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Knockdown by *MSH2* and *EPCAM* siRNA Suppress Wnt/*β-catenin* Pathway in HCT116 Cell Line

Abstract **–** Small interfering RNA (siRNA) has the potential as a therapeutic approach against selective pathways in colorectal cancer (CRC). *EPCAM*, a transmembrane glycoprotein mediating cell adhesion, was known to be involved in suppressing Wnt/*β-catenin* pathway, an important pathway for tumour progression in colon cancer cells. *EPCAM* deletions caused a transcriptional read-through that may silence its neighbouring gene, *MSH2*. This study aimed to investigate the effect of co-siRNA targeted genes, *MSH2* and *EPCAM*, in colon cancer cell line, HCT116, and their effect in regulating the Wnt/*β-catenin* pathway. Methods: Pre-designed siRNA of *MSH2* and *EPCAM* were transfected into HCT116 cells. The cells were divided into six group of treatments: untreated cell group, cells treated with negative control siRNA, *MSH2*-siRNA treated cells, *EPCAM*-siRNA treated cells, cells treated with both *EPCAM* and *MSH2*-siRNA, and cells treated with transfection reagent (mock control). The mRNA and protein expression following the individual and combined siRNA treatments were assessed by quantitative polymerase chain reaction and Western blot. Results: The mRNA and protein expression levels of *MSH2, EPCAM* and *β-catenin* were reduced in the individual *MSH2* and *EPCAM*-siRNA treated samples as compared to the untreated sample. Further reduction of mRNA and protein expressions for *MSH2, EPCAM* and *β-catenin* were identified in combined siRNA treatments. Conclusion: Reduction of *β-catenin* expression by simultaneous silencing of *MSH2* and *EPCAM* suggested that these genes may play a role in supressing the Wnt/*β-catenin* pathway in cancer cells.

Keywords *– Small interfering RNA (siRNA), colorectal cancer (CRC), MSH2, EPCAM, β-catenin*

1. INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of death [1, 2]. The most common hereditary colon cancer is Lynch syndrome (LS) which is caused by germline mutations in any of the four mismatch repair (MMR) genes namely *MLH1*, *MSH2*, *MSH6* and *PMS2* [3] and to date, *EPCAM* gene was also reported to be associated with LS [4]. *EPCAM* functions in intracellular signalling, differentiation and proliferation of normal and cancer tissues [4]. This was suggested by the *EPCAM* signalling that is activated by intra-membrane proteolysis in which the extracellular domain is flowed off and the intracellular domain (EpICD) is then released into the cytoplasm. In the cytoplasm, *EPCAM* formed a nuclear complex with transcriptional regulators *β-catenin* and Lef, both are parts of Wnt signalling pathway [5]. Germline deletions in the last exon of the non-MMR gene, *EPCAM*, may silence its neighbouring gene, *MSH2*, through promoter hypermethylation [6]. Previous study has shown that in *EPCAM*-silenced colon cancer cells, the expression level of *β-catenin* was decreased, and the result showed that silencing of *EPCAM* could inactivate the Wnt/*β-catenin* pathway in tumour cells [7]. Hence, *EPCAM* was identified as a principal target for the treatment of tumours, through the mechanism of suppressing the Wnt/*β-catenin* pathway. *EPCAM* deletions cause a transcriptional read-through and silenced the *MSH2* gene [8]. The mechanism of promoter hypermethylation happens when a number of methyl groups bound to the *MSH2* promoter region, and consequently decrease the expression of *MSH2* protein products in the

epithelial cells. Considering the major role of *MSH2* protein in DNA repair mechanism, the loss of this protein may subsequently affect the process of DNA repair, and mistakes accumulated upon cell division [4].

Wnt signalling pathway has become one of the major pathway in CRC and LS predisposition with about 65% overactivation in CRCs associated to LS and activating *β-catenin* mutations were identified in approximately 40% of these tumours [9]. Although CRC was commonly triggered by the alterations in the signalling components of the Wnt signalling pathway [10], the relationship between *MSH2* and *EPCAM* genes towards the effect of associated pathway is yet to be discovered. Therefore, this study aimed to investigate the effect of *MSH2* and EPCAM *siRNA* mediated gene knockdown in regulating the Wnt/β-catenin pathway.

2. MATERIALS AND METHODS

2.1 Culture of HCT116 Cell Line

HCT116 cell line was purchased from RIKEN BioResource Centre, Japan, and was established according to manufacturer's protocol. The cell suspension was centrifuged in complete culture medium (DMEM supplemented with 1.0 g/l of glucose, L-glutamine, sodium pyruvate and 10% FBS) for centrifugation at 1000 rpm for 3 minutes at room temperature. Followed by centrifugation and resuspension, the cell suspension was seeded into two 25 cm^2 culture flasks and incubated at 37°C in humidified incubators with 5% CO_2. The growth of cells was maintained daily and fresh culture medium was changed for every two days or when necessary. Sub-culturing was carried out when the cells reached 80% confluence. When the cells reached 80% confluence or 80% of the flask surface area was covered by the cells, sub-culturing was carried to ensure continuous growth of cells during their exponential growth. The first few cultures were sub-cultured with split ratio of 1:2 to 1:3 depending on the growth of cells. Before sub-culturing, the culture medium was discarded, and the adherent cells were washed with 1 ml of PBS. After two to three washing steps, the cells were digested with 0.25% trypsin-EDTA for 5 minutes at 37°C in humidified incubators with 5% CO_2. Upon confirmation of cell detachment, 1 ml of complete culture medium was added and gently dispersed by pipetting over the surface of the cell layer to deactivate the trypsin activity. The trypsin and cell suspension were mixed thoroughly and transferred into 15 ml centrifuge tube prior to centrifugation at 1200 rpm for 5 minutes at room temperature. Supernatant was then discarded, and the cell pellet was re-suspended in 1 ml of complete culture medium. The cell suspension was sub-divided according to appropriate split ratio into the new culture flasks containing fresh complete culture medium. The cells were then maintained at 37°C in humidified incubators with 5% CO_2.

2.2 SiRNA Preparation and Transfection

Hiperfect® Transfection Reagent (Qiagen, Denmark) was used for siRNA transfection of MSH2 and EPCAM genes into the HCT116 cells. SiRNA was prepared in an RNAse-free environment. The target sequence of four pre-designed siRNAs (FlexiTube GeneSolutions, Qiagen, Denmark) for each *MSH2*, *EPCAM* and negative control siRNA were listed in Table 1. RNase-free water in a total volume of 100 µl was added to 1 nmol of lyophilized siRNA for MSH2 and EPCAM genes to obtain a final stock concentration of 10 µM. For the negative control siRNA, a total volume of 250 µl of RNase-free water was added for a final stock concentration of 20 µM. For the preparation of 1 µM working solution, 2 µl of siRNA was added to the stock solution for a final volume of 20 µl. All siRNAs were stored at -20°C until further use.

Table 1. List of siRNA sequences for MSH2 and EPCAM gene (FlexiTube GeneSolution siRNA, Qiagen)

***MSH2*_siRNA ID**	**Target sequence**
Hs_*MSH2*_6	TCCAGGCATGCTTGTGTTGAA
Hs_*MSH2*_5	CCCATGGGCTATCAACTTAAT
Hs_*MSH2*_10	AAGAAGATGCAGTCAACATTA
Hs_*MSH2*_12	TTGGATATTACTTTCGTGTAA
***EPCAM*_siRNA ID**	**Target sequence**
Hs_ *EPCAM*_6	AACTATATAATTTGAAGATTA
Hs_ *EPCAM*_5	CTGGATCATCATTGAACTAAA
Hs_ *EPCAM*_10	AGGGAACTCAATGCATAACTA
Hs_ *EPCAM*_9	TTTGTGAATAATAATCGTCAA

Before transfection, the cells were harvested in 1 ml complete culture medium. The viability of cells was assessed by dye exclusion test using Trypan Blue solution (GIBCO®, Invitrogen™, Canada). Approximately 60 000 cells were seeded in each well of 24-well plate containing 500 µl of complete

culture medium. The cells were then incubated overnight under normal growth condition at 37°C with 5% CO_2. The samples were divided into six groups with different treatments at a total siRNA concentration of 40 nmol per group of samples. The six groups were untreated cell group (Group 1), cells treated with negative control siRNA (Group 2), *MSH2*-siRNA treated cells (Group 3), *EPCAM*-siRNA treated cells (Group 4), cells treated with both *EPCAM* and *MSH2* siRNA (Group 5) and cells treated with HiPerfect Transfect Reagent which served as a mock control (Group 6). Each group of samples were run in triplicates. A set of four different siRNAs for each gene were used. A total of 37.5 ng (5 nmol) of each siRNA were diluted in 100 µl DMEM culture medium without serum and 3 µl of HiPerfect Transfect Reagent was added to the diluted siRNA. After gently mixed, the mixture was incubated for 10 min at room temperature for the formation of transfection complexes. The complexes were dispensed dropwise into each well and the plate was gently swirled to ensure uniform distribution of complexes throughout the cells.

2.3 RNA Extraction and cDNA Synthesis

RNA extraction was performed after 48 hours of transfection by using a commercial RNA extraction kit, RNeasy Mini Kit (QIAGEN, Germany). The protocols of RNA extraction were carried out according to the manufacturer's protocols. The quality of the RNA was assessed by gel electrophoresis. Synthesis of cDNA was performed by using a commercial kit, SensiFAST™ cDNA Synthesis Kit (Bioline, USA) according to manufacturer's protocols. A total of 1 µg RNA served as an initial concentration for cDNA synthesis followed by the following conditions: 25 °C for 10 minutes (primer annealing) followed by reverse transcription at 42 °C for 15 minutes and the inactivation step at 85 °C for 5 minutes with a final hold at 4 °C. The final volume of 20 µl cDNA in RNase-free was stored in -20 °C until further use.

2.4 Gene Expression using Quantitative Real-time PCR

The relative quantification of gene expression for *β-actin* and the targeted genes, *MSH2*, *EPCAM* and *β-catenin* was performed using Stratagene Mx3000P qPCR System (Agilent Technologies, USA). cDNA was diluted in 10-fold dilution of 5 different concentrations with the initial concentration of 100 ng/µl to further determine the PCR efficiency of each targeted gene. Real-time PCR (qPCR) amplification was carried out using commercial kit, Quantinova™ SYBR® PCR Kit (QIAGEN, Germany)**.** The kit was used in combination of real-time predesigned primers of Quantitect Primer Assay (QIAGEN, Germany). The reaction mastermix was prepared in 96 well plate according to the proposed reaction setup according to the manufacturer's protocol. *β-actin* was used as a reference gene for the relative quantification of MSH2, EPCAM and β-catenin genes. The reaction mixture for *β-actin*, *MSH2*, *EPCAM* and *β-catenin* was prepared in a total volume of 20 µl containing 10 ng of cDNA, 10 µl of 1X SYBR Green PCR buffer, 2 µl of 1X QN ROX reference dye, 1X primer assay and RNAse free water. The samples were run in triplicates for each set of the primers. The conditions for the amplification were 95 °C for 2 min for the initial heat inactivation, denaturation at 95 °C for 5 sec and 45 cycles of combined annealing and extension at 60 °C for 10 sec followed by dissociation stage at 95 °C for 1 min, 55 °C for 30 sec and 95 °C for 30 sec.

2.5 Relative Quantification Analysis and Statistical Analysis

Relative quantification analysis was performed to determine the expression of the targeted genes (*MSH2*, *EPCAM* and *β-catenin*) in all samples by using comparative double ΔCt method. Statistical analysis was performed by SPSS® statistical package, version 24.0 (SPSS Inc., Chicago, IL, USA). All data were presented as mean ± SD. The statistical comparison of more than two groups in this experiment was tested using one-way ANOVA. $P<0.05$ was considered significant.

2.6 Western Blot Analysis

Protein extraction was performed by using RIPA Buffer (Nacalai Tesque, Japan) according to the manufacturer's protocols. The cells were washed twice with cold PBS and 1X RIPA buffer was added to the culture depending on the number of cells in the culture flask. The protein concentration in a sample was measured using Bradford protein assay. Each sample replicates (n=3) for each treatment were pooled separately with concentration of 20 µg. The protein samples and the standard marker subsequently subjected to 10% SDS-PAGE electrophoresis at 100 V for 80 minutes. The samples were transferred to PVDF membrane and was run at 25 V for 2 hours prior to

incubation in 5% blocking buffer for 1 hour at room temperature. The membrane was then incubated with 5% blocking buffer mixed with an appropriate dilution of each primary antibody; *MSH2* (1:2000), *EPCAM* (1:2000) and *β-catenin* (1:1800) and *β-actin* (1:7500), for overnight at 4°C with gentle agitation. After an overnight incubation, the primary antibody was discarded. The membrane was washed twice at room temperature with TBS-T buffer followed by one time washing with TBS buffer. The membrane was then incubated with Goat Anti-Mouse IgG secondary antibody conjugated to horseradish peroxidase (HRP) in 5% blocking buffer at 1:20 000 dilutions for 1 hour with gentle agitation. The samples were visualized using chemiluminescent detection kit (Nacalai Tesque, Japan).

2.7 Densitometry Analysis

The intensity of the protein bands was semi-quantitatively analysed by Image J tool (NIH, Bethesda, MD, USA) (http://rsb.info.nih.gov/ij/index.html) to compare the intensity of each protein band across different samples. A profile plot for each band that represented the density of the band was then created by using the Image J tool. The relative protein level of each sample was then calculated based on the following equation [11]:

$$\text{Relative protein level} = \frac{\text{Intensity of GOI}_{\text{(treated sample)}}/\text{Intensity of GOI}_{\text{(non-treated sample)}}}{\text{Intensity of } \beta\text{-actin}_{\text{(treated sample)}}/\text{Intensity of } \beta\text{-actin}_{\text{(non-treated sample)}}} \times 100\%$$

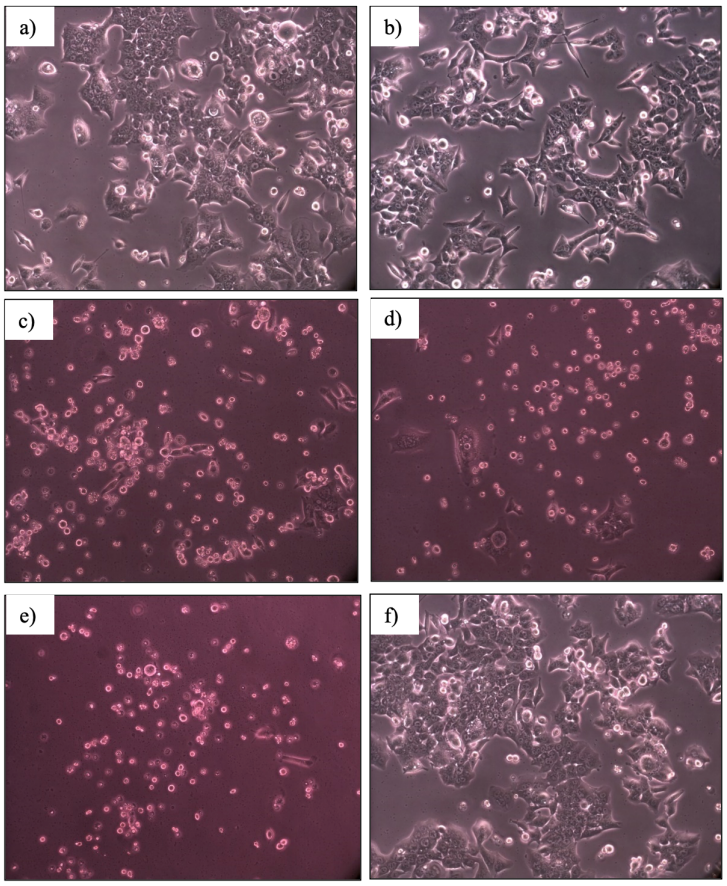

Figure 1. Representative light microscopy images of HCT116 cells at 48 hours after transfection. (a) Growth and morphology in untreated cell group (Group 1). (b) Growth and morphology of cells treated with negative control siRNA (Group 2). (c) Growth and morphology of *MSH2*-siRNA treated cells (Group 3). (d) Growth and morphology of *EPCAM*-siRNA treated cells (Group 4). (e) Growth and morphology of cells treated with both *EPCAM* and *MSH2* siRNA (Group 5). (f) Growth and morphology in mock control (Group 6)

3. RESULTS

3.1 Effect of siRNA Transfection

The effect of *MSH2* and *EPCAM* on the Wnt/*β-catenin* was investigated by transfecting the HCT116 cell lines with siRNA against the *MSH2* and *EPCAM* gene. The comparison of cell growth and morphology in each group of treated samples were observed after 48 hours of treatment (Figure 1). The number of attached cells in the *MSH2* and *EPCAM* treated cells or *MSH2* and *EPCAM* knockdown cells as well as cells which were simultaneously treated by both genes were found to decrease notably. From the microscopic observation, the cells in Group 1, Group 2 and Group 6 were appeared as epithelial in shape as compared to the group of cells treated with the targeted genes. The morphology of cells in Group 3, Group 4 and Group 5 were observed to change into rounded bodies with a few smaller cells in epithelial shape. In the treated samples, the detached cells were observed to be aggregated with a few cells remained attached to the surface of the flask. As compared to Group 3 and Group 4 samples, the cells in Group 5 displayed the highest cell aggregation with a few numbers of cells attached on the surface of the flask.

3.2 Gene Expression Analysis of *MSH2* and *EPCAM* Knockdown

The gene expression level of *MSH2*, *EPCAM* and *β-catenin* in six different groups were determined by comparing the $2^{-\Delta\Delta Ct}$ values of the target genes normalized to the housekeeping genes, *β-actin* with respective to the control group. The expression level of individual gene knockdown, *MSH2*-siRNA treated group and *EPCAM*-siRNA treated group and simultaneous double gene knockdown, *MSH2*+*EPCAM*-siRNA treated group were shown to be statistically significant with *p*-value<0.05 as compared to the control (Figure 2). *MSH2*-siRNA significantly inhibited the *MSH2* mRNA expression at the level of 0.17±0.04 (*p*-value=0.005) as compared to the untreated one (Figure 2a). Meanwhile, a slight decrease which was not much altered were observed for mRNA expression level in negative control siRNA with no significant difference compared to the untreated control. A notable knockdown of *MSH2* expression was also observed by *EPCAM*-siRNA at the level of 0.25±0.05 (*p*-value=0.012) (Figure 2a). Similarly, *EPCAM* expression level was inhibited by both *MSH2*-siRNA and *EPCAM*-siRNA with the level of 0.25±0.03 (*p*-value=0.007) and 0.11±0.04 (*p*-value=0.010), respectively (Figure 2b). A significant knockdown was also observed in *β-catenin* in which both *MSH2*-siRNA and *EPCAM*-siRNA has reduced *β-catenin* expression at the level of 0.34±0.07 (*p*-value=0.044) and 0.32±0.04 (*p*-value=0.016), respectively (Figure 2c). The effect of simultaneous gene expression was determined in these targeted genes. The mRNA expression level in these three targeted genes were observed to have significantly reduced by *MSH2*+*EPCAM*-siRNA (Figure 2).

3.3 Protein Expression Analysis of *MSH2* and *EPCAM* Knockdown

Protein expression was observed after 48 hours of transfection, including the untreated control. Reduced band intensity was observed for *MSH2*-siRNA and *EPCAM*-siRNA treated samples that indicated less protein was expressed for these three targeted genes as compared to untreated control. Reduced band intensity of *MSH2*+*EPCAM* siRNA treated samples were further observed that may indicate the possible effect of simultaneous gene knockdown resulted in low expression of the two targeted proteins (Figure 3). The percentage of *MSH2* protein level transfected by the individual *MSH2*-siRNA and *EPCAM*-siRNA were reduced at 33% and 32% respectively (Figure 4a). Increased reduction of protein level was observed in the simultaneous knockdown of *MSH2* and *EPCAM* siRNA with the percentage protein level of 14% (Figure 4a). For *EPCAM* gene, the protein level was also identified to decrease in both targeted siRNA by 44% in *MSH2*-siRNA transfected sample and 43% of protein level in the *EPCAM*-siRNA transfected sample (Figure 4b). Reduced protein level was identified in both genes transfected sample with the remaining protein level of 10% (Figure 4b). In addition, for *β-catenin* gene, the *MSH2* and *EPCAM*-siRNA transfected sample was decreased to 38% and 33% respectively (Figure 4c). Notable reduction of *β-catenin* protein level was observed in the simultaneous gene knockdown with the percentage remaining protein level of 5% (Figure 4c).

4. DISCUSSION

Germline deletion that cause the *MSH2* inactivation was considered a novel findings in the predisposition of LS [4, 5]. including a novel large duplication of *MSH2-EPCAM* previously reported in a LS patient [12]. However, the association of these two genes in the pathway associated to CRC and LS have not yet been elucidated. Previous study showed that *EPCAM* was suggested as an important target gene that can disrupt the mechan-

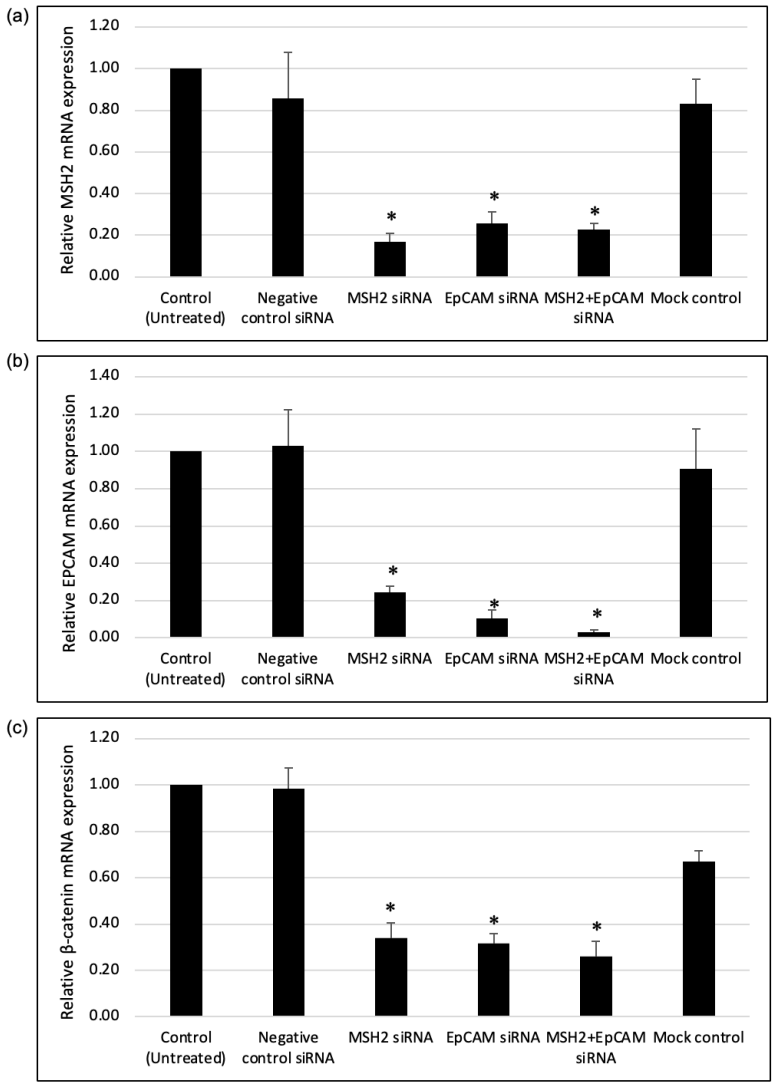

Figure 2. Effect of different siRNA transfection on mRNA expression level. (a) The relative mRNA expression level of *MSH2* normalized with *β-actin.* (b) The relative mRNA expression level of *EPCAM* normalized with *β-actin*. (c) The relative mRNA expression level of *β-catenin* normalized with *β-actin*. Values represented mean value ± standard error (SE). **p*<0.05 compared to untreated cell group (n=3)

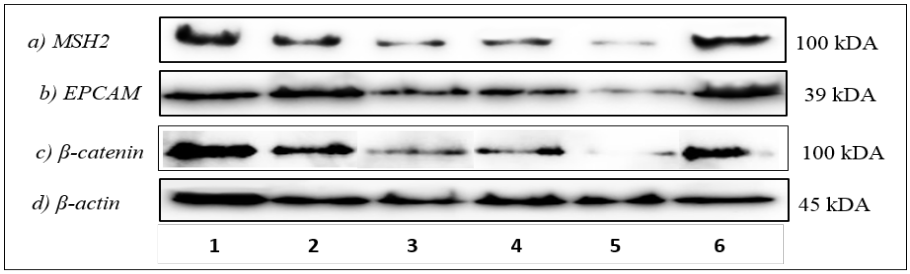

Figure 3. Western blot results of *β-actin, MSH2, MSH6* and *β-catenin* with their respective size of protein. (a) Protein band of *MSH2* with the size of 100 kDA. (b) Protein band of *EPCAM* with the size of 39 kDA. (c) Protein band of *β-catenin* with the size of 100 kDA. (d) Protein band of internal reference, *β-actin* with the size of 45 kDA. 1-control, 2-Negative control, 3- Cells transfected with *MSH2*-siRNA, 4- Cells transfected with *EPCAM*-siRNA, 5- Cells transfected with *MSH2*+*EPCAM*-siRNA, 6- Mock control

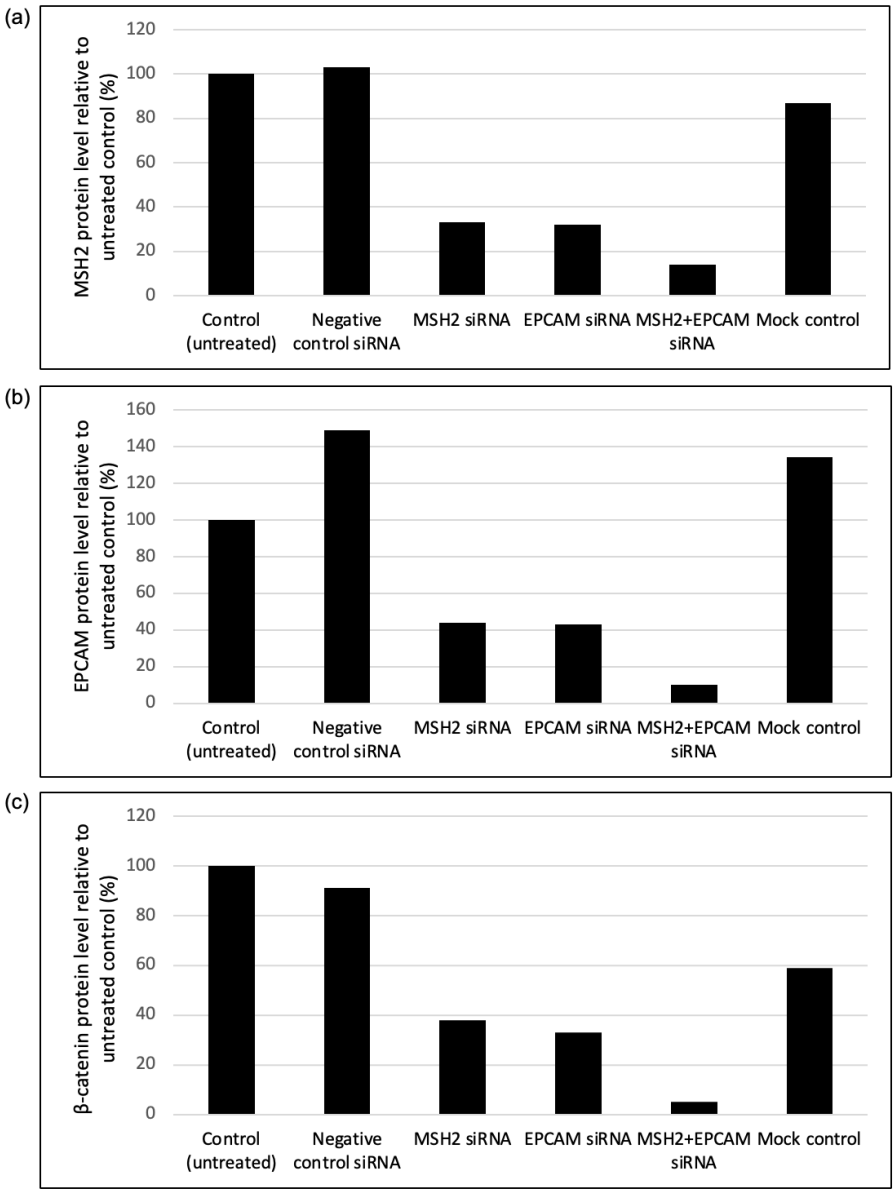

Figure 4. Percentage protein level of different siRNA transfected sample including the untreated control sample by densitometric analysis with *β-actin* as internal control. (a) Protein level of *MSH2* protein. (b) Protein level of *EPCAM* protein. (c) Protein level of *β-catenin* protein

-ism of Wnt/*β-catenin* pathway in HCT116 cells [7] but tumour cells commonly involved multiple genetic and epigenetic alterations and single inhibition of one tumour associated gene as a therapeutic strategy may inadequate to inhibit the development of tumour [13]. SiRNA-mediated RNAi was used over other RNAi-based technique due to its unrestricted choice of targets [14] and ability to target a specific gene [14]. Due to the specificity of siRNA, the role of the siRNA itself has contributed to the advantage in the area of personalized medicine in cancer therapy [15].

The present study was carried out in colon cancer cell line, HCT116 to evaluate the role of *MSH2* and *EPCAM* gene in modulating the Wnt/*β-catenin* pathway using siRNA target gene.

The resulted gene silencing after siRNA transfection was evaluated by the morphological changes of the cells, analysis of gene expression and protein expression by Western blot analysis

which further verified the efficient gene knockdown in the mRNA and protein level. Result from morphological analysis displayed that the cells treated with *MSH2* and/or *EPCAM* showed a prominent cell shrinkage and death as compared to untreated cells, negative control treatment and cells treated with transfection reagent only. Microscopically, it was observed that individual treatment of *MSH2* and *EPCAM* and also combination treatment of *MSH2* and *EPCAM* could induce apoptosis, and inhibit cell growth, suggesting a promising synergistic effect of *MSH2* and *EPCAM* on anti-proliferation activity. The expression of individual gene knockdown has reduced the mRNA and protein expression of *MSH2*, *EPCAM* and *β-catenin* in *EPCAM*-siRNA and *MSH2*-siRNA transfected cells. This finding was concordant with previous study that reported a significant reduction of proliferative activity and reduced mRNA and protein expression in *EPCAM*-siRNA transfected in HCT116 cells [7]. The expression level of *β-catenin* was also reduced in the *EPCAM*-siRNA transfected cells [7]. *MSH2* knockdown was previously carried out in other colon cancer cell lines, SW480 using shRNA-mediated gene silencing which resulted in the reduction of cell proliferative activity and decreased mRNA expression [16].

The simultaneous knockdown of *MSH2* and *EPCAM* may also be associated with the epigenetic modifications that usually occurs at the promoter region or enhancers of tumour-suppressor genes that often cause tumorigenesis [17]. *MSH2* methylation was reported as disease specific due to the absence of *MSH2* methylation in normal tissues as well as in sporadic CRC cases [18]. Promoter region of *MSH2* was suggested as a target of aberrant methylation in LS due to the presence of high level of promoter methylation [18]. Previous study demonstrated the role of 3'-end *EPCAM* deletion that may cause *MSH2* methylation in patients with no LS germline mutations in *MSH2* gene [8]. Due to the downstream position of *MSH2* gene to *EPCAM* gene, gene silencing by transcriptional read-through of a neighbouring gene could represent a general mutational mechanism and also caused by a second somatic hit that inactivates *MSH2* in tumours with *EPCAM* deletion [8].

A significant reduction of *β-catenin* expression was observed in the simultaneous gene knockdown of *MSH2* and *EPCAM* which suggested that the silencing of these two genes may interrupt the activation of Wnt/*β-catenin* pathway in cancer cells. It has been well known that the degradation of Tcf/*β-catenin* complex formation decreased *EPCAM* gene expression in normal human hepatocytes culture and HCC cell lines [19] and human colon cancer line [7]. The key transcription regulator of Wnt signalling pathway is *CTNNB1* gene which was identified to encode for *β-catenin* [20]. The mechanism took place when the phosphorylation of *β-catenin* by CK1α followed by GSK3β mediated phosphorylation of the destruction complex and targeting *β-catenin* for ubiquitination and subsequent proteolysis [21]. However, the subsequent degradation of *β-catenin* will be avoided by the point mutations at these amino acids by hindering the *β-catenin* from being phosphorylated [20]. Mutations in *CTNNB1* and *AXIN2* in CRC were mostly arise in tumours with MMR genes; *MLH1, MSH2* or *PMS2* inactivation [22]. Although previous *in-vivo* study has showed that the deficiency of *MSH2* led to an enhance of *β-catenin* activity and cellular hyperproliferation in colon epithelial cells, however, this activity was dependent on gut microbes [23].

Wnt pathway played an important role as molecular signalling in various cancers and due to frequent abnormality of Wnt activation in CRC, it has been proposed as one of the key pathway in CRC predisposition [24, 25]. The activation of Wnt signalling commonly occurs by the presence of genetic alterations in *APC, β-catenin gene, AXIN1* and *AXIN2* that further caused *β-catenin* to be accumulated in the cytoplasm [22]. Increase of cell migration inducing hyaluronan binding protein (CEMIP) reported to contribute to the carcinogenesis of CRC. Knockdown of CEMIP inhibited the cell proliferative activity in HCT116 and led to an inactivation of Wnt/β-catenin/Snail pathway [26]. In addition, an oncogene, Wnt family member 10A (Wnt10a), that previously associated with the carcinogenesis of renal cell carcinoma, was also shown to supress the Wnt/β-catenin signaling pathway by using Wnt10a-siRNA and LGK-974 inhibitor in HCT116 cells [27].

Despite of the broad potential of siRNA-based therapy, there are few limitations of using siRNA for clinical application. Firstly, siRNA is unstable under physiological conditions as it is easily degraded by nucleases in the serum [14]. Secondly, the role of kidney in siRNA clearance that has been reported in several animal studies [28], besides the off-target effects of siRNA, that could result to unexpected phenotypes [14]. Chemical modifications and suitable delivery

methods are crucial to bring siRNA to its target site without detrimental effects [14].

In conclusion, based on the findings in the current *in-vitro* study, the profound effect of the simultaneous gene knockdown of *MSH2* and *EPCAM* gene to the reduction of *β-catenin* expression may indicate the ability of these two genes to become co-target genes in regulating the Wnt/*β-catenin* pathway. This combined siRNA approach has also suggested to be a new therapeutic approach in the treatment of CRC as well as LS through suppression of Wnt/*β-catenin* pathway. Nevertheless, although the expression of these genes has been demonstrated *in-vitro*, future *in-vivo* studies need be done to further elucidate their molecular mechanism and clinical significance in CRC predisposition.

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AUTHORS' CONTRIBUTIONS

WK Wan Juhari performed the experiment, drafted the manuscript, performed the analysis. KB Ahmad Amin Noordin have been involved in the conception of idea, drafting the manuscript, performed the analysis and revising it critically for important intellectual content. WF Wan Abdul Rahman, AZ Zakaria, WMM Wan Muhamad Mokhter, BA Zilfalil involved in the conception of idea and drafting of the manuscript. All authors approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

Not Applicable.

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