

Original Research Article

Circular RNA-EPHB4 as a Potential Biotarget in Colorectal Cancer: A Preliminary Analysis

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Abstract: Colorectal cancer (CRC) is among the top causes of cancer-related deaths. With the advent of new biomedical technologies, new therapeutic targets are being actively discovered. Circular RNAs (circRNAs) are characterized by the absence of covalently closed-loop structures at the 3' and 5' ends. Recent studies have shown that circRNAs are abnormally expressed in tumor tissues. Certain circRNAs have been reported to have potential as cancer biomarkers. Among all the circRNAs previously discovered, circEPHB4 seemed promising. Therefore, this study is aimed to determine the function of circEPHB4 in CRC. By utilizing in-silico prediction tools, qPCR and cell-based assays, we were able to provide preliminary insights into the role of circEPHB4. The expression of both circEPHB4 and linear EPHB4 were upregulated in our Malaysian CRC tissues and commercial cell lines as compared to the non-cancerous counterparts. Reduction of both circEPHB4 and EPHB4 had effects on wound healing, drug response and clonogenic abilities of SW-480 and HCT-116 cells. Our initial findings suggest that circEPHB4 may be involved in the regulation of CRC though further in-depth studies are needed.

Keywords: Colorectal cancer; circular RNA; EPHB4

1. Introduction

Colorectal cancer (CRC) is ranked worldwide as the third most prevalent cancer^[1]. Colorectal cancer incidence and mortality rates are still on the rise in most countries including highly developed nations^[1]. In Malaysia, CRC is the second most diagnosed cancer in males

and third in females^[2]. Most patients are diagnosed at a late stage; therefore, it may not be effective in eradicating cancer through the treatment offered. There is a need to find biomarkers or targeted therapies that can identify CRC earlier, understand disease progression and improve the clinical outcome.

Multiple potential molecular markers include proteins, mRNAs, micro RNAs (miRNAs), and long noncoding RNAs (lncRNAs) have been reported. Apart from the standard biomarkers, a newly discovered type of non-coding RNA, the circular RNAs have been shown to have advantageous properties as biomarkers compared to the other types of RNA^[3]. Circular RNAs (circRNAs) are a class of closed RNA loops that lack the termination 5' cap and 3' polyadenylated tail structure of linear RNAs^[4,5]. Apart from that, circRNAs are known to be more stable and are have longer half-lives^[6,7], thus making them ideal targets for biomarker identification. Functionally, circRNAs have been reported to be involved in multiple processes including chemoresistance^[8] and migration processes in multiple cancers such as CRC^[9,10], prostate cancer^[11,12], hepatocellular carcinoma^[13] and others. On top of that, dysregulation of circRNAs have been widely reported in CRC^[14].

Erythropoietin-producing hepatocellular (Eph) Type-B receptor 4 (EphB4) is a member the largest family of receptor tyrosine kinases (RTKs)^[10,15] that consists of 14 types of receptors in human^[16]. EPHB4 is reported to be involved in a number of cancer-promoting effects including angiogenesis, invasion, and metastasis^[17]. From previous studies, it has been suggested that high EPHB4 expression enhances migratory abilities in CRC cells, leading to increase rate of metastasis^[18]. EPHB4 was also be found in a circular RNA form^[19]. A recent study by Tan *et al.*, has shown that circEPHB4 could have functional effects in hepatocellular carcinoma^[19]. Previously, our group has identified several potential circRNAs in chemoresistant HCT-116 cells^[9], as well as in the corresponding extracellular vesicles^[20], nevertheless, we did not perform any assessment on circEPHB4. Therefore, in this study, we aimed at determining the level of circEPHB4 in our Malaysian CRC patients as well as perform preliminary assays to assess its potential as a biotarget.

