required to shed light on their role in CRC tumorigenesis. Therefore, this study aims to identify the significantly deregulated miRNAs in CRC tumorigenesis. (2) Methods: Three upregulated miRNAs (hsa-miR-20a-5p, hsa-miR-21-5p, and hsa-miR210-3p) from 14 significant differentially expressed miRNAs (DEMs) were chosen from microarray profiling to be validated in plasma. Bioinformatics analyses showed that these miRNAs generally contributed to tumorigenesis, but only hsa-miR-20a-5p and hsa-miR-21-5p were specifically linked to CRC. Only two miRNAs showed a positive correlation when compared to their expression in plasma. However, further analysis showed that all three miRNAs in plasma were significantly different between the early and advanced stages of CRC. ROC curve analysis was used to evaluate miRNAs’ diagnostic performance in the early and advanced stages. (3) Results: Collectively, hsa-miR-20a-5p showed the highest discriminative value.
(AUC= 0.82, sensitivity = 86%, and specificity= 88%) in discriminating early CRC, while both hsa-miR-21-5p and hsa-miR-210-3p give a perfect performance for advance CRC. In addition, the performance of all miRNAs’ combinations also gives a perfect performance for diagnosis in both early and advanced CRC, except the combination of hsa-miR-20a-5p and hsa-miR-210-3p. (4) Conclusions: A few potential miRNA panels as CRC biomarker is needed for better prediction of disease. The reflective circulating miRNAs can be contributed to by minimal invasive screening tools.

**Keywords:** MicroRNA (miRNA); Colorectal cancer (CRC); Biomarker; Receiver Operating Curve (ROC); Target prediction

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1. Introduction

Colorectal cancer (CRC) is a type of gastrointestinal cancer that was reported to be the third most frequent cancer after lung and breast cancer worldwide [1] and the second cancer leading to death in Malaysia [2]. In the last decade, over one million people regardless of sex, were diagnosed with CRC, and nearly half of the cases, have resulted in death [3]. Western countries presented the highest cases of CRC, followed by Asian countries [1,3]. The incidence is influenced by genetic inheritance, dietary, smoking habits, and alcohol intake [4-5]. According to a study conducted by Magaji and colleagues, the incidence of CRC varies by ethnic group in Malaysia, with Chinese having the highest incidence (27.35 per 100 000), followed by Malays (18.95 per 100 000), and Indians (17.55 per 100 000). CRC incidence and mortality rates in Malaysia were 21.32 and 9.79 per 100,000 people, respectively. The 5-year survival rates in Malaysia ranged from 31.2% to 77%, with Chinese patients having the lowest survival rate compared to Malay and Indian patients [6].

The majority of cases were over the age of 50, with a late stage and a poor prognosis [7]. This is because of late disease manifestation and poor screening tests [8]. More than half of the participants in a Malaysian public awareness campaign were aware of the symptoms of CRC. Nevertheless, this did not lead to an increase in screening activity after the campaign [9]. According to researchers, the economic burden will probably rise over time as a result of the growing trend of CRC in Malaysia. Indirectly, this will portray CRC’s management as being exclusive and only accessible to those with higher incomes [8]. Countries with low to middle incomes may have the lowest survival rates; therefore, having a low-cost diagnostic tool may help with early detection and successful management of CRC [10]. Another alternative, a clinical researcher would develop a screening protocol for patients with Lynch Syndrome who have a higher risk of developing CRC [11].

Early detection of CRC has been challenging. The screening test is conducted in two modes: examining the stools or inspecting the colon [12]. Due to the difficulties in preparation, neither of these two modes is appealing to the general public. Since then, molecular screening has been used to detect altered DNA in cancer cells or hemoglobin in stool (e.g., FOBT, fecal DNA, and FIT). However, clinical evidence revealed that it had low sensitivity and
specificity, and researchers are still working to improve its efficacy [12]. Although stool-based screening tools appear to have more advantages, there are technical and thorough implementation challenges. For example, the requirement for a special buffer and multistep lab analysis may pose the complexity of this type of screening approach [13]. Additionally, a colonoscopy is a method of colon inspection that can detect and remove precancerous polyps. The ability to fully visualize the entire colon is another benefit of colonoscopy over another tool of a similar nature (flexible sigmoidoscopy). Patients might experience an uncomfortable situation, though, because of the tedious setting. [14].

