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# Comprehensive Analysis of microRNA Methylation Profiles and Determination of Their Functional Significance in Colorectal Cancer: A Study Protocol

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**Abstract:** Colorectal cancer (CRC) is a leading cause of cancer-associated fatalities globally. Despite the notable progress in diagnostics and patient care, challenges persist in areas such as early detection, prognostic variable identification, metastatic disease treatment, and personalised treatment options. In CRC, microRNAs, a type of short non-coding RNA, are found to be deregulated and can significantly influence its onset and progression. However, previous microRNA research primarily focused on expression levels to ascertain their biological significance, leaving the microRNA methylation landscapes in CRC patients largely unexplored. This study aims to investigate the genome-wide methylome profiles of microRNA and clarify their roles in CRC. We will analyse the microRNA methylation profiles from our in-house data of more than 100 CRCs and correlate the differentially methylated microRNAs with clinicopathological features. To understand the biological significance of methylated microRNAs, we will perform pathway enrichment analysis and

molecular dynamics simulations to examine the binding of argonaut protein to the differentially methylated microRNAs and the structural changes involved. Furthermore, we will conduct functional studies to determine the roles of selected microRNAs. CRC cell lines with hypermethylated microRNA of interest will be treated with a demethylating agent, followed by cell-based assays. Subsequent transcriptome-wide microRNA gene target identification and protein profiling will be performed to interrogate the molecular pathways affected by demethylation of the microRNA of interest. Methylation signifies a major transformation in cancer detection, as widespread epigenetic changes could potentially provide superior early-stage cancer detection and classification compared to somatic mutations. Effective CRC diagnosis ensures timely and appropriate treatment for patients, thereby improving their quality of life. The insights gained from this study could be applied to individualized health diagnostics, disease prognostication, and treatment monitoring.

**Keywords:** Colorectal cancer; microRNAs; methylation profiles; differential methylation; personalized health diagnostics; precision medicine; SDG 3 Good health and well-being

## 1. Introduction

Colorectal cancer (CRC) stands as one of the most prevalent cancer diagnoses around the world. The impact of this issue is not limited to individuals, as it also puts a significant economic burden on healthcare systems. The cost of managing new CRC cases is estimated at RM62 million annually [1]. While there have been considerable improvements in diagnostic procedures and patient care, numerous obstacles remain. These involve early diagnosis, pinpointing prognostic factors, devising effective treatments for metastatic conditions, and tailoring treatment strategies to individual patients [1, 2]. A local study highlighted the high cost of CRC, indicating that provider costs range from RM13,672 for stage I to RM27,972 for stage IV [3].

Cancer poses a significant health challenge globally, with over 18.1 million new cases identified in 2018, and this number is anticipated to grow in the forthcoming decade, especially in less developed and developing countries [4]. In Malaysia, CRC is the most frequently diagnosed cancer, and the prevalence is 21.3 cases for every 100,000 individuals [5]. Despite numerous studies revealing molecular changes involved in the development of CRC [6-12], the prognosis for advanced stages of this disease remains poor. The search for biomarkers that could offer precise guidance in managing and treating CRC remains ongoing within the medical community. Therefore, there is a significant demand for reliable prognostic and predictive biomarkers, and one potential source of these biomarkers could be the tumor's epigenome [13]. Researchers aim to unlock the full potential of the tumor epigenome by employing a diverse array of techniques, ranging from the simplicity of molecular assays like RT-PCR [14] to the complexity of multi-omic analyses [15-17]. This comprehensive approach is anticipated to significant contribute to the ongoing pursuit of more effective and innovative cancer diagnostics and therapies [18-23].

MicroRNAs are small, non-coding RNAs, approximately 22 nucleotides in length, that regulate gene expression in a variety of eukaryotic organisms [24]. These single-stranded RNAs function by associating with specific target mRNAs, predominantly through partial correspondence with sequences located in the 3' untranslated region (UTR). This interaction leads to either mRNA degradation or inhibition of translation [24]. MicroRNAs are crucial in regulating numerous cellular processes, such as cell proliferation, metabolism [25], apoptosis, angiogenesis [26], cell cycle and differentiation, by simultaneously modulating the expression of several genes [24, 27, 28]. In human cancers, it has been observed that microRNA expression profiles can vary significantly between tumors and the normal tissues, among various tumor types, and even within the same tumor exhibiting different phenotypes [17, 29-32]. MicroRNAs play a crucial role in tumorigenesis, acting oncogenes or tumor suppressors that function to promote or inhibit cancer development, respectively [33-35].