2. Materials and Methods

2.1. Cell Culture

Six human colorectal cancer cell lines (SW480, CCD-112-CoN, CCD-18-CoN, COLO-205, HCT-116, and HT-29) were purchased from American Type Culture Collection (ATCC). These cells were maintained in RPMI-1640 medium (Invitrogen, USA) except for CCD-112-CoN and CCD-18-CoN, which were cultured in Dulbecco's Modified Eagle Medium, DMEM (Invitrogen, USA). All media were supplemented with 10% fetal bovine

serum (Gibco, USA). The cultures were grown in an incubator at 37°C in a 5% CO₂-containing humidified atmosphere.

2.2. Collection of the tissue specimens

All the CRC tissues and normal colonic tissues were obtained from the UKM Medical Molecular Biology Institute (UMBI) Biobank, this study was conducted following the 1964 Helsinki declaration and its later amendments. Prior to sample collection and storage, written informed consent was obtained from all patients. All of the tumor tissue was diagnosed histopathologically by an experienced pathologist. The study was conducted using only tumor tissues containing >80% cancerous cells and normal tissues (non-tumor tissue) containing <20% necrotic cells. Tumor and non-tumor tissue samples were obtained from CRC patients during surgical resection of the tumor. A subset of tissues from these patients was previously used to detect a specific long non-coding RNA in a different study^[21]. Demographic data of the patients is shown in **Table 1**.

Table 1. Demographic information of the Malaysian CRC specimens used in this study.

	CRC Specimens (%)
Overall	26 (100)
Age	
<70	17 (65.4)
>70	9 (34.6)
Gender	
Male	15 (57.7)
Female	11 (42.3)
Race	
Malay	16 (61.5)
Chinese	10 (38.5)
Stage	
Dukes A	3 (11.5)
Dukes B	11 (42.3)
Dukes C	12 (46.2)

2.3. Total RNA Isolation and Quantitative Real-Time RT-PCR (qRT-PCR)

Total RNA from cells was extracted using the RNeasy Mini Kit (Qiagen, Germany) as described. While, for total RNA from CRC tissue was isolated AllPrep DNA/RNA/miRNA Isolation Kit (Qiagen, Germany) according to the manufacturer's protocol. The amount and purity of the RNA were measured with Nanodrop 1000 (Thermo Fisher Scientific, USA). The miRNA-specific primer sequences and circular RNA levels of

EPHB4 were designed independently. The primer sequence for mRNA EPHB4 was designed using the PrimerBank database^[22] other primer sequences for circEPHB4 were designed using the web tool Circinteractome^[23]. The complementary DNA (cDNA) was synthesized using the RT2 First Strand Kit (Qiagen, Germany) based on the manufacturer's instructions. The expression level of circEPHB4 and EPHB4 in primary CRC tissue and secondary CRC cell lines were measured using the Quantinova qPCR Mastermix (Qiagen, Germany) on a CFX96 Touch™ Real-Time PCR Detection System (Biorad, USA). The qRT-PCR results were analyzed and the relative RNA expression of CT (threshold cycle) value, which was then converted to fold changes. Fold change of gene was calculated by the equation $2^{-\Delta\Delta Ct}$.

2.4. Prediction of the circRNA-miRNA-mRNA Expression Network and Function Analysis

The corresponding miRNA response elements (MREs) were predicted using the web-based interface, Circinteractome^[23]. Target miRNAs were obtained from the Starbase database, using default settings^[24]. The expression of the miRNAs was obtained from the Mir-TVdatabase^[25]. The target genes targeting the selected miRNA were obtained from the FunRich tool^[26] where the further biological pathway was determined. Furthermore, the enrichment network (ORA) was also determined using the Webgestalt platform^[27] and the KEGG database^[28].

2.5. Transfection of Colorectal Cancer Cell

Two types of siRNAs (Si-linear EPHB4 and Si-circEPHB4) were designed and synthesized by Dharmacon (Thermo Fisher, USA). The cells were seeded in 24-well plates at a density of 5×10^4 /well and cultured to a confluency of 80-90%. Cells were transfected with control or gene-specific siRNAs using jetPRIME® (Polyplus-transfection SA, USA) as described by the manufacturer.