Since its discovery, microRNAs have been closely related to tumorigenesis [15-17]. Few findings have discovered the mechanism of miRNAs in tumorigenesis [18-21], including in CRC [22-24]. This short non-coding RNA class regulates the gene expression at the post-transcriptional level and acts as either oncomiR or tumor-suppressor-miR according to its sequence complementarity [25-26]. MiRNAs have great potential as minimally invasive biomarkers due to their stability in blood samples even though they are exposed to extreme pH value, extended storage, and several freeze-thawing [27-28]. Despite its robustness in findings, none are firmly promised as an established biomarker for CRC screening.

Hence, this study intended to identify a list of deregulated miRNAs in CRC tissue as potential biomarkers. Panels of significant miRNAs were validated in plasma samples and analyzed for their expression correlation with tissue miRNAs. Bioinformatics analysis was used to predict the selected miRNAs for their targeted genes and pathways. Finally, to assess their diagnostic performance as a CRC biomarker, receiver operating characteristics (ROC) analysis was used to determine their sensitivity, specificity, and area under the curve (AUC). Other circulating data from the TCGA database were also compared to evaluate each miRNA’s performance in other populations.

2. Materials and Methods

2.1. Patient sample recruitment

In the initial stage of the study, we collected plasma and CRC mucosa (both adjacent non-tumoral (NT) and tumoral (T) tissues) from eight (n = 8) people who had been diagnosed with CRC during the operative procedure. In the meantime, during the second phase of the study, plasma from eight independent CRC groups was recruited for each early stage (n=8) and advanced stage (n=8) of CRC cases. Since this study is a matched case-control study, we also recruited healthy controls (HC, n=8). All recruited individuals are those who came to our center study, Hospital Universiti Sains Malaysia and fulfilled the study’s inclusion criteria. The listed criteria can be seen in supplementary material, S1. This study was approved by The Human Research Ethics Committee of USM (JEPeM), USM: USM/JEPeM/18050239, and written informed consents were obtained by patients themselves and their relatives, voluntarily. All procedures are carried out following the Helsinki Declaration's relevant guidelines and regulations under the 'Informed Consent' section.
Total RNA extraction and microarray profiling for tissue sample. Total RNA was extracted using the RiboExTM, GeneAll (Seoul, South Korea) from ~80-100mg of both tumoral and non-tumoral CRC mucosa. The RNA quality was assessed by Agilent 2100 Bioanalyzer for RNA integrity number (RIN). Sample with RIN, 6-10 were standardized to 50ng and labeled with Cyanine 3-pCp for the hybridization step using miRNA Complete Labelling and Hyb kit (Agilent, Valencia, CA). Agilent SureScan Microarray Scanner (G4900DA) was used to scan miRNA expression on hybridized chips. All procedures were done according to the manufacturer’s protocol.

2.2. Selection of DEMs

Significant DEMs were analyzed using Agilent Genespring Analysis software version 14.9.1 by applying percentile shift for normalization. This study measured the probe-based miRNAs expression which was filtered based on an upper cut-off of 100% and a lower cut-off of 20%, to avoid saturated probes and background noise. The volcano plot is set to filter miRNAs that have fold change (FC) ≥ 2 and p-value < 0.05. The FC value was obtained by calculating the difference between the pair of tissue types (T versus NT) using a moderated T-test with Benjamin-Hochberg multiple testing correction. Only three (hsa-miR-20a-5p, hsa-miR-21-5p, and hsa-miR-210-3p) of the nine upregulated miRNAs were chosen for further investigation in this study.

2.3. Bioinformatic prediction analysis

This study analyzed the disease association of selected miRNAs retrieved from miRCancer databases (http://mircancer.ecu.edu). MiR-TV (https://mirtv.ibms.sinica.edu.tw/) was accessed to obtain the list of predicted target genes by miRNA. Other functional analyses such as gene ontology (GO) and predicted pathway were enriched from g.Profiler (https://biit.cs.ut.ee/gprofiler/gost).