The precise mechanism that leads to microRNA deregulation in cancer remains unclear. However, numerous studies have indicated that epigenetic mechanisms [36-38], particularly DNA methylation, play a vital role in regulating the expression of microRNAs [39, 40]. DNA methylation, a biological process, attaches methyl groups (CH3) to the cytosine ring, creating 5-methylcytosine (5mC) [41]. This process may epigenetically control the expression of microRNAs through the methylation of DNA in CpG islands located within promoter regions, and disturbances in these mechanisms could lead to changes in microRNA expression, which could then modify gene and protein expression, ultimately contributing to the progression of cancer [42].

Despite the established research into microRNA gene methylation, with scholarly articles on this subject having been published approximately a decade ago, there is still a knowledge gap, especially in CRC. The majority of the existing data is derived from CRC cell lines instead of patient specimens [43, 44]. To the best of our understanding, only a small number of studies have conducted comprehensive profiling of microRNA methylation across the entire genome in cancer. The main research focus in this area has been centered around a specific group of microRNAs that are known to exhibit hypermethylation in CRC. Some of these microRNAs are miR-34b/c, miR-124, miR-133b, and miR-324, among others [45-47]. Although epigenome-wide microRNA methylation profiles exist for other cancers [48-50], only one study has been conducted in the context of CRC thus far [51]. A recapitulation of microRNA methylation's role in CRC was already delineated in our previous work [52]. The fact that only one study has conducted global microRNA methylation profiling in CRC using high-throughput methods such as microarray amplifies the significance of our study and highlights the need for more comprehensive research in this area.

The objective of this research is to delve into the genome-wide methylome profiles of microRNA and their roles in CRC. We aim to identify the distinctive pattern of methylated microRNAs in CRC and establish a correlation between the significantly differentially methylated microRNAs and clinicopathological features such as stage, lymph node or distant organ metastasis, age, gender, and histological subtype. Furthermore, we seek to elucidate the pathways regulated by the differentially methylated microRNAs and the mechanisms

involved. Lastly, we plan to interrogate the roles of selected differentially methylated microRNAs in the development and progression of CRC. Through these objectives, we hope to gain a deeper understanding of the role of microRNA methylation in CRC.

Our research is guided by several hypotheses. We hypothesize that the microRNAs in CRC differ from those in the normal colon due to a distinct methylation signature. We also postulate that the significantly differentially methylated microRNAs are associated with clinicopathological features such as stage, lymph node or distant organ metastasis, age, gender, and histological subtype. Furthermore, we believe that various cancer-related genes and pathways are regulated by the differentially methylated microRNAs via altered target inhibitory effects and structural changes in the argonaut (AGO) protein complex. Lastly, we propose that the differentially methylated microRNA plays significant roles in CRC development and progression, influencing cancer cells' viability, migration, invasion, and colony-forming ability.

To validate these hypotheses, our research will seek to answer several key questions. Why does the signature of the microRNA methylome in CRC differ from the normal colon? How do the significant differentially methylated microRNAs correlate with the clinicopathological features? How do the differentially methylated microRNAs influence the cancer pathways? And finally, how significant are the roles of the methylated microRNAs in CRC development and progression? Through these inquiries, we aim to shed light on the role of microRNA methylation in CRC.

## 2. Materials and Methods

### *2.1. Data Acquisition*

Methylation microarray data from 104 CRCs based on Illumina Infinium Human Methylation 450K will be retrieved from our internal database. The inclusion and exclusion criteria are as follows:

- Inclusion criteria: Malaysian patients diagnosed with CRC, consented to specimen collection and future research, histological subtypes include the most frequently identified, for example, adenocarcinoma and mucinous carcinoma of the colon, and patients with methylation microarray datasets already available in our laboratory.

- Exclusion criteria: Malaysian patients diagnosed with cancer(s) other than CRC, did not consent for specimen collection and future research, histological subtypes other than adenocarcinoma and mucinous carcinoma of the colon, and patients without in-house methylation microarray datasets.

The clinicopathological data will be retrieved manually from various reports and records available at the Record Unit (UKM Medical Center) upon obtaining ethical approval.

### *2.2. Cell Lines and Culture Condition*

Eight CRC cell lines (SW 480, SW 48, COLO 320DM, COLO 205, HCT 116, HT 29, SW 1463 and SW 1116) and three normal colon cell lines (CCD 33 Co, CCD 112 CoN and CCD 841 CoN) will be obtained from UMBI's cell bank and will be maintained in McCoy's 5A (Gibco) or DMEM (Gibco) at 37°C and 5% $CO_2$ in the incubator (Galaxy 170R, Eppendorf). All culture media will be supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% Penicillin-Streptomycin.

### *2.3. Nucleic Acid Extraction and Determination of Nucleic Acid Purity*

The DNA and total RNA will be extracted using the AllPrep DNA/RNA/microRNA Universal Kit (Qiagen) [53]. The purified DNA and RNA will be stored at -20°C and -80°C in aliquots to avoid repeated freezing and thawing. The purity of nucleic acid yield will be determined by using a NanoDrop ND-2000 Spectrophotometer (Thermo Fisher) at 260 nm (A260) and 280 nm (A280) absorbance, using the A260/A280 ratio, with a sample volume of 1.5 μL. The acceptable range of purity for DNA and RNA is 1.8 to 2.0 and 2.0 to 2.2, respectively.

### *2.4. MicroRNAs Expression Study*

For microRNA expression profiling, cDNA will be synthesized using miRCURY LNA RT Kit (Qiagen). qPCR will be performed on the Biorad CFX96 Real-Time PCR System (Biorad). Primers will be selected from miRCURY LNA microRNA PCR Assay lists and qPCR will be performed using miRCURY LNA SYBR Green PCR Kit (both from Qiagen). U6 snRNA and SNORD38B will be used as the housekeeping controls. The fold change will be calculated using the $2^{-\Delta\Delta CT}$ Method [54].

### *2.5. Bisulfite Sequencing*

The bisulfite conversion process will be carried out on a DNA sample of 250 ng using the EZ DNA Methylation-Gold™ Kit from ZYMO Research. This will be conducted following the instructions provided by the manufacturer. Following this, bisulfite PCR amplification will be performed, followed by direct sequencing and cloning. This method is particularly effective for detecting methylation at multiple CpG sites, even in cases where only partial methylation is present on a CpG island. To elaborate, primers that target specific regions of interest will be designed using the Methyl Primer Express Software v1.0 from Thermo Scientific. Methylation PCR will then be carried out using the EpiMark Hot Start Taq DNA Polymerase and Deoxynucleotide Solution Mix from New England Biolabs. This will be followed by standard agarose gel electrophoresis.

The unpurified PCR products will then be cloned into the pJET1.2/Blunt vector from Promega and transformed into competent cells. The cells containing the ligated vectors will then be selected on agar plates containing ampicillin/X-gal/IPTG using blue/white screening selection. In this process, blue colonies represent an empty vector, while white colonies indicate

vectors that have been inserted with the target PCR product. The white colonies will be selected and cultured in an LB medium. Plasmids containing the target DNA will be purified and subjected to standard sequencing analysis [55]. This comprehensive approach allows for a detailed examination of methylation patterns, contributing to our understanding of the role of methylation in various biological processes.

### *2.6. Data Analysis*

#### *2.6.1. Microarray Methylation Data Analysis*

Microarray methylation data analysis will be performed using the CHAMP Bioconductor packages [56]. The raw intensities will undergo normalization using the Subset-quantile Within Array Normalization (SWAN) method. This step is crucial to mitigate the technical biases inherent in the probe design before proceeding with further statistical analysis. Following normalization, we will extract the β values. We will then conduct a statistical analysis on these β values using t statistics from the limma Bioconductor package. From the significant differentially methylated loci, we will manually extract the list of microRNAs. We have set a threshold for statistical significance at a delta beta of |0.2| and an adjusted P-value of less than 0.05.

#### *2.6.2. Pathway Enrichment Analysis*

Pathway enrichment analysis will be carried out using DAVID [57]. DAVID is a comprehensive tool that enables researchers to decipher the biological significance of extensive miRNA lists through the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis in a systematic and integrated manner. The threshold for statistical significance will be set at $P < 0.05$.

#### *2.6.3. Molecular Dynamics Simulations*

To explain the biological significance of methylated microRNAs, molecular simulations will be performed to investigate the binding of argonaut protein to the differentially methylated microRNAs and determine the effect on the protein structure caused by the methylation. The simulation was adapted from Konno et al. [58]. The X-ray structure of the AGO2/RNA complex will serve as the reference for investigating the binding of methylated and non-methylated microRNAs to the human Argonaut2 (AGO2) protein (PDB ID: 4OLB and 4W5N). Both structures will be retrieved from the Protein Data Bank (https://www.rcsb.org) [59]. Firstly, we will replace the RNA-binding bases with the corresponding bases found in each microRNA. Molecular dynamics simulations will, therefore, be conducted under organic conditions (1 atm and ~37 ° C) for each complex microRNA structure. The energy minimisations will be carried out after thermodynamic experimentation to estimate the docking of the structures. Both computations will be conducted with AMBER 99 force field using the AmberTools19 program (http://ambermd.org/) [60].

### *2.6.4. General Statistical Analyses*

In addition, the statistical significance of qPCR experiments will be analysed using the t-test for p-values of less than 0.05. The test will be performed on GraphPad Prism (GraphPad Software, Inc.). For correlation analysis with clinicopathological features and functional analyses, all statistical analyses will be performed using the same software. Experimental data will be presented as mean ± standard deviation (SD). Correlation analysis with clinicopathological features will be analysed using the chi-square or Fisher Exact tests. The statistical analysis for cell viability will be analysed by a two-way ANOVA test, whereas the t-test will be used to analyse the remaining cell-based assays. The threshold for statistical significance is established as $P < 0.05$. To ensure the reproducibility of the results, all cell-based assay experiments for cell-based assays will be conducted in triplicates.

### *2.6.5. Bisulfite Sequencing Analysis*

Bisulfite sequencing data will be analysed using Bioedit [61] and BISMA [62].

## *2.7. Drug Sensitivity Assay*

The cells will be placed in 96-well plates at a density of $1x10^4$ cells per well and treated with the demethylating agent 5-Aza-2′-Deoxycytidine (Sigma) for 72 hours. The concentration of the agent will vary, ranging from 10 to 100 mM. To ensure optimal conditions, the culture media will be refreshed every 24 hours, with a renewal of 5-Aza-2′-Deoxycytidine. All agents will be initially dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque) and subsequently diluted in the culture media for the experiment. Cells that are not treated will serve as a control group. For these control cells, DMSO (Nacalai Tesque) will be added to the cell preparation at a final concentration of 0.2%.

## *2.8. Over-expression Study of MicroRNA*

Selected microRNA and empty vector will be purchased from Origene. Cells will be cultured in 6-well plates, transfected using 5 μL of TurboFectin Transfection Reagent (Origene) and 2 μg of target plasmid per well. After 4 hours, the transfection medium will be replaced with a normal medium, and the cells will be cultured for an additional 20 - 42 hours for various assays as indicated [63].

## *2.9. Cell-based Assays*

The first step in our functional experiments will be to determine cell viability using the XTT assay from Biotium. This will be done 72 hours after treatment or transfection, following the instructions provided by the manufacturer. The fluorescence of the samples will be measured using a microplate reader, specifically the SkanIt RE for Varioskan Flash 2.4 (Thermo Fisher Scientific) [64]. The excitation and emission wavelengths used for this measurement will be 560 nm and 590 nm, respectively. This part of the experiment will be performed six times to ensure the accuracy of our results.

Following the viability assay, we will conduct a colony-forming or clonogenic assay. This is a quantitative technique used *in vitro* to assess the ability of a single cell to grow into a large colony through clonal expansion. This measure of clonogenic activity is particularly important when studying stem cells, which can give rise to undifferentiated cancer cells. In the colony formation assay, 500 cells will be plated in a 6-well plate with complete media after 72 hours post-treatment. The plate will be gently swirled to ensure an even distribution of cells. These cells will then be incubated at 37°C with 5% $CO_2$ for 10 days, during which the media will be replaced every three days. On the tenth day, the media will be removed and the cells will be washed twice with PBS. The colonies will then be fixed with 50% cold methanol for 10 minutes, air-dried, and stained with a 0.5% crystal violet solution for 30 minutes. To remove any excess stain, the plate will be washed three times with tap water. Images of the stained plates will be captured for further analysis. Any cell colonies containing more than 50 cells will be counted. This procedure will be repeated three times for each treatment to ensure the reliability of our findings.

Cell migration is a fundamental process in development and physiological repair, and it is also implicated in various pathological conditions, including cancer invasion, metastasis, and angiogenesis. Evaluating the migration potential is essential for understanding the molecular mechanisms involved in cancer. In this experiment, cells that have been treated will be collected 72 hours post-treatment and seeded at a density of approximately 3 to 7 × $10^5$ cells/mL in each well. Cells that have yet to be treated will serve as controls. The cells will be incubated at 37°C with 5% $CO_2$ to allow them to attach. A monolayer of cells that is approximately 70-80% confluent should be formed after 24 hours. At this point, a small linear scratch will be made in the monolayer of cells by gently scraping the surface with a sterile pipette tip. The dish will then be examined under an inverted microscope, and images will be captured. Observations will be made by taking images at several time points over the next few hours (0h, 24h, 48h, and 72h). The percentage of wound healing will be measured using the T scratch software.

The ability of malignant tumour cells to invade normal surrounding tissue contributes significantly to cancer morbidity and mortality. Invasiveness includes many distinctive cellular functions involving adhesion, motility, detachment, and proteolysis of the extracellular matrix. Metastatic cells produce several proteolytic enzymes with increasing expression of certain cell surface protease receptors. To assess the invasive potentials of selected microRNA, the CytoSelect™ 24-Well Cell Invasion Assay (CellBiolabs) with an insert polycarbonate membrane of 8 µm pore size will be used. The assay will be carried out according to the manufacturer's protocol [64].

### *2.10. Transcriptome-wide microRNA Target Identification*

MISSION Target ID Library (Sigma) containing 16,922 cDNA targets will be purchased from Sigma Aldrich to identify transcriptome-wide human microRNA gene functional targets. It is a pool of plasmids, each with a single human cDNA inserted into the 3′-UTR of a thymidine kinase-zeocin fusion protein (TKzeo) [65]. Zeocin will be used to select

stably transfected cells. A kill curve experiment, for example, exposing untransfected cells to increasing amounts of zeocin to determine the minimum lethal dose of the cancer cell lines, will be performed. The minimum concentration of zeocin that causes complete cell death after the desired time will be used for that cell type in the subsequent experiments. A total of $2x10^7$ stable cells obtained from the kill curve experiment will be transfected with the MISSION Target ID Library and allowed to recover for 3-5 days [65]. After selection for stable integration and cell expansion, the cells will be treated with Ganciclovir and cells that survive this selection will be used for the subsequent experiment. Subsequently, genomic DNA will be isolated from the cell lines after transfection and PCR will be performed with primers provided (Forward 5′ ACGACGTGACCCTGTTCATC 3′ and Reverse 5′ TAAAACGACGGCCAGTGAAT 3′). These primers flank the cDNA insert sites and only inserts are amplified. The PCR products will be sequenced for identification. Gene targets will be identified by BLAST alignment of sequences with the human transcriptome (for known transcripts) and the human genome (for novel transcripts).

### *2.11. Targeted Antibody Array*

The cells that have been treated or transfected for 72 hours will be lysed in Pierce RIPA buffer (Thermo Fisher Scientific) with Halt Protease Inhibitor Cocktail (100X) (Thermo Fisher Scientific) at 1:100 concentration. Protein concentrations will be determined by Bradford assay (Bio-rad) [66]. To parallelly determine the relative levels of selected human cancer-related proteins using the Human XL Oncology Array (R&D Systems, USA), 200 µg protein extracts will be incubated with the membranes according to the manufacturer's instructions [67]. HRP-conjugated streptavidin antibodies and chemiluminescent detection reagents provided by the manufacturer will be used to visualize the protein. The significant proteins identified will be further validated using Jess Simple Westerns™ System (R&D Systems).

## 3. Conclusions

This research is set to make a significant contribution to the understanding of microRNA methylome in CRC, with the potential of unveiling new biomarkers that could be harnessed for the development of novel drugs. This could, in the long run, pave the way for more effective treatments and enhanced patient care. This research stands out for several reasons. Firstly, it will be the first to describe the methylation landscape of microRNAs in Malaysian CRC patients. Secondly, unlike previous CRC studies that focused on specific microRNAs, this research will cover a wide range of microRNAs at a genome-wide level. Lastly, it aims to extensively investigate the functional roles of various methylated microRNAs, an area that has yet to be thoroughly explored. The expected impact of this research on society, economy, and nation is substantial. It will lead to a deeper understanding of microRNA methylome in CRC, thereby revealing potential biomarkers that could be utilised for the creation of new drugs. This aligns with the Sustainable Development Goal 3 (SDG 3). In the longer term, this research could lead to more effective treatments and improved patient care, thereby enhancing the quality of life for CRC patients.

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## References

1. Sw E, Ms N, S A, *et al*. Economic evaluation of monoclonal antibody in the management of colorectal cancer. J Cancer Res Ther 2013; 1(1): 34-39.
2. Sim AAXH, Cheam JY, Law JW-F, *et al*. The ameliorative role of probiotics in 5-fluorouracil induced intestinal mucositis. Prog Microbes Mol Biol 2023; 6(1): a0000339.
3. Azzani M, Dahlui M, Ishak WZW, *et al*. Provider Costs of Treating Colorectal Cancer in Government Hospital of Malaysia. Malays J Med Sci 2019; 26(1): 73-86.
4. Chan PF and Abd Hamid R. An overview of breast cancer: Classification and related signaling pathways. Prog Microbes Mol Biol 2021; 4(1): a0000194.
5. Azizah AM, Nor Saleha IT, Noor Hashimah A, *et al MALAYSIAN NATIONAL CANCER REGISTRY REPORT 2007-2011*. 2016, Putrajaya, Malaysia.
6. The Cancer Genome Atlas. Comprehensive Molecular Characterization of Human Colon and Rectal Cancer. Nature 2012; 487(7407): 330-337.
7. Naumov VA, Generozov EV, Zaharjevskaya NB, *et al*. Genome-scale analysis of DNA methylation in colorectal cancer using Infinium HumanMethylation450 BeadChips. Epigenetics 2013; 8(9): 921-934.
8. Baharudin R, Ab Mutalib N-S, Othman SN, *et al*. Identification of Predictive DNA Methylation Biomarkers for Chemotherapy Response in Colorectal Cancer. Front Pharmacol 2017; 8(47).
9. Ishak M, Baharudin R, Tan LT-H, *et al*. Landscape Of HOXA Genes Methylation in Colorectal Cancer. Prog Microbes Mol Biol 2020; 3(1): a0000085.
10. Abdul S-N, Ab Mutalib N-S, Sean KS, *et al*. Molecular Characterization of Somatic Alterations in Dukes' B and C Colorectal Cancers by Targeted Sequencing. Front Pharmacol 2017; 8: 465.
11. Mohd Yunos RI, Ab Mutalib NS, Khor SS, *et al*. Whole exome sequencing identifies genomic alterations in proximal and distal colorectal cancer. Prog Microbes Mol Biol 2019; 2(1): a0000036.
12. Nasir SN, Ishak M, Mutalib NSA, *et al*. Circular RNA-EPHB4 as a potential biotarget in colorectal cancer: A preliminary analysis. Prog Microbes Mol Biol 2022; 5(1): a0000249.
13. Mohamed SS, Ahmad A, Ab Mutalib NS, *et al*. A Panel of Three MicroRNA Signatures as a Potential Biomarker for CRC Screening Based on Stages and Functional Prediction Using Bioinformatic Analysis. Prog Microbes Mol Biol 2023; 6(1): a0000343.
14. El Aje R, Naya A, Khoaja A, *et al*. A Study Comparing Immunohistochemistry, Fluorescence in situ hybridization, GeneXpert® Breast Cancer STRAT4 Assay for Determining Hormone Receptor, Human Epidermal Growth Factor Receptor 2, Ki67 Status in Invasive Breast Carcinoma in Moroccan women. Prog Microbes Mol Biol 2023; 6(1): a0000332.
15. Azmi NS, Samah AA, Sirgunan V, *et al*. Comparative analysis of deep learning algorithm for cancer classification using multi-omics feature selection. Prog Microbes Mol Biol 2022; 5(1): a0000278.