2.6. Wound Healing Assay

Cells were transiently transfected with the siRNAs and harvested at 48 hours after transfection. The ibidi Culture-Inserts (ibidi GmbH, Germany) were placed on a cell culture surface and cells were seeded at 5×10^5 cells/ml in each of the ibidi Culture-Insert well (70 μ l into each well) according to the manufacturer protocol. The dish was viewed under the inverted microscope (Nikon, Japan) and the image was captured. The observation process was started by taking images several times throughout the following hours (0 h, 24 h, 48 h and 72 h). The percentage of wound healing (%) was measured using the T scratch software^[29].

2.7. Clonogenic analysis

After transfection, 5000 cells were plated in a 6-well plate with complete media. The cells were grown in ~10 days until cells in control plates have formed colonies with substantially good size (50 cells per colony is the minimum for scoring) with media replacement every 3 days. After that, the media was removed, and cells were washed twice with PBS. The colonies were fixed with Acetic acid/methanol 1:7 (vol/vol) for 10 min, dried and stained with 0.5% crystal violet solution for 2 hours at room temperature. Images of the stained plates were captured, and each treatment was performed in triplicates.

2.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism v7 (GraphPad Software, USA). The quantitative data were presented as the mean \pm standard deviations (SD). Differences were considered statistically significant at values of $p < 0.05$.

3. Results

3.1. Both *CircEPHB4* and *Linear EPHB4* were up Regulated in CRC Tumor Tissue and CRC Cell Lines

We examined the expression of both *circEPHB4* and *linear EPHB4* in our cohort of CRC tissues and cell lines. As shown in **Figure 1A**, the average expression of both *circEPHB4* and *linear EPHB4* was upregulated in CRC tissues as compared to non-cancerous colonic tissues. Nevertheless, within the CRC group, *linear EPHB4* had a significantly higher expression than *circEPHB4* ($p=0.03$). Interestingly, for *circEPHB4*, from five of our normal colon samples, only two samples had detectable expression ($CQ < 38$). Similarly, in the cell lines, the expression of both *circEPHB4* and *linear EPHB4* was upregulated in CRC cell lines (SW480, HCT116 and COLO205) as compared to the non-cancerous colon cell lines (CCD-112-CoN and CCD-18-CoN) as displayed in **Figure 1B**.