2.4. MiRNA validation by reverse transcriptase quantitative PCR (RT-qPCR) for plasma sample

Total RNA from plasma samples were extracted using MiRNeasy serum/plasma kit, QIAGen (Hilden, Germany), and their quality was assessed by microDrop™ plate. RT-qPCR was used to validate the selected miRNAs expression profile that was obtained from microarray data in plasma samples. The extracted RNAs were standardized to 100ng before reverse transcripted to cDNA using iScript™ cDNA synthesis (Bio-Rad, 170-8891). Mastermix from SensiFAST™ SYBR® Hi-ROX (BIOLINE, BIO-92005) is used and qPCR reaction was set up using 7300 System SDS v1.3.1 (Applied Biosystems) StepOne qPCR machine. For each run, no template control and no reverse transcriptase were also included. The qPCR technical setup can be seen in Supplementary material (S2-5). The primer of selected miRNAs used were as follow; hsa-miR-20a-5p (MIMAT0000075, 5’-CTACCTGCACTATAAGCAC-3’), hsa-miR-21-5p (MIMAT0000272, 5’-
GTCTGTCAATTCATAGGTCAT-3’) and hsa-miR-210-3p (MIMAT0000267, 5’-TCAGCCGGTGTGCAC-3’). Melt curve and standard curve were observed for primers specificity and efficiency. The standard curve was done with 2-fold dilution for five-times serial with one no template control.

2.5. Statistical test

Descriptively, the Mann-Whitney U test was used to compare the median age of the CRC group and the healthy group. While the Fisher Exact test was used to associate the gender of participants and disease status. Spearman correlation was used to assess the relationship of the selected miRNA expression in tissue with their expression in the plasma of the same individuals. The Kruskal-Wallis test was used to determine the significant difference between groups. The miRNAs expression analysis was analyzed using the $2^{\Delta\Delta Cq}$ method. Expression level was normalized to cel-miR-39-3p. Data are expressed as mean ± standard deviation. The experiment was done in triplicate for each biological control. The diagnostic performance of the miRNAs was determined using the receiver operating curve (ROC). The potential performance of both individual and combined miRNAs was analyzed to enrich the ROC analysis on miRNAs’ potential mechanism. To evaluate their validation further, we compared the differential potential of each miRNA in both tissue and circulating data from TCGA retrieved using CancerMIRNome (jialab-ucr.org).

3. Results

3.1. Demographical and clinicopathological characteristics

Demographical characteristics illustrate that there is no significant association of gender to CRC ($p>0.05$) (Supplementary material: Table S6). However, the age of CRC cases is significantly different from healthy groups ($p < 0.05$). 54.2% of CRC groups were diagnosed in the advanced stage (stage III: 29.0% and IV: 25.2%) and 41.7% of them reported metastasis to the liver/lung. The majority of cases (95.8%) presented with adenocarcinoma.

3.2. MiRNA profiling analysis in the tissue sample

We have evaluated the miRNA profiling data using a heatmap (Figure 1. A) and a Volcano plot (Figure 1. B) to discover the potential list of significant DEMs expressed in CRC tissue. The microarray panel used in this study consisted of 2,548 genes. However, after using the Moderate T-test with Benjamin Hochberg correction, only 14 filtered DEMs remained. Figure 1(C) shows all the DEMs with their respective FC. This study discovered 9 upregulated miRNAs (hsa-miR-192-5p, hsa-miR-20a-5p, hsa-miR-21-5p, hsa-miR-210-3p, hsa-miR-215-5p, hsa-miR-223-3p, hsa-miR-23a-3p, hsa-miR-24-3p, and hsa-miR-29a-3p) and 5 downregulated miRNAs (hsa-miR-3653-3p, hsa-miR-3945, hsa-miR-5090, hsa-miR-5196-5p and hsa-miR-6133). These DEMs were miRNAs that passed the filter FC ≥ 2 and $p$-value < 0.05.
3.3. Bioinformatic analysis

MiRNAs' functional enrichment analyses were performed to elucidate the potential biological function of selected DEMs. The association of miRNAs with the type of cancer based on the miRCancer database and the top ten potential target genes for each miRNA based on the miR-TV database were presented in Table S7. Meanwhile, Table S8 listed the potential GO and predicted biological pathways for each miRNA obtained from the g:Profiler database. The g:Profiler database presented the GO (molecular function, cellular component, and biological process) and top ten list of predicted pathways from three other databases; namely KEGG, REACTOME, and WikiPathways.

3.4. MiRNA expression analysis in the plasma sample

This study analyzed the correlation between the expression of selected miRNAs in CRC tissue and their counterpart plasma using the Spearman correlation (Figure 2). Among the selected miRNAs, only hsa-miR-20a-5p and hsa-miR-21-5p have a positive correlation of expression between tissue and plasma. Hsa-miR-21-5p has a higher potential as a biomarker due to its stronger correlation ($r=0.838$) compared to hsa-miR-20a-5p ($r=0.204$). On the other hand, a negative correlation of hsa-miR-210-3p ($r=-0.746$) indicates that the upregulation of this miRNA in CRC tumoral tissue was inversely correlated with its expression in plasma.

Next, the fold change expression of each selected miRNA in plasma was evaluated using the 2-$\Delta\Delta$Cq method (Figure 3) in both early and advanced groups of CRC (earCRC; $n=8$ and advCRC; $n=8$) and HC group ($n=8$). The analysis discovered that all the selected potential miRNAs have significant differential expression compared to HC. The miRNA quantification is too low (Cq > 35) to be quantified by RT-qPCR.

3.5. Diagnostic performance of selected miRNAs

ROC analysis was run to determine the capability of these miRNAs in screening the CRC (Figure 4). Among these three miRNAs, hsa-miR-20a-5p presented the highest discriminative power with an AUC value of 0.82 (95% CI: 0.56-1.00), with 86% sensitivity and 88% specificity in predicting the CRC cases for the early stage. On the contrary, in predicting the CRC cases with advanced stage, both hsa-miR-21-5p and hsa-miR-210-3p presented a perfect score for AUC = 1.0 (1.0-1.0), with 100% sensitivity and specificity. Thus, hsa-miR-20a-5p can be thought of as a potential CRC biomarker for a single panel in detecting early cases, and either hsa-miR-21-5p or hsa-miR-210-3p as a panel in detecting CRC with advanced cases.

This study also performed the ROC curve analysis for a combination of potential miRNAs in both CRC groups (early and advanced stage) against HC. This study evaluated the performance of combinations of two miRNA panels (Figure 5.A. hsa-miR-21-5p and hsa-miR-210-3p; Figure 5.B. Hsa-miR-20a-5p and hsa-miR-210-3p and Figure 5.C. Hsa-miR-20a-5p and hsa-miR-21-5p) and combinations of all three miRNAs (Figure 5.D). The trend
of AUC values can be observed from the data presented in Figure 5. The results showed that only the combination of hsa-miR-20a-5p and hsa-miR-210-3p has the lowest discriminative power when tested in the early stage of the CRC group, with AUC values of 0.82 (95% CI: 0.92-1.0), 71% sensitivity, and 75% specificity. In contrast, the rest showed perfect performance in both the early and advanced stages of CRC.

We compared the differential potential of each selected miRNA from the circulating TCGA database. The TCGA data presented a pooled sample of CRC (n=14) against healthy (n=6). Figure 6 shows both the miRNA differential expression (I. A -I. C) and their ROC respectively (II(A)-II(C)). Based on the circulating TCGA data presented, not only their differential expressions were not significant but also (Figure 6. II(A)-II(C)) have poor discriminative power with AUC below 0.7.

Figure 1. (A) The miRNAs expression analysis using probe-based detection. Color gradient expression of clustered CRC tissue (T represents tumor tissue and NT represents non-tumoral tissue) using heatmap. The red-gradient color indicates upregulated FC, meanwhile, the blue-gradient color indicates downregulated FC. (B) The volcano plot showed the filtered DEMs by FC ≥ 2 and p-value < 0.05. Each dot represents miRNA. Red dots represent upregulated miRNAs, while blue dots represent downregulated miRNAs. (C) The expression level of all DEMs with their respective FC value. Data are the mean ± standard deviation.
Figure 2. Spearman correlation shows the correlation of miRNA expression in tissue against plasma. (A) Correlation of hsa-miR-20a-5p (B) correlation of hsa-miR-21-5p and (C) correlation of hsa-miR-210-3p. Asterisk (*) indicates for significant p-value.

Figure 3. Validation of selected miRNAs in plasma of early CRC (earCRC, n=8), advanced stage of CRC (advCRC, n=8), and HC (n=8) group. Asterisk (*) note indicates for significant p-value.
Figure 4. ROC curve of selected miRNAs for the early and advanced stage of CRC groups.

Figure 5. ROC analyses of several combinations of miRNA panels in the early and advanced stages of the CRC group.
4. Discussion

Circulating miRNAs are the potential to be a minimally invasive biomarker for cancer screening. The study of circulating miRNAs first started in 2008, when a study observed the presence of miR-141, miR-149, miR-299-5p, and miR-136b in the serum of pregnant mothers [29]. In CRC, several studies found panels of miRNAs that are relevant to be associated with CRC [30–33]. For example, a study by Zhang and team discovered that miR-17-5p and miR-135b in body fluids were related to CRC [34]. Even though many circulating miRNAs have been proposed as promising screening biomarkers for CRC, none of them have been reported clinically as well-established biomarkers in screening and diagnosing CRC. This is possibly due to the divergence of the miRNA mechanism and the targets themselves.

To the best of our knowledge, this study is the first that reports the upregulated expression of hsa-miR-210-3p in CRC tissue although it has a negative correlation in plasma. This was supported by the bioinformatics analysis in the current study that found no previous studies reported the association of hsa-miR-210-3p with CRC, but other cancers. Hsa-miR-
210-3p is known as hypoxamirs, a type of miRNA that specifically will be upregulated under the condition of low oxygen, such as in cancer, myocardial infarction, ischemia, and preeclampsia \cite{35-41}. Therefore, we postulated that hsa-miR-210-3p presented at all stages of cancer due to its mechanism, since it presented in other types of cancer as well. Unlike hsa-miR-20a-5p and hsa-miR-21-5p, both of them were reported to be associated with CRC tumorigenesis, as illustrated in Table S7.

We presented the list of ten targeted genes for each of the miRNAs that were retrieved from miR-TV, an integrative database that compiled the findings from miRDB, targetScan, and miRanda, in Table S7. As we discovered that none of the genes was found to be overlapped among each miRNA. As hsa-miR-20a-5p targeted Forkhead Box Q1 (FOXQ1) genes, a study has reported high expression of FOXQ1 in CRC tissue and its potential mechanism by targeting the HB-EGF/EGFR pathway, which promoted CRC invasion and metastasis \cite{42}. Meanwhile, hsa-miR-21-5p and hsa-mi-210-3p were reported to target Fibroblast growth factor receptor (FGFR) family genes; FGFR18 and FGFR1 respectively. Generally, FGFR family genes promote cancer cell proliferation, differentiation, and invasion. However, they have distinct functions for each sub-family gene \cite{43}. A review study revealed that CRC patients were likely to express FGFR genes frequently \cite{44}. The identification of targeted genes and their mechanism is crucial as they are capable of providing insight into the reliability of the candidate miRNAs as biomarkers for specific cancers. This study did not examine the alteration of miRNA-genes according to CRC lesions, as Yunos and colleagues claimed that there is a difference in gene alteration between the lesions \cite{45}.

Furthermore, this study also enriched the analysis with the bioinformatic prediction on GO and biological pathways for each selected miRNA (Table S8). Based on GO data, hsa-miR-20a-5p is involved in the biogenesis of binding proteins, such as cytokine receptor activity, glycosaminoglycan binding and interleukin 8 receptor binding, and angiogenesis-related processes, such as blood vessel development and blood vessel morphogenesis. Meanwhile, hsa-miR-21-5p is mostly reported for metalloendopeptidase activity, such as metalloendopeptidase inhibitor activity and regulation of metalloendopeptidase activity. Metalloendopeptidase is derived from endopeptidase and metallopeptidase activity. A bioinformatics study by Meng and team discovered that metallopeptidase protein was significantly upregulated in tumor cells compared to normal cells. Notably, it was found higher in colon cancer, lung adenocarcinoma, and uterine corpus endometrial carcinoma due to its association with disease stages, clinical findings, mutations, and microsatellite instability \cite{46}.

The g:Profiler, which compiled biological pathway predictions from KEGG, REACTOME, and WikiPathways, showed a list of predicted pathways for both hsa-miR-20a-5p and hsa-miR21-5p. According to KEGG and WikiPathways, hsa-miR-20a-5p is involved in cancer pathways such as pancreatic cancer, bladder cancer, and gastric cancer. Besides, REACTOME showed more specific pathways, such as signaling by FGFR3 fusions in cancer and MAPK1 (ERK2) activation. A few researchers have reviewed the crosstalk of
growth factors and MAPK pathways, especially in colorectal cancer. They showed that MAPK mostly responded to growth factor receptors and contributed to cell proliferation \[^{47-50}\]. Another miRNAs study also showed the association of MAPK pathways with other deregulated miRNAs, such as miR-139-5p \[^{51}\].

Our correlation analysis discovered that hsa-miR-20a-5p and hsa-miR-21-5p were upregulated in both tissue and plasma, although the FC value was slightly different. The discrepancy of gene expression using microarray and RT-qPCR has been discussed by Draghici and team (2016), where they assumed that the difference in the detection mechanism of the tools used resulted in different quantification. Microarray measured the gene expression by \(~70-90\)% changes different from RT-PCR to prevent false detection by the probe \[^{52}\]. This was also supported by Koltai & Weingarten-Baror (2008) who proposed that different measurement tools have different accuracy, different reproducibility rates, and different detection for sensitivity and specificity \[^{53}\].