16. Azman NS, Samah AA, Lin JT, *et al*. Support Vector Machine–Recursive Feature Elimination for Feature Selection on Multi-omics Lung Cancer Data. Prog Microbes Mol Biol 2023; 6(1): a0000327.
17. Gerovska D, Larrinaga G, Solano-Iturri JD, *et al*. An Integrative Omics Approach Reveals Involvement of BRCA1 in Hepatic Metastatic Progression of Colorectal Cancer. Cancers (Basel) 2020; 12(9): 2380.
18. Lee L-H, Ser H-L, Law JW-F, *et al*. IDDF2021-ABS-0123 Streptomyces pluripotens MUSC 135T as a treasure trove for anti-colon cancer and anti-MRSA agents. Gut 2021; 70(Suppl 2): A44-A45.
19. Chew S-S, Tan LT-H, Law JW-F, *et al*. Targeting gut microbial biofilms—a key to hinder colon carcinogenesis? Cancers (Basel) 2020; 12(8): 2272.
20. Ong YS and Tan LT-H. Cancer, natural products and nanodrug delivery systems. Prog Microbes Mol Biol 2020; 3(1): a0000089.
21. Tan LT-H, Chan K-G, Pusparajah P, *et al*. Mangrove derived Streptomyces sp. MUM265 as a potential source of antioxidant and anticolon-cancer agents. BMC Microbiol 2019; 19: 1-16.
22. Tan LT-H, Chan K-G, Pusparajah P, *et al*. Targeting membrane lipid a potential cancer cure? Front Pharmacol 2017; 8: 12.
23. Goh JXH, Tan LTH, Goh JK, *et al*. Nobiletin and Derivatives: Functional Compounds from Citrus Fruit Peel for Colon Cancer Chemoprevention. Cancers (Basel) 2019; 11(6).
24. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116(2): 281-297.
25. Wai Hon K, Zainal Abidin SA, Othman I, *et al*. Insights into the Role of microRNAs in Colorectal Cancer (CRC) Metabolism. Cancers (Basel) 2020; 12(9): 2462.
26. Soheilifar MH, Grusch M, Neghab HK, *et al*. Angioregulatory microRNAs in colorectal cancer. Cancers (Basel) 2019; 12(1): 71.
27. Liao W-T, Li T-T, Wang Z-G, *et al*. microRNA-224 promotes cell proliferation and tumor growth in human colorectal cancer by repressing PHLPP1 and PHLPP2. Clin Cancer Res 2013; 19(17): 4662-4672.
28. Mo JS, Park WC, Choi S-C, *et al*. MicroRNA 452 regulates cell proliferation, cell migration, and angiogenesis in colorectal cancer by suppressing VEGFA expression. Cancers (Basel) 2019; 11(10): 1613.
29. Calin GA and Croce CM. MicroRNA-Cancer Connection: The Beginning of a New Tale. Cancer Res 2006; 66(15): 7390-7394.
30. Zhang Y and Wang J. MicroRNAs are important regulators of drug resistance in colorectal cancer. Biol Chem 2017; 398(8): 929-938.
31. Lye K-L, Tan LT-H, and Yap H-M. Insight of microRNA role in Colorectal Cancer. Prog Microbes Mol Biol 2020; 3(1): a0000083.
32. Yusof AM, Tieng FYF, Muhammad R, *et al*. In-depth characterization of miRNome in papillary thyroid cancer with BRAF V600E mutation. Prog Microbes Mol Biol 2020; 3(1): a0000043.
33. Svoronos AA, Engelman DM, and Slack FJ. OncomiR or Tumor Suppressor? The Duplicity of MicroRNAs in Cancer. Cancer Res 2016; 76(13): 3666-3670.
34. Koong X, Look K, Abdullah ADI, *et al*. Therapeutic Targeting of MOAP-1 in Cancer: A Systematic Review of Current Approaches and Future Directions. Prog Microbes Mol Biol 2023; 6(1): a0000387.
35. Ab Mutalib N-S, Ismail I, and Ser H-L. Molecular profiling and detection methods of microRNA in cancer research. Prog Microbes Mol Biol 2020; 3(1): a0000099.

36. Gregorova J, Vychytilova-Faltejskova P, and Sevcikova S. Epigenetic Regulation of MicroRNA Clusters and Families during Tumor Development. Cancers (Basel) 2021; 13(6): 1333.
37. Humphries B, Wang Z, and Yang C. MicroRNA Regulation of Epigenetic Modifiers in Breast Cancer. Cancers (Basel) 2019; 11(7): 897.
38. Angius A, Cossu-Rocca P, Arru C, *et al*. Modulatory Role of microRNAs in Triple Negative Breast Cancer with Basal-Like Phenotype. Cancers (Basel) 2020; 12(11): 3298.
39. Kaur S, Lotsari-Salomaa JE, Seppänen-Kaijansinkko R, *et al MicroRNA Methylation in Colorectal Cancer*, in *Non-coding RNAs in Colorectal Cancer*, O. Slaby, et al., Editors. 2016: Cham. p. 109-122.
40. Wang S, Wu W, and Claret FX. Mutual regulation of microRNAs and DNA methylation in human cancers. Epigenetics 2017; 12(3): 187-197.
41. Malpeli G, Innamorati G, Decimo I, *et al*. Methylation Dynamics of RASSF1A and Its Impact on Cancer. Cancers (Basel) 2019; 11(7): 959.
42. De Palma FDE, D'Argenio V, Pol J, *et al*. The Molecular Hallmarks of the Serrated Pathway in Colorectal Cancer. Cancers (Basel) 2019; 11(7): 1017.
43. Lujambio A, Calin GA, Villanueva A, *et al*. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci 2008; 105(36): 13556-13561.
44. Lujambio A, Ropero S, Ballestar E, *et al*. Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 2007; 67(4): 1424-1429.
45. Toyota M, Suzuki H, Sasaki Y, *et al*. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 2008; 68(11): 4123-4132.
46. Lv LV, Zhou J, Lin C, *et al*. DNA methylation is involved in the aberrant expression of miR-133b in colorectal cancer cells. Oncol Lett 2015; 10(2): 907-912.
47. Grady WM, Parkin RK, Mitchell PS, *et al*. Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. Oncogene 2008; 27(27): 3880-3888.
48. Oltra SS, Peña-Chilet M, Vidal-Tomas V, *et al*. Methylation deregulation of miRNA promoters identifies miR124-2 as a survival biomarker in Breast Cancer in very young women. Sci Rep 2018; 8(1).
49. Zhang J, Shi K, Huang W, *et al*. The DNA methylation profile of non-coding RNAs improves prognosis prediction for pancreatic adenocarcinoma. Cancer Cell Int 2019; 19(107).
50. Roy R, Chatterjee A, Das D, *et al*. Genome-wide miRNA methylome analysis in oral cancer: possible biomarkers associated with patient survival. Epigenomics 2019; 11: 473–87.
51. Patil N, Abba ML, Zhou C, *et al*. Changes in Methylation across Structural and MicroRNA Genes Relevant for Progression and Metastasis in Colorectal Cancer. Cancers (Basel) 2021; 13(23): 5951.
52. Baharudin R, Rus Bakarurraini NQ, Ismail I, *et al*. MicroRNA Methylome Signature and Their Functional Roles in Colorectal Cancer Diagnosis, Prognosis, and Chemoresistance. Int J Mol Sci 2022; 23(13): 7281.
53. Nazarie WFWM, Yusof AM, Tieng FYF, *et al*. Differential gene expression analysis of papillary thyroid carcinoma reveals important genes for lymph node metastasis. Prog Microbes Mol Biol 2022; 5(1): a0000269.
54. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.) 2001; 25(4): 402-408.

