TGF-β and SMAD2/4 Expression in Non-metastatic and Metastatic Colorectal Cancer Patients

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Abstract. Colorectal cancer (CRC) is the third most common and second cancer with the highest mortality rate in the world. The leading cause of death in colorectal cancer patients is cancer that has metastasized, with the most common site of metastasis being the liver. One of the signaling that regulates malignancy of cancer cells is TGF-β/Smad. Through activation of the Smad2/3/4, TGF-β regulates the EMT Transcription factors to activate Epithelial Mesenchymal Transition (EMT) program. Tumor cells that have undergone EMT have migratory, invasive, and metastatic phenotypes. This study aims to know the differences mRNA expression of TGF-β, Smad2, and Smad4 in metastatic colorectal cancer and non-metastatic groups using real time PCR method. The results showed TGF-β and Smad2 expression in metastatic CRC was higher in the metastatic group than in the non-metastatic group. In contrast,Smad4 expression was found to be higher in the non-metastatic group. The results suggest that TGF-β/Smad signaling pathway has a role in promoting metastasis and severity in CRC patients.

1 Introduction

Colorectal Cancer (CRC) is the third most common cancer in the world and the second with the highest mortality cases in the world. Based on data from the Global Burden Of Cancer Study (GLOBOCAN) from WHO, it is estimated that more than 1.93 million incidents and 940 thousand colorectal cancer deaths will occur in 2020 [1]. The incidence of colorectal cancer in 2020 represents 10% of the global cancer incidence (total of 19.29 million new cases) and 9.4% represents all cancers that cause death (total of 9.96 million deaths). According to GLOBOCAN, the incidence of colorectal cancer in Indonesia in 2020 reached 34,189 and is the fourth highest incidence compared to other types of cancer. In general, CRC cases in Indonesia are at a high stage, this is due to a lack of public awareness regarding cancer [2]. The prevalence of colorectal cancer has also been reported to continue to increase globally in recent years. The incidence of CRC was also gradually rising in Indonesia [3].

The increased incidence of colorectal cancer is associated with increased exposure to risk factors resulting from shifts in lifestyle and diet towards westernization [4]. Furthermore, previous studies revealed a strong correlation between metabolic dysfunction and the

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prevalence of colorectal cancer. Metabolic syndrome is an independent risk factor for colorectal cancer and has been linked to patients CRC recurrence [5]. Approximately 70-75% of colorectal cancer cases occur sporadically and are associated with modifiable risk factors, whereas 25-30% of cases are associated with non- modifiable risk factors such as genetic factors, history of polyps/adenomas, family history of colorectal cancer or hereditary risk. (Lynch syndrome or familial adenomatous polyposis [6]. Sporadic colorectal cancer cases are caused by the increasing prevalence of modifiable risk factors such as lifestyle, namely smoking habits, alcohol consumption, unhealthy eating patterns, low physical activity, obesity, and others [7].

The main cause of death in colorectal cancer patients is cancer that has metastasized and it is reported that half of all colorectal cancer patients have metastases [8]. The most common site of colorectal cancer metastases is the liver and is present in 70% of colorectal cancer patients compared to the lungs, lymph nodes, and peritoneal [9]. This is caused by colorectal cancer cells that spread hematogenously through the portal circulation and often make the liver the first site of metastasis. Only 20% of metastatic colorectal cancer patients were reported to have survived more than 5 years after diagnosis [10]. The presence of metastases increases the aggressiveness of tumor cells and reduces patient survival and prognosis.

Many carcinomas initiation, growth, and invasiveness are regulated by TGF-β. Metastasis of colorectal cancer facilitated by Transforming Growth Factor-β (TGF-β) through Epithelial Mesenchymal Transition (EMT), angiogenesis, immunosuppression and stemness [11]. TGF-β/Smad signaling that induces EMT are closely related to tumor invasion and metastasis [12]. The canonical TGF-β signaling pathway is triggered by the transcription factors Smad2/Smad3 which bind to the TGF-β serine/threonine kinase receptor, then phosphorylate and interact with Smad4. The Smad2/Smad3/Smad4 complex accumulates in the nucleus and binds to target gene promoters to regulate gene transcription including the EMT regulatory transcription factor. EMT disrupts cell adhesion, loss of apical-basal polarity, remodeling of the cytoskeleton and acquisition of mesenchymal cell characteristics such as increased migratory capacity, invasion of other tissues, and accompanied by high resistance to apoptosis. In addition, overexpression of the TGF-β gene is associated with the formation of neoplastic stem cells in the tumor stroma, decreased immune response, triggering the process of carcinogenesis and EMT. In the tumor microenvironment of CRC, increased TGF- β expression levels are crucial. The function of anti-tumor immune cells is inhibited by TGF-β signaling, which creates an immunosuppressive milieu and controls the development of CRC. In CRC cells, TGF- signaling activation may encourage cancer invasion and metastasis [13].

Metastasis in colorectal cancer is the greatest challenge for successful treatment. The most effective treatment for patients with metastatic colorectal cancer is surgery, but only a subset of patients are suitable for initial surgery. It is reported that about 30% of colorectal cancer patients recover after surgery and 70% of disease recurs or metastasizes during the first 2 years after treatment [14]. This is exacerbated by the fact that colorectal cancer patients can potentially have micrometastases that are not detected during primary tumor surgery. Therefore, finding candidate metastatic biomarkers and colorectal cancer prognosis is increasingly important. Measurement of potential markers in primary tumors by identifying patients who have the potential to have metastases can improve risk stratification and selection of appropriate treatment [15]. Metastatic gene expression profiling in the early stages of colorectal cancer is also very necessary to prevent the development of colorectal cancer and improve patient survival rates[16]. Therefore, there is a need for molecular screening studies that measure the expression of TGF -β/Smad to determine differences in EMT mechanisms and the metastatic potential of colorectal cancer.

2 Research Method

2.1 Ethical Submission and Sample Preparation

Submission of Ethics and Sample Preparation for this study has obtained permission from the Medical and Health Research Committee, Faculty of Medicine, Gadjah Mada University, Central General Hospital dr. Sardjito Yogyakarta with number: KE/FK/0938/EC/2021. The samples used were colonic tissue from non-metastatic colorectal cancer patients (18 samples) and metastases (10 samples) diagnosed with colorectal cancer by a team of doctors at the RSUP. Dr. Sardjito, Yogyakarta through clinical examination and CT-Scan. Baseline data on patient characteristics such as age, BMI, tumor stage, were measured in this study by the RSUP medical team. Dr. Sardjito, Yogyakarta. This study used the qPCR method to measure the expression of the TGF-β1, Smad2, and Smad4 genes in these two groups of patients.

2.2 Total RNA Isolation

The RNA isolation procedure was carried out based on the QIAzol Lysis Reagent (QIAGEN) kit protocol as follows: Trizol 500 was addedμL in each microtube containing 0.01 g of sample, homogenized with a sonicator for ±30 seconds, centrifuged at 12,000 xg for 10 minutes at 4 °C. The supernatant was transferred to a microtube containing 200 ml of chloroformμL and inverted then left on ice for 10 minutes, centrifuged at 12,000 xg for 10 minutes. Transferred colorless supernatant ± 200μL to a microtube containing 600μL isopropanol, inverted and left at room temperature for 10 minutes. Centrifuge at 12,000 xg for 10 minutes. Furthermore, the supernatant was discarded, added 200μL 70% ethanol without mixing and centrifuged at 7,500 xg for 5 minutes, discard the supernatant, dry the tube containing the pellets for ± one hour, add 50μL RNAse Free Water, and stored the results of RNA isolation at – 4 °C.

2.3 Reverse Transcription

Reverse transcription was carried out by two-step and the cDNA synthesis procedure was carried out according to the ReverTrace qPCR-RT Master Mix (TOYOBO) kit protocol as follows: The RNA template was incubated at 65°C for 5 minutes with a thermal cycler machine, prepared a mixture of 4X DN Master Mix and gDNA remover with a ratio of 88μL : 1.8μL, prepared DNAse I cocktail (4X DN Master Mix = 2μL, Template RNA = 2μL, Nuclease Free water = 4μL), incubated the DNAse I cocktail at 37°C for 5 minutes, the reverse transcription cocktail was prepared (DNAse I cocktail = 8μL, 5X RT Master Mix = 2μL, put the cocktail into the GeneAmp® PCR System 9700 machine (Thermo Scientific) with an incubation program at 37°C for 15 minutes, incubation at 50°C for 5 minutes, incubation at 98°C, incubation until the temperature reached 4°C, then the cDNA results were stored at -4°C.

2.4 Quantitative PCR (qPCR)

The qPCR procedure was carried out based on the SYBR Green Real-time PCR (Bioline) kit protocol as follows: RNA templates, gene primers, and RNAse Free Water were prepared. Prepared 2x SensiFAST SYBR® No-ROX Mix = 5μL per sample, forward gene primer = 0.8μL per sample, reverse gene primer = 0.8μL per sample, template RNA = 1μL, RNAse Free Water = 2.4μl per sample, mixed reaction, then machine qPCR (Analytic Jena qtower^3) programmed with a cycle (Pre-denaturation = 2 minutes at 95 °C, Denaturation = 5 seconds

at 95 °C, Annealing/extension = 30 seconds at 60 °C). The target gene primers used are listed in Table 1. The relative expressionof the target gene is obtained based on the calculation of $2^{\Delta Ct}$ with ΔCt which is the difference between the Ct housekeeping gene and target gene values in relative quantification. Differences in target gene expression between the two groups were analyzed using t-tests. Significance is indicated by $p<0.05$. Data obtained then visualized using GraphPad Prism software, then the data was analyzed descriptively.

Table 1. Primers used for qPCR

Genes	Forwards (5'-3')	Reverse (5'-3')
β-actin	CATGTACGTTGCTATCCAGG C	CTCCTTAATGTCACGCACGAT [17]
TGF-β1	AAGTGGACATCAACGGGTTC	GTCCTTGCGGAAGTCAA TGT [18]
Smad2	TCATAGCTTGGATTTACAGCCAG	TTCTACCGTGGCATTTCGGTT [19]
Smad4	AAGGCCTAGCACCACCTTAG	AGCCTTAAACTCTGACCTGT [20]

3 Results and Discussion

This study used 18 samples of non-metastatic CRC and 10 samples of metastatic CRC. Based on these data, there were 9 male patients in the non-metastatic CRC group out of 18 patients and 6 out of 10 patients in the metastatic CRC group. GLOBOCAN data in 2020 shows that the ratio of colorectal cancer incidence in men and women is 19.8, in men it is 23.4, and in women it is 16[1]. This shows that men have a higher incidence of colorectal cancer than women. The high incidence of colorectal cancer in men is related to the susceptibility of men to exposure to CRC risk factors such as smoking habits, consumption of alcoholic beverages, visceral fat, and unhealthy diet patterns. In addition, the protective role of estrogen in women is thought to play a role in reducing the incidence of colorectal cancer in women. In general, estrogen acts as a protective hormone for cancