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Differences in the Expression of β-Catenin Nucleus/Cytoplasm Ratio e-Cadherin and n-Cadherin and Correlation of β-Catenin Cytoplasm and Cadherin in Model of Duke D Stage Colorectal Cancer Cell Line

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Abstract

BACKGROUND: β-catenin has a critical role in the homeostasis processes. Wnt/β-catenin signaling mainly involved in the modulation of cancer cell development.

AIM: This research aimed to investigate the differences of β-catenin expression observed in the form of nucleus–cytoplasm ratio, the differences of E-cadherin and N-cadherin expressions, and the correlation between N-cadherin and E-cadherin and β-catenin cytoplasm in Dukes D stage colorectal cancer (CRC), which is an advanced stage and has experienced metastasis.

MATERIALS AND METHODS: This study followed an experimental research design. The processes of culture manufacturing and subculture preparation of Dukes D stage CRC cell line model were performed before the administration of β-catenin, E-cadherin, and N-cadherin antibodies. The next process was staining using fluorescein-5-isothiocyanate and rhodamine, and observations were performed using a confocal microscope. The number of cells was counted, and the intensity of antibody expression based on the arbitrary unit was measured.

RESULTS: There was a significant difference between the expression of β-catenin nucleus and cytoplasm expression ($p = 0.00$), as well as between E-cadherin expression and N-cadherin expression ($p = 0.00$). In addition, a correlation also existed between an increased N-cadherin expression and decreased E-cadherin expression and β-catenin cytoplasm in Dukes D stage CRC, but the results were not significant ($p = 0.837$ and $p = 0.108$).

CONCLUSION: In advanced-stage CRC (Dukes D), the Wnt signaling proved to be active and was characterized by a high expression of β-catenin nucleus, it activates the target gene. Similarly, at the Dukes D stage, N-cadherin expression increased whereas E-cadherin expression decreased in which causing the translocation of β-catenin into the nucleus.

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer. It is among the top three causes of cancer incidence worldwide [1]. CRC was also mentioned as the fourth leading cause of cancer death [2]. Data show that the worldwide prevalence of CRC in 2013 was equal to 9% of all types of cancer [3]. The modality of handling CRC, as well as other cancers, is divided into surgery, chemotherapy, and radiotherapy. This modality is based on the clinical stage of CRC [4], [5].

As it is known, the failure rate of chemotherapy due to resistance is fairly high [6]. Chemotherapy resistance is caused by many factors. One theory that has been investigated so far is the existence of gene mutations that occur in two types of CRC, namely, familial adenomatous polyposis and hereditary non-polyposis CRC [7]. Besides, several signaling pathways also cause genetic mutations in CRC and further aggravate the occurrence of chemotherapy resistance [8]. The other factor involved in the progression of CRC is the alteration of gut microbiota. The modern lifestyle, high protein intake, increasing branched-chain fatty acid, etc., proved to be responsible for CRC induction by acting as pro-carcinogenic and promote inflammation. This dysbiosis is also presumed to be responsible for the genetic mutations of CRC [9].

β-catenin, a multifunctional protein, has a central role in the process of homeostasis in the human body. Wnt/β-catenin signaling is a classic pathway involved in the modulation of cancer cell development, namely, proliferation, resistance, differentiation, motility, adhesion, and apoptosis of cancer cells. The signal transducers that influence β-catenin (phosphorylation/degradation) activity are

glycogen synthase kinase 3β, Axin, and adenomatous polyposis coli [10], [11], [12].

Wnt signaling and adhesion processes mediated by cadherin are known to be involved in both embryonic development and cancer progression. Recent research demonstrated that there is a crosstalk between Wnt signaling and cadherin-mediated cell adhesion [13], [14]. Cadherin mediates cell adhesion through the extracellular domain and connects the actin cytoskeleton to catenin through the cytosolic domain. Cadherin is involved in the regulation of inter-cell adhesion, such as morphogenetic modulation and the differentiation process during cell development. Epithelial cells express E-cadherin, whereas mesenchymal cells express several cadherin variations, such as N-cadherin, R-cadherin, and cadherin-11. Cadherin is very important in the development of cell polarity and cell selection during embryonic [13], [15].

E-cadherin is a complex that exists in the cytoplasm of cancer cells and functions to form an actin cytoskeleton to strengthen cell adhesion. It is responsible for maintaining cell polarity so that it is considered as a marker of cancer cells that have not undergone metastasis [14]. Contrarily, N-cadherin experiences increased regulation of mesenchymal cells, where these cells are more motile (moving) and less polarized than the epithelial cells. N-cadherin is expressed in several cell types, such as neuron cells, endothelial cells, stromal cells, and osteoblasts [13], [15].

In an advanced-stage CRC, it has been observed that most would experience epithelial-mesenchymal transition (EMT) [13]. During the process, switching between E-cadherin and N-cadherin occurs, which is known as cadherin switching; it indicates a decrease in E-cadherin regulation followed by an increase in N-cadherin regulation when cancer cells undergo EMT [14]. However, in some circumstances, E-cadherin expression does not change significantly, but cells experience an increase in N-cadherin expression. In some types of cancer, E-cadherin shifts to N-cadherin, but conversely, N-cadherin shifts to E-cadherin in some other cancers [14], [15]. Transforming Growth Factor-β (TGF-β) is the other factor involved in the progression of EMT in CRC. Initiation of $Smad_4$ by TGF-β will make a complex with multiple transcription factors (e.g., Snail-1, Slug, or Twist) and promote activation of target genes and triggering proliferation of these cancer cells [16].

In general, cadherin is known to negatively regulate the Wnt pathway by separating β-catenin from the T-cell factor (TCF) transcription into the plasma membrane. The signal transduction of β-catenin is activated if Wnt/β-catenin protein binds to the TCF protein on the cell surface [15]. Another theory states that proteolytic cleavage of cadherin releases β-catenin and sequentially induces Wnt signal. Cadherin cleavage inside the cell is carried out by protease caspase-3 or presenilin and outside the cell by ADAM10. As a result of cadherin cleavage, cell adhesion becomes loose; thus, β-catenin is released and translocated into the nucleus and activates the target gene [14].

Based on the evidence mentioned, the researchers hypothesized that at the Dukes D stage CRC, a β-catenin translocation occurs from the cytoplasm into the nucleus. At the Dukes D stage CRC, there is also an interaction between β-catenin cytoplasm and cadherin, where an increase in N-cadherin expression occurs, followed by a decrease in E-cadherin expression. Until now, there are no research aimed at comparing β-catenin cytoplasm and nucleus and E-cadherin and N-cadherin and investigating the correlation between both of them, especially in the Dukes D stage CRC. Thus, in this study, the researchers aimed to investigate the differences of β-catenin expression observed in the form of nucleus–cytoplasm ratio, the differences of E-cadherin and N-cadherin expressions, and the correlation between N-cadherin and E-cadherin and β-catenin cytoplasm in Dukes D stage CRC.

Materials and Methods

Samples and specimens

The sample in this study was obtained from the Dukes D CRC cell line model. Cell lines were ordered from the American Type Culture Collection (ATCC) through the Santacruz provider. Dukes D stage CRC cell type was HCT-116 (ATCC CCL-247).

Antibodies and reagents

The primary antibodies used were mouse monoclonal immunoglobulin (Ig)G β-catenin, mouse monoclonal IgG E-cadherin, and mouse monoclonal IgG N-cadherin ordered from the Santacruz provider. No secondary antibodies were used in this study.

Cultivation procedure

The immunocytochemical procedure was preceded by the manufacture and propagation of cell cultures. Vials containing cell cultures were defrosted carefully in a water bath at 37°C. The O-ring was maintained and closed tightly to reduce the chance of contamination. Defrosting must be performed quickly, about 2 min. The vials were removed from the water bath immediately after the vial contents have melted, and decontamination was performed by dripping or spraying 70% ethanol to the vial contents. Aseptic technique must be used when performing all actions in this process. The melted cells in the vials were transferred into the centrifuge tubes containing a complete culture medium and rotated at 125 × G for 5–7 min. The pellet cells were then resuspended into

a complete medium and then poured into new culture flasks. The culture was incubated at 37 °C without CO_2.

Fixation and staining

The staining process was preceded by fluorochrome-conjugated administration, which is a secondary antibody, followed by incubation at room temperature for 1–2 h. Nucleus staining using rhodamine dyes was performed as needed. To see the intensity of β-catenin expression, double staining was performed using fluorescein-5-isothiocyanate (FITC) to see the overall β-catenin and rhodamine to see β-catenin in the cell nucleus. To observe E-cadherin and N-cadherin, rhodamine staining was also performed. Next, incubation was carried out for 2–5 min at room temperature. Washing with phosphate-buffered saline was performed once. Next, mounting medium containing an anti-shadow fluorescent agent was added. The coverslip was carefully placed on top, and the unused mounting medium was removed. Observations were performed using a confocal laser-scanning microscope (CLSM) with a 400× magnification and put in a Fluoview Ver4.2 viewer computer and processed with ImageJ 1.50 software. A sample of 10 cells was taken and observed per one field of view. The number of cells was counted and the color intensity/luminescence measured using the arbitrary unit (AU).

Statistical analysis

All data were expressed as mean ± SD for the three independent experiments. The differences between the two examination groups were analyzed using Statistical Product and Service Solution software, IBM Statistics 20, with a 0.05 significance level and a 95% confidence level.

Ethical statement

The ethics approval of this research was obtained from the Health Research Ethics Committee of the Faculty of Medicine, Brawijaya University Malang, Indonesia (07/EC/KEPK-S3/01/2019). This research was conducted from November 2019 to April 2020 at the Central Laboratory of Biological Sciences, Brawijaya University, Malang, and the Biomedical Laboratory, Faculty of Medicine, Brawijaya University, Malang.

Results

Comparison between β-catenin nucleus and cytoplasm

The intensity of β-catenin expression in Dukes D stage CRC cell line was distinguished using double staining, namely, FITC for overall β-catenin staining and rhodamine for nucleus staining. Double staining was performed to differentiate the expression of β-catenin nucleus and cytoplasm. The observation results revealed that the intensity of the β-catenin nucleus appeared brighter compared with that of the cytoplasm (Figure 1). This is demonstrated by the calculations using the AU as shown in the histogram.The differences in the intensity expression of β-catenin nucleus and cytoplasm are presented in a histogram and calculated based on the AU. The mean intensity expression of β-catenin nucleus is 1521.069 AU, whereas the mean intensity expression of β-catenin cytoplasm is 774.2415 AU (Figure 2).The normality test for β-catenin data was conducted using the Shapiro–Wilk test, where β-catenin nucleus and β-catenin cytoplasm data were normally distributed ($p = 0.155$ and $p = 0.091$). Therefore, an independent t-test was conducted to determine the relationship between β-catenin nucleus and cytoplasm, and a significant difference was observed ($p = 0.000$).

Comparison of E-cadherin and N-cadherin

The observations revealed that the luminescence expression was very weak in cells that received E-cadherin antibodies compared with cells that received N-cadherin antibodies (Figure 3). This is demonstrated by the results of the calculations based on the AU.

The differences in the intensity of E-cadherin and N-cadherin expressions are presented in a histogram and are calculated based on the AU. The mean intensity of E-cadherin expression was 44.394 AU, whereas the mean intensity of N-cadherin expression was 514.8415 AU (Figure 4). The data normality test of N-cadherin was conducted using the Shapiro–Wilk test, and normally distributed data ($p = 0.153$) were obtained. E-cadherin data were found to be not normally distributed ($p = 0.153$). Therefore, the Mann–Whitney U test was conducted to determine the relationship between E-cadherin and N-cadherin, and a significant difference was obtained ($p = 0.000$).

Comparison between β-catenin cytoplasm and N-cadherin expression

Before calculating the correlation between β-catenin cytoplasm and N-cadherin, the data normality test was conducted, and apparently normally distributed data were obtained; thus, Pearson's correlation test was conducted. The results of Pearson's correlation test revealed a positive correlation between β-catenin cytoplasm and N-cadherin, where increased expression of β-catenin cytoplasm was followed by an increased expression of N-cadherin ($r = 0.075$), but the correlation was not significant ($p = 0.837$). In the diagram presented in Figure 5a, β-catenin cytoplasm expression is displayed as ordinate and N-cadherin expression as

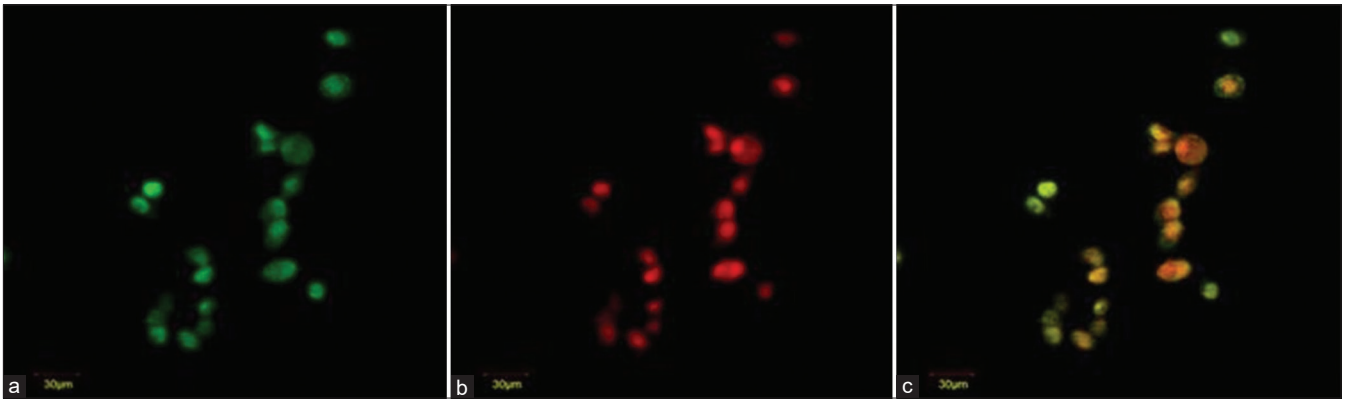

Figure 1: Immunofluorescence images of β-catenin in Dukes D stage line. β-catenin immunofluorescence uses fluorescein-5-isothiocyanate (green) (a) staining. Cell nucleus staining uses rhodamine (red), (b) If β-catenin is present in the nucleus and cytoplasm, the color will be orange, (c) Imaging was performed using confocal laser scanning microscope (400× magnification, a red line scale 30 = μm)

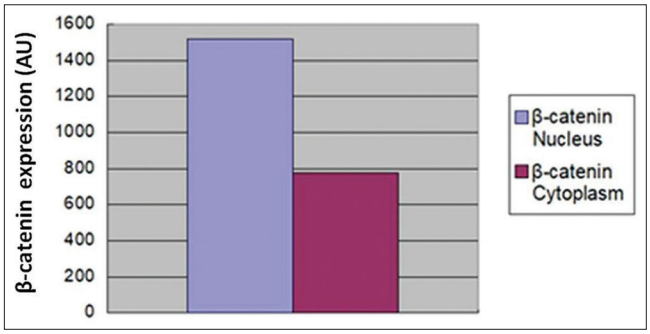

Figure 2: The intensity of β-catenin nucleus and cytoplasm expression. The results are expressed as Standard Error of Measurement ± SEM and show a significant difference between the two (p = 0.000)

axis. From the diagram, there is a cross line from the lower left to the upper right. This indicates a positive correlation between β-catenin cytoplasm expression and N-cadherin expression.

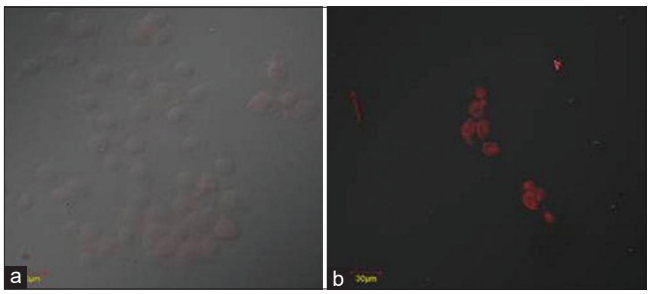

Figure 3: Immunofluorescence images of E-cadherin and N-cadherin. Immunofluorescence uses rhodamine staining (red). Light intensity on E-cadherin antibody is very weak (a) compared with light intensity on N-cadherin antibody (b) Imaging was performed using confocal laser scanning microscope (400× magnification, a red line scale 30 = μm)