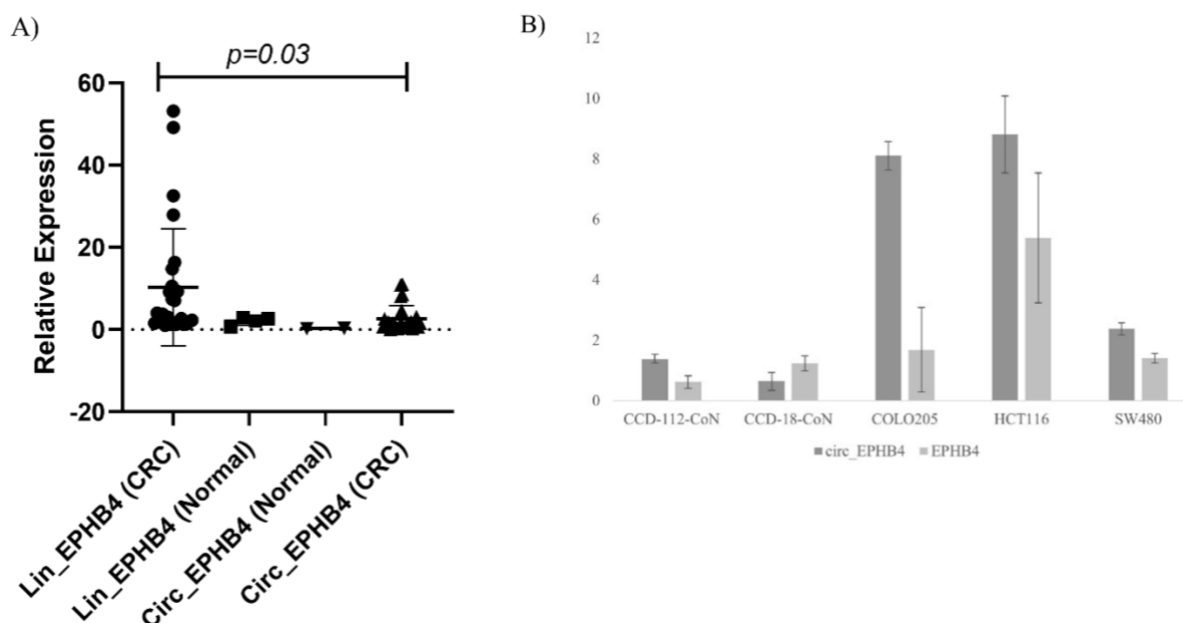


Figure 1. Relative expression of both linear EPHB4 and circEPHB4 in A) our cohort of CRC tissues and normal colonic tissues, and B) a panel of commercially available CRC cell lines and normal colon cells. Gene ontology and pathway enrichment analyses of the predicted target genes for circEPHB4.

3.2 CircRNA-miRNA-mRNA Co-expression Network for CircEPHB4

Since circRNAs have been reported to sponge to miRNAs^[3], we decided to identify potential target miRNAs for circEPHB4. The prediction of miRNA target sites was conducted using the web tool StarBase^[24] using the default parameters. There were 7 miRNAs predicted to bind to circEPHB4, as shown in **Table 2**. Due to our limited number of clinical samples, we were unable to verify the expression of these miRNAs. Nevertheless, we used the Mir-TV database^[25], from the TCGA cohort to get an overview of the expression of selected miRNAs. Out of the 7 miRNAs, only 4 were expressed in the COAD dataset, as shown in **Figure 2**. From the four miRNAs, three miRNAs had a lower expression in CRC tissues as compared to the normal tissues, which are hsa-miR-193a-5p, hsa-miR-3605-5p and hsa-miR-193b-3p. Afterward, the functional analysis for the target genes was further conducted using the FunRich web tool^[26]. We performed target prediction to three of the identified miRNAs. The FunRich tool identified 252 possible target genes; however, no significant biological pathways identified using this set of genes, as shown in **Figure 3A**. When the same set of genes were subjected to an enrichment network (ORA) using the Webgestalt^[27] platform, some of the most statistically enriched pathways include MicroRNAs in cancer and the glioma pathway as shown in **Figure 3B**. **Figure 3C** shows the distribution of the target genes based on the gene ontology- molecular function, biological process and cellular components.

Table 2. Predicted target miRNAs to hsa_circ_001730 (circEPHB4) based on the Miranda database. The table was obtained from the Starbase database^[24].

miRNAid	miRNAname	geneID	chromosome	clipExpNum	RBP	merClass	miRseq
MIMAT0019761	hsa-miR-4677-3p	hsa_circ_0001730	chr7	1	AGO1-4	8mer	ucaucaAGAA ACCAGAGU GUCu
MIMAT0003245	hsa-miR-580-3p	hsa_circ_0001730	chr7	1	AGO1-4	7mer-m8	ggauuacuaAG UAGUAAGA GUu
MIMAT0000459	hsa-miR-193a-3p	hsa_circ_0001730	chr7	2	AGO1-4	7mer-m8	ugaccuGAA ACAUCCGG UCAa
MIMAT0002819	hsa-miR-193b-3p	hsa_circ_0001730	chr7	2	AGO1-4	7mer-m8	ucGCCUGA A---ACU- CCCGGUCAa
MIMAT0017982	hsa-miR-3605-3p	hsa_circ_0001730	chr7	1	AGO1-4	7mer-m8	gaucUCCUGU CCAUUGUG CCUCc
MIMAT0004614	hsa-miR-193a-5p	hsa_circ_0001730	chr7	1	AGO1-4	7mer-m8	agUAGAGCG GGCGUUUC UGGGu
MIMAT0004909	hsa-miR-450b-5p	hsa_circ_0001730	chr7	1	AGO2	8mer	aaaAGUCCU UGUAUAAC GUUUu

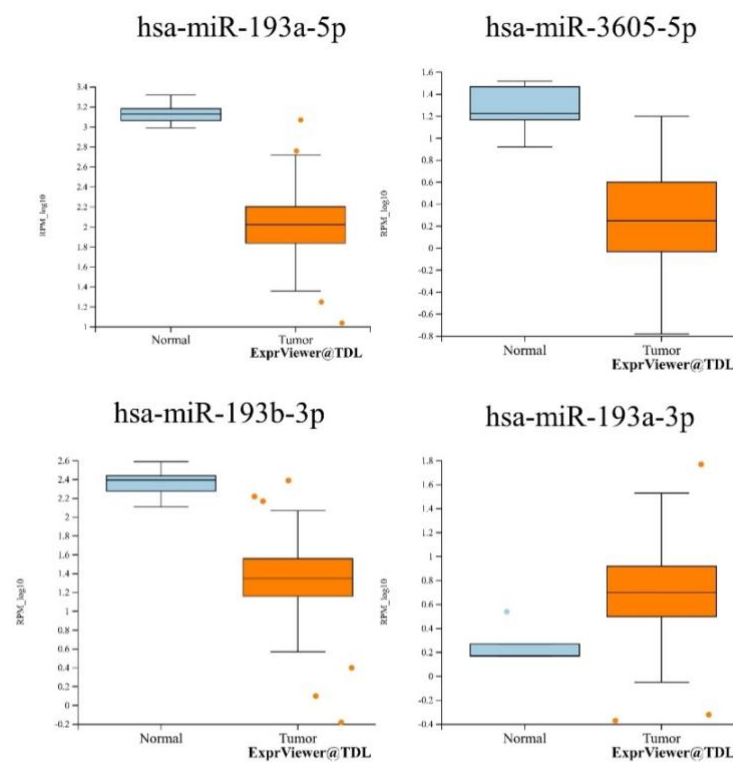


Figure 2. Expression of 4 miRNAs that were predicted to bind to circEPHB4. Data obtained using the mir-TV^[25] database utilizing the TCGA (COAD) dataset.

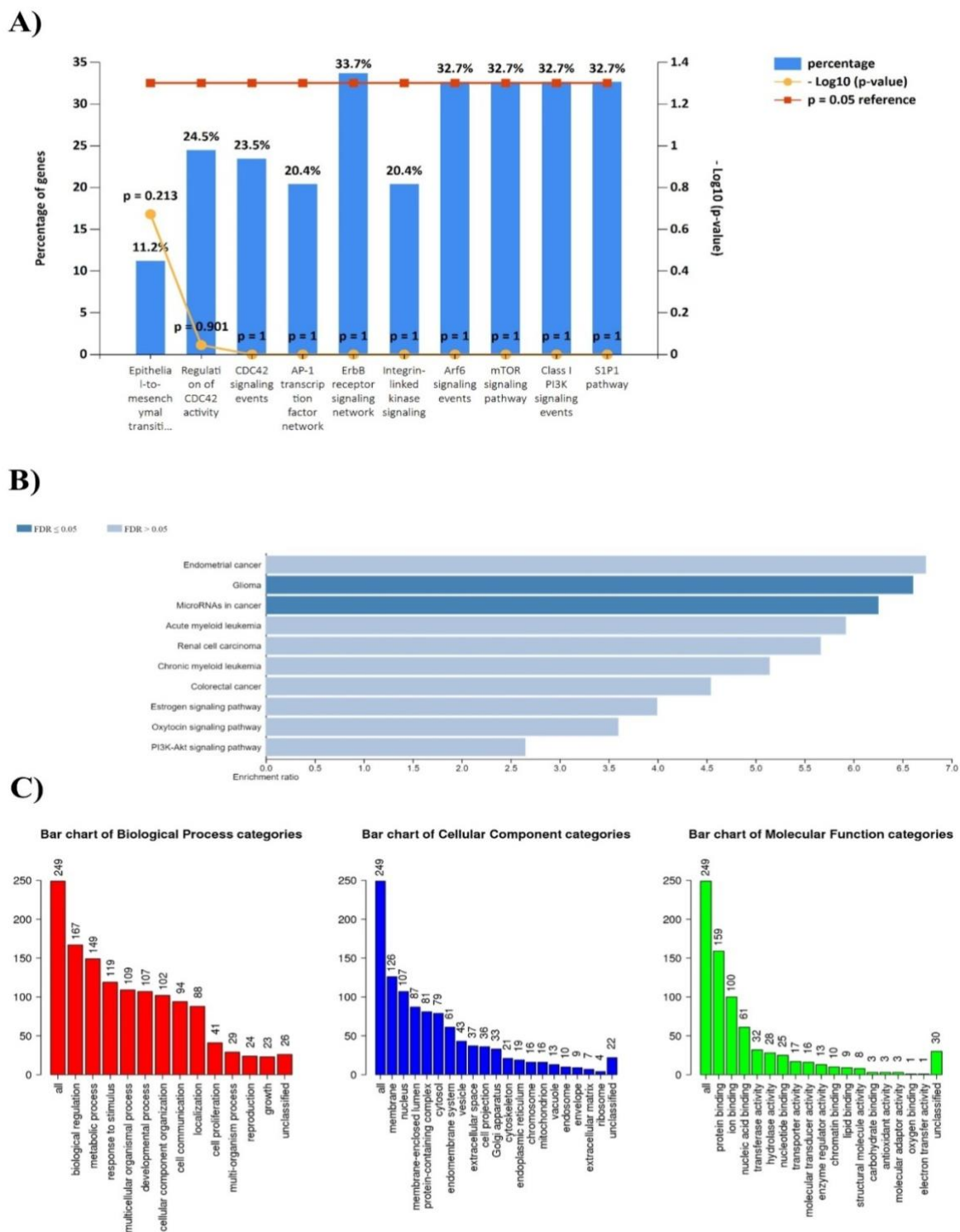


Figure 3. A) Biological pathways that were predicted from the target genes of three selected miRNAs using the FunRich tool. B) Enriched network of the same target genes using the Webgestaltweb-based tool. C) Gene ontology analysis of the target genes from the Webgestaltweb.

3.3 CircEPHB4 and Linear EPHB4 Affect the Wound Healing Process in HCT-116 and SW480 Cells

Now that we have shown that circEPHB4 may be involved in cancer-related processes, we performed two preliminary cell-based assays. Firstly, we performed the wound healing analysis to determine the migration ability of the cells upon the reduction of circEPHB4 and EPHB4. As shown in **Figure 4A**, that knockdown of circEPHB4 significantly reduced the wound healing efficiencies in both SW480 and HCT116 cell lines. However, the knockdown of linEPHB4 only significantly reduced the closure of wound in the SW480 cell line. The second assay was to determine the clonogenic ability of the cells upon knockdown of our target. As shown in **Figure 4B**, there was no statistically significant difference in the number of colonies between si-CircEPHB4 and si-EPHB4 in both cell lines.

4. Discussion

EPHB4 is a subfamily of receptor tyrosine kinase (RTKs) that regulates key biological processes including cell differentiation, proliferation and motility. The expression of EPHB4 in CRC has been conflicting, for instance an earlier study by Stephenson *et al.* showed that EPHB4 was up-regulated in CRC^[30]. On the contrary, a study by Davalos *et al.*, showed the opposite regulation in CRC^[31]. In this study, we validated the expression of both circEPHB4 and EPHB4 in primary CRC tissues and secondary cell lines. We showed that the expression of both transcripts was upregulated in diseased samples as compared to the non-cancerous controls. It was also observed that the linear EPHB4 had a higher expression than the circEPHB4. Interestingly, a study by Bachmayr-Heyda showed that there is a global reduction of circRNAs in CRC^[32]. Nevertheless, this study showed that expression of a circular RNA located at the chr7:100410369-100410830 region was up-regulated in CRC tissues^[32]. This circRNA corresponds to the same location as circEPHB4 (hsa_circ_0001730). Interestingly, a study by Tan *et al.*, showed that circEPHB4 is downregulated in hepatocellular carcinomas^[19]. This shows that the expression of circEPHB4 is heterogenous and is likely cancer-dependent, nevertheless a larger sample size is needed to further confirm this observation.

It is known that circRNAs regulate the cellular machinery by sponging to multiple miRNAs, hence we identified the predicted miRNA targets for circEPHB4 using the web tool CircInteractome and Starbase. We postulate that there should be a negative correlation between the expression of circEPHB4 and the selected miRNAs. We then selected 2 miRNAs with the highest probability of binding to our circRNA of interest, which is hsa-miR-193a and has-miR-193b. Through the TCGA dataset in the OncoMir database, both of these miRNAs were downregulated in CRC tissues. These miRNAs have previously been reported as being involved in the progression of cancer. For instance, hsa-miR-193a was shown to be deregulated in several cancers including CRC. Although from our analysis using the TCGA dataset showed that hsa-miR-193a-3p was upregulated in CRC, several other studies showed

an opposite pattern. For instance, in a study by Mamoori *et al.*, the expression of hsa-miR-193a-3p was significantly downregulated in 70% of the tested CRC tissues^[33].

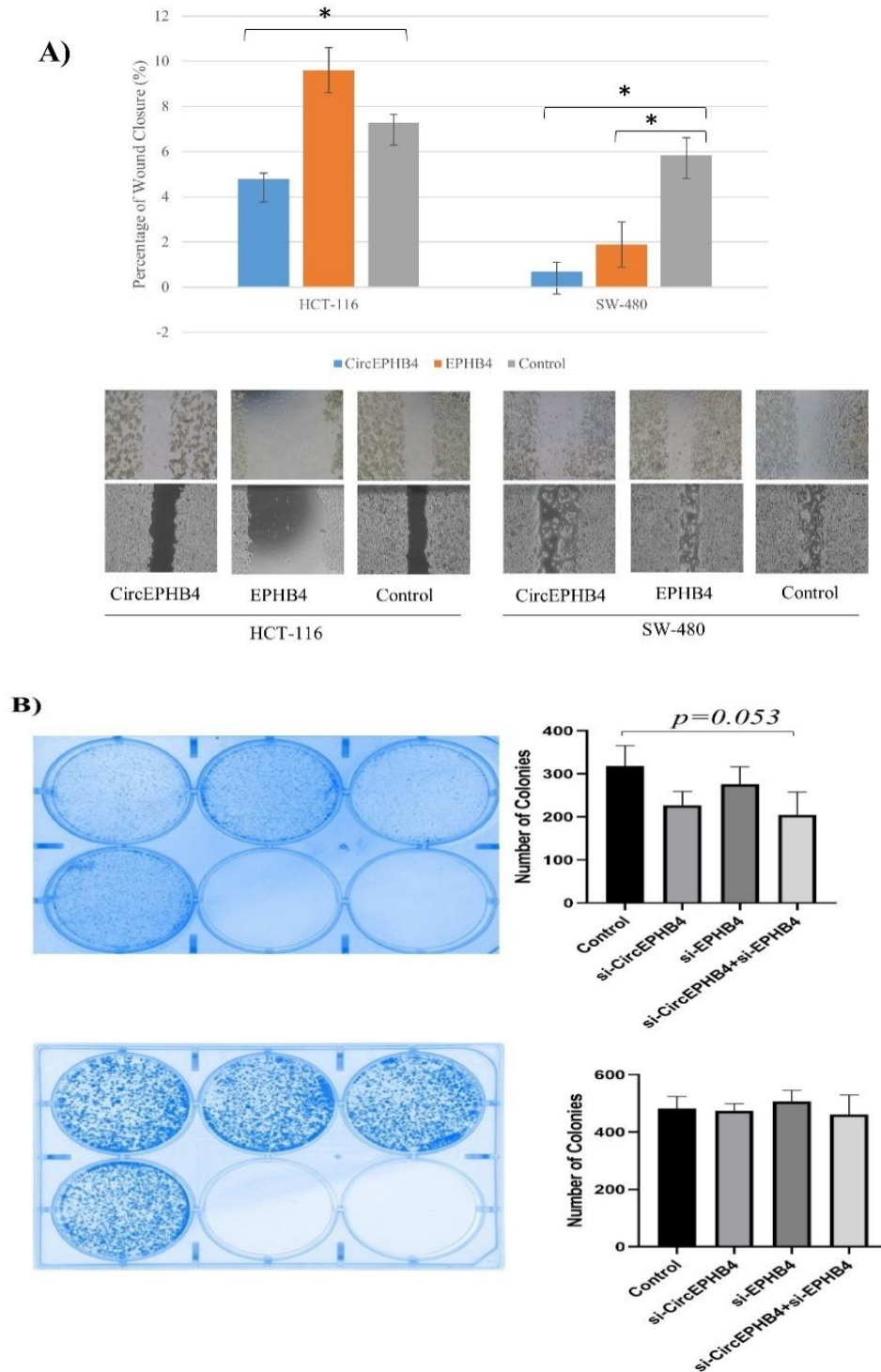


Figure 4. A) Wound healing analysis of HCT-116 and SW-480 cells and B) Clonogenic assay upon knockdown of circEPHB4 and linear EPHB4. (Upper Left: Control, Upper Middle: si-circEPHB4, Upper Right: si-EPHB4, Lower Left: si-circEPHB4+si-EPHB4).

The authors show that the up-regulation of hsa-miR-193a-3p was able to impede cell proliferation and induce cell death in CRC cells *in vitro*^[33]. Apart from that, a study by Zhang *et al.*, has shown that the expression of hsa-miR-193a-5p was downregulated in CRC tissues, and even more reduced in samples with metastasis^[34]. This shows that the expression of this miRNA family is heterogeneous and needs further validation. Similarly for hsa-miR-193b, the relative expression was reduced in CRC tissues as compared to normal mucosa^[35]. The expression of hsa-miR-193b was significantly associated with TNM stages and lymph node invasion^[35]. A similar study by Guo *et al.*, also showed that hsa-miR-193b was significantly downregulated in CRC^[36]. The authors also show that this miRNA targets STMN1 in CRC^[36]. For hsa-miR-3605-5p, there were limited studies correlating this miRNA to cancers, thus making this an interesting target to further explore on.

Although we did not find any significant biological pathway from the FunRich tool, we were able to obtain two significant pathways from the KEGG database. The miRNAs in cancer pathway had an enrichment ratio of 6.2546 with the target genes. Most of the detected genes are well-known cancer-related genes such as KRAS, PTEN and PAK4. Therefore, we postulate that circEPHB4 could be involved in the tumorigenesis process in CRC. To ascertain this, we performed two basic cell-based assays, the wound healing assay and clonogenic assay. From our data, we showed that circEPHB4 could indeed affect the migration process of two CRC cell lines. Further validation using other methods is needed to confirm this effect. Nevertheless, circEPHB4 did not affect the clonogenic ability of the cells, showing a selective effect of circEPHB4.

5. Conclusion

In summary, we have shown that circEPHB4 and linear EPHB4 were indeed upregulated in CRC tissues and CRC cell lines. Through *in-silico* analysis, we showed that circEPHB4 could interact with three possible miRNAs and downstream pathways include cancer-related pathways. Apart from that, we were able to show that circEPHB4 could affect the migration process in CRC cell lines, though more assays are needed to confirm this. In the future, it will be necessary to explore the molecular mechanism of circEPHB4 involving the development and progression of CRC and more work is needed to understand the mechanism underlying the regulatory effects. Additionally, this study is mainly based on *in-vitro* experiments, and the results require to be further verified by *in-vivo* experiments. Overall, circEPHB4 is a promising biotarget in CRC that warrants further attention.

Author Contributions: NA - conceptualize the study, SNN, NA, MI - performed the experiments and data analysis, IS, MIR, NSAM - provided materials, NA, NSAM, RJ - provided critical input and feedback.

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Conflicts of Interest: We declare that we have no conflict of interest.

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