Aside from that, the correlation analysis in Figure 2 shows that hsa-miR-210-3p is inversely correlated (r = -0.746, p < 0.05) between plasma and tissue. Although this study is the first study to mention the significant expression of hsa-miR-210-3p in tissue profiling as presented in Figure 1, however, due to its low expression in plasma validation, this miRNA is unlikely to be chosen as a CRC biomarker (Figure 2). According to the findings of a study conducted by Eslamizadeh and colleagues (2018), the abundance of circulating miRNAs in body fluid was influenced by mass production and was exported out of tissue \[^{54}\]. However, the current study discovered results that contradicted to theory by Eslamizadeh et al., where the expression of hsa-miR-210-3p in blood circulation was lower than its counterpart in tissue. Aside from that, another study conducted by Xu and the team, postulated that the cells might only allow certain miRNAs to be released at certain stages of the cell cycle and only a certain group of miRNAs originating from tumor tissue \[^{55}\].

Knowing that multiple miRNAs may attach to a single mRNA and/or that a single miRNA may be able to target multiple mRNAs of the same pathway, it is crucial to evaluate the diagnostic performance of miRNAs’ combination \[^{56-57}\]. The combination of all miRNAs gives a perfect score in discriminating early cases of CRC, except for the combination of two miRNA panels (hsa-miR-20a-5p and hsa-miR-210-3p) (Figure 5. B(I)). Surprisingly, ROC analysis on combinations of miRNA panels in predicting the advanced cases of CRC showed that all the test provides a perfect score for each panel. This can be interpreted as, these combinations of three potential miRNAs would give a good panel of biomarkers for CRC, either for early cases or advanced cases. Apart from predicting disease diagnosis, miRNA has the potential to be used as a therapeutic agent; however, there is currently no database containing this information \[^{58}\]. That is, researchers are still working to determine the future potential of this miRNA by elucidating its potential role. MiRNA also has the capability to collaborate with another group of non-coding RNA (e.g; circular RNA (circRNA)) in joining the cancer-related pathway. A few findings show the involvement of CircHIPK3 \[^{59}\] and circEPHB4 \[^{60}\] in CRC progression.
As compared to the circulating TCGA database (Figure 6), these three miRNAs seem unlikely to have good performance as biomarkers, non-significance expression, and low power in discriminating disease. These datasets were obtained from GSE39845 of GEO/ArrayExpress Accession where they obtained the miRNAs set from whole blood samples. Although the study was done from the same population [61] as the current study, however, they are differences in the list of significant DEMs and their performance also differed from the current findings. We first postulated that it might be from the different samples used but other researchers suggested that there was no significant difference between the types of samples used [62]. Therefore, we concluded that the diversity of miRNAs expression depends on the type of population ethics as most of the samples from the study come from the Chinese population, meanwhile, this current study recruited all Malay population.

5. Conclusions

Efficiently, having a single panel of biomarkers seems to cut costs, however, due to the divergence of miRNA’s mechanism, it is suggested to have a few other panels to support the detection evidence. Although all the miRNAs selected were significantly identified in CRC tumoral tissue, we assumed that not all the significant DEMs were necessarily associated with CRC. However, this study was only able to validate those potential biomarkers on a small population scale making it one of the limitations. A similar approach could be taken in a larger independent cohort study, which could broaden the analysis into diagnostic, prognostic, and predictive markers for CRC.

Supplementary Materials: The following files are available online on the journal website.

Author Contributions: Conceptualization, Mohamed SS, Ahmad A, Ab Mutalib NS, Din TADAAT, Md Salleh MS and Zakaria Z; Methodology, Mohamed SS, Ahmad A, and Ab Mutalib NS; Software, Mohamed SS and Ab Mutalib NS; Validation, Mohamed SS and Ahmad A; Formal analysis, Mohamed SS, Ahmad A, Ab Mutalib NS, Din TADAAT and Md Salleh MS; Investigation, Mohamed SS, Ahmad A, and Din TADAAT; Resources, Ab Mutalib NS, Zakaria AD and Zakaria Z; Data curation, Mohamed SS and Ahmad A; Writing—original draft preparation, Mohamed SS and Ahmad A; Writing—review and editing; Mohamed SS, Ahmad A, Ab Mutalib NS, Din TADAAT, Md Salleh MS Zakaria A.D, and Zakaria Z.

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