Comparison between β-catenin cytoplasm and E-cadherin expression

Before investigating the correlation between β-catenin cytoplasm and E-cadherin, data normality test was conducted. It turns out that the data were not normally distributed; thus, Spearman's correlation test was conducted. Spearman's correlation test results revealed a negative correlation between β-catenin cytoplasm and N-cadherin, where increased expression of β-catenin cytoplasm was followed by a decreased expression of E-cadherin (r = −0.539), but the correlation was not significant (p = 0.108). This can be observed in the scattered diagram presented in Figure 5b, where β-catenin cytoplasm expression is displayed as ordinate and E-cadherin expression as axis. From the diagram, there is a line that crosses from the top left to the bottom right. This indicates a negative correlation between β-catenin cytoplasmic expression and E-cadherin expression.

The correlation between β-catenin cytoplasm and E-cadherin is explained in the table below. There is a positive correlation between β-catenin cytoplasm expression and N-cadherin (r = 0.075), but the correlation was not significant (p = 0.837). Conversely, there is a negative correlation between β-catenin cytoplasm expression and E-cadherin (r = −0.539), but the correlation is also not significant (p = 0.108) (Table 1).

Table 1: Correlations between N-cadherin and E-cadherin expressions and β-catenin cytoplasm

Table of Correlation	N-cadherin expression (AU)		E-cadherin expression (AU)	
	r	p-value	R	p-value
β-catenin cytoplasm	0.075	0.837	–0.539	0.108

AU: Arbitrary unit

Discussion

Dukes D stage (or C2 stage in modified Dukes) CRC is the final stage of CRC, in which the cancer cells have undergone distant metastases, such as to the liver, bones, and lungs. At this stage, tumor cells are said to experience migration, invasion, and metastatic activity, and the rate of failure or chemotherapy resistance is also increasing, which is marked by a 5-year survival rate of only 6.6% compared with Dukes A stadium that is 93.2% [17], [18]. The Previous studies demonstrated

that in Dukes D stage, cancer cells have undergone distant metastases, and the activation of Wnt/β-catenin signaling also has an increased regulation marked by the entry of β-catenin into the nucleus and activates transcription genes, such as Snail, Slug, Twist, and ZEB. Increased activation of Wnt signaling is followed by a decrease in E-cadherin expression and an increase in N-cadherin expression, which is known as "cadherin switching" [13], [15].

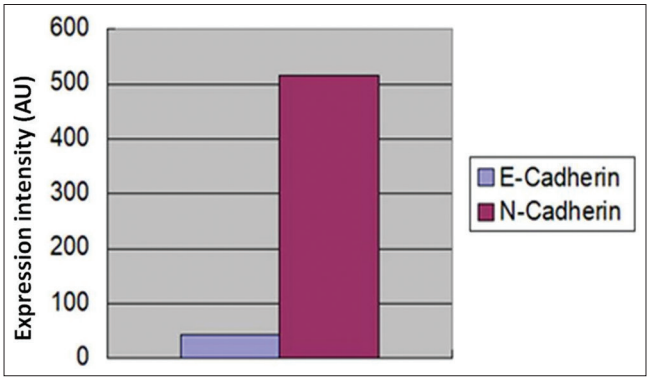

Figure 4: The intensity of E-cadherin and N-cadherin expressions. The results that are expressed as mean of Standard Error of Measurement ± SEM show a significant difference between the two (p = 0.000)

In this study, Dukes D stage CRC cell line that received monoclonal antibody β-catenin exhibited differences in the expression intensity of β-catenin nucleus compared with β-catenin cytoplasm. This difference was observed by the immunofluorescence method using CLSM, which resulted in a higher mean expression intensity of β-catenin nucleus compared with the mean intensity of β-catenin cytoplasm expression (1521.069 AU vs. 774.2415 AU), and the result was significant (p = 0.00) (Figures 1 and 2). From these data, it can be concluded that at Dukes D stage CRC, the expression of β-catenin in the nucleus is higher than that of β-catenin in the cytoplasm, although there are still β-catenin contained in the cytoplasm, but with low expression. Gao *et al*. who compared the levels of β-catenin membranes, cytoplasm, and nucleus, found that a high level of β-catenin nuclei is correlated with the stage of CRC that had undergone metastasis [19]. Another study by Yoshida *et al*. in 201 CRC patients, whose β-catenin expressions were examined, revealed that the expression of β-catenin nucleus is correlated with a poor CRC prognosis, characterized by lymphatic invasion, venous invasion, and lymph node metastases [20]. The criteria above are following Duke D stage CRC.

This study also obtained data on Dukes D stage CRC cell line that received monoclonal antibodies E-cadherin and N-cadherin that show differences in the expression intensities of E-cadherin and N-cadherin. These differences were observed by the immunofluorescence method using CLSM that showed the mean intensity of E-cadherin expression versus the average intensity of N-cadherin expression (44.394 AU vs. 514.8415 AU), and the results were significant (p = 0.00) (Figures 3 and 4). From these data, it can be concluded that in Dukes D stage CRC, the expression of E-cadherin is much lower than that of N-cadherin, and even the intensity of E-cadherin expression is almost invisible; this is evidenced by the absence of fluorescence from the observed cell line (Figure 3a). Almost the same as the results of this study, a study by Ye *et al*. [21] on 37 isolated CRC samples found a correlation between E-cadherin and N-cadherin levels and CRC stage according to Dukes. The study concluded that at the Dukes C/D stage, the E-cadherin expression was lower than that at the A/B stage, whereas the N-cadherin expression at the Duke C/D stage was higher than that at the Duke A/B stage. Kim *et al*. [22], in a meta-analysis of 50,000 samples of CRC patients observed since 1986 and examined for the CDH1 (E-cadherin) levels, concluded that loss of E-cadherin expression is closely related to infiltration undergone by tumor cells. Research conducted by He *et al*. also found an association between a decrease

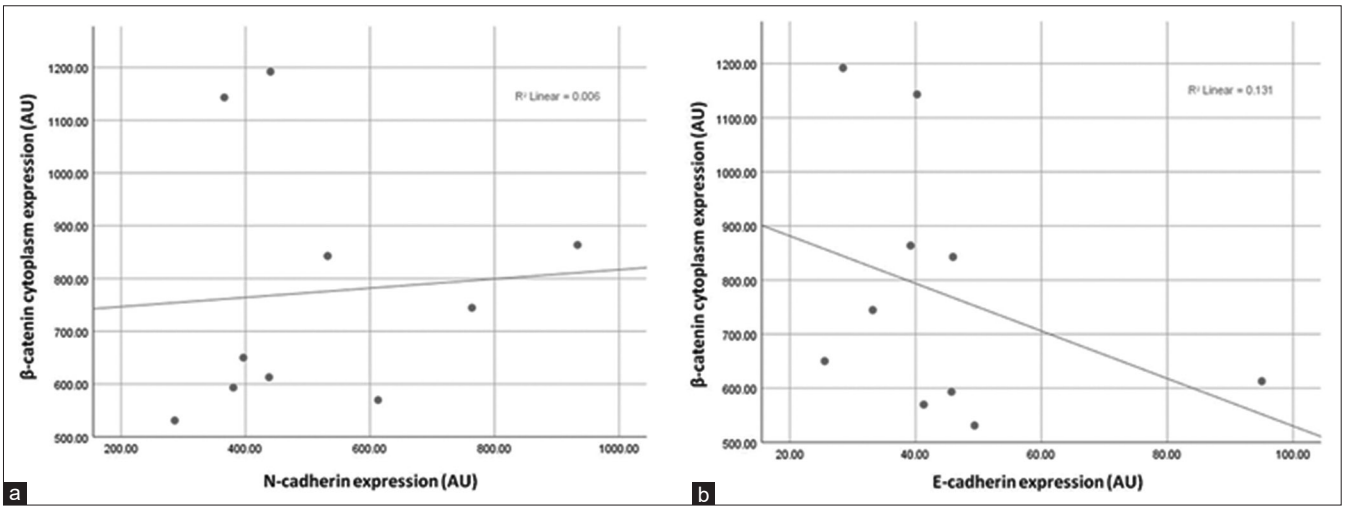

Figure 5: A correlation between β-catenin cytoplasm and N-cadherin (a) The results reveal a positive correlation, where an increase in β-catenin cytoplasm is followed by an increase in N-cadherin. A β-catenin cytoplasm and E-cadherin correlation (b) The results reveal a negative correlation, where the increase in β-catenin cytoplasm is followed by a decrease in E-cadherin

in E-cadherin regulation and the final stage and poor prognosis of CRC [23]. Research conducted by Li *et al*. concluded that expression of CDH1 (E-cadherin) patients and CDH1-160C/A polymorphism is associated with the decreased risk of CRC in the Chinese Han population [24]. A similar result of the research conducted by Sun *et al*., who concluded that CDH1-160C/A polymorphism may become a susceptible predictor for risk of CRC in Caucasians [25].