55. Li Y and Tollefsbol TO. DNA methylation detection: bisulfite genomic sequencing analysis. Methods Mol Biol 2011; 791: 11-21.
56. Tian Y, Morris TJ, Webster AP, *et al*. ChAMP: updated methylation analysis pipeline for Illumina BeadChips. Bioinformatics (Oxford, England) 2017; 33(24): 3982-3984.
57. Huang Da W, Sherman BT, and Lempicki RA. Systematic and Integrative Analysis of Large Gene Lists Using David Bioinformatics Resources. Nat Protoc 2009; 4(1): 44–57.
58. Konno M, Koseki J, Asai A, *et al*. Distinct methylation levels of mature microRNAs in gastrointestinal cancers. Nat Comm 2019; 10(1): 1-7.
59. Burley SK, Bhikadiya C, Bi C, *et al*. RCSB Protein Data bank: Tools for visualizing and understanding biological macromolecules in 3D. Protein Science: A Publication of the Protein Society 2022; 31(12): e4482.
60. Case DA, Aktulga HM, Belfon K, *et al*. AmberTools. J Chem Inf Model 2023; 63(20): 6183-6191.
61. Hall T. BioEdit : a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999; 41: 95-98.
62. Rohde C, Zhang Y, Reinhardt R, *et al*. BISMA - Fast and accurate bisulfite sequencing data analysis of individual clones from unique and repetitive sequences. BMC Bioinformatics 2010; 11(1): 230.
63. Lauda Tomasi M, Cossu C, Spissu Y, *et al*. S-adenosylmethionine and methylthioadenosine inhibit cancer metastasis by targeting MicroRNA 34a/b-methionine adenosyltransferase 2A/2B axis. Oncotarget 2017; 8(45).
64. Ibrahim FF, Jamal R, Syafruddin SE, *et al*. MicroRNA-200c and microRNA-31 regulate proliferation, colony formation, migration and invasion in serous ovarian cancer. J Ovarian Res 2015; 8(1): 56.
65. Coussens MJ, Forbes K, Kreader C, *et al*. Genome-wide screen for miRNA targets using the MISSION target ID library. J Vis Exp 2012(62): e3303.
66. Tan LTH, Chan CK, Chan KG, *et al*. Streptomyces sp. MUM256: A Source for Apoptosis Inducing and Cell Cycle-Arresting Bioactive Compounds against Colon Cancer Cells. Cancers (Basel) 2019; 11(11).
67. Kang Y-T, Hsu W-C, Ou C-C, *et al*. Metformin Mitigates Nickel-Elicited Angiopoietin-Like Protein 4 Expression via HIF-1α for Lung Tumorigenesis. Int J Mol Sci 2020; 21(2): 619.