There is a study that examined soluble E-cadherin (sE-cadherin) in the cell line SW480 which was induced by TGF-β1 to become EMT. The result of this study was the expression of serum sE-cadherin level that was increased in the CRC cell which already undergone EMT. This result was different compared with usual research that expression of E-cadherin will decrease in the CRC with EMT process [26]. In this study, a correlation test between β-catenin cytoplasm expression and E-cadherin and N-cadherin was also conducted. The correlation between N-cadherin and β-catenin cytoplasm can be explained by the theory that N-cadherin that binds with FGF receptors increases interactions with endothelial and mesenchymal cells, whereas N-cadherin that binds with PDGF receptors interact with β-catenin, increasing cell motility, followed by an endocytosis process against E-cadherin [13].

Other interactions between cadherin and β-catenin occur in the cytoplasm. Cadherin cleavage by protease enzymes, such as ADAM1 and Presenilin-1, releases β-catenin from the destruction complex and undergoes translocation. The translocation of β-catenin into the nucleus is followed by the activation of target genes, such as SNAIL1, SLUG, TWIST, and ZEB, and subsequently leads to more metastatic cancer cells [14]. Chen *et al*. [27] in their study concluded that Wnt/β-catenin signaling decreases E-cadherin regulation and, conversely, improves N-cadherin regulation and supports the EMT process. The absence of a significant correlation between β-catenin cytoplasm and E-cadherin and N-cadherin in this study is probably because most of the β-catenin has been translocated into the nucleus, as evidenced by the higher intensity of expression of β-catenin nucleus compared with that of the cytoplasm (1521.069 AU vs. 774.2415 AU).

In advanced-stage CRC, many transduction signals are proven to play a role in the processes of migration, invasion, proliferation, and metastasis. Wnt signaling has been proven to be activated, and β-catenin has been translocated into the nucleus and activates the target gene in Dukes D stage CRC. In Dukes D stage CRC, “cadherin switching” also occurs, where E-cadherin expression decreases, and N-cadherin expression increases, and is correlated with β-catenin cytoplasm. Interactions between β-catenin and cadherin occur in the cell membranes through binding with several receptors and in the cytoplasm through the release of β-catenin from the destruction complex. A recent study using HOXB8, a transcription factor that induces STAT3 revealed that in cell line HCT116 (Duke D stage of CRC), the depletion of HOXB8 will decrease the expression of E-cadherin and increase the expression of vimentin, N-cadherin, Twist, ZEB1, and ZEB2. This study also concludes that HOXB8 protein will promote the proliferation and metastasis of CRC [28].

Conclusion

Further research is required to ensure the differences in the expression of β-catenin and cadherin at other CRC stages (Dukes A, B, and C) and also to find the influencing factors on the increase or decrease in the expression of β-catenin, E-cadherin, and N-cadherin. Another further research is needed to look for some potential biomarkers instead of three proteins above. New potential biomarkers, such as p-STAT3, ALR, and PIAS3 are proven associated in the early stage of CRC. These proteins were activated by gut microbiota, which has been a very long time already known involved in gastrointestinal cancer [29]. In the future, research of new chemopreventive drugs based on the activities (promoting or inhibiting) Wnt signal or cadherin is also very important to be explored. Silibinin, the most active flavinolignan has been revealed that it can suppress HCT-116 (Duke D stage of CRC) proliferation and migration and induced apoptosis. This drug can attenuate EMT through decreasing β-catenin gene, N-cadherin, and vimentin expression [30].

There are still many signaling pathways involved in the progression of CRC which are still not be revealed. We still need to be challenged with many researches for one purpose to decreasing the morbidity and mortality of this cancer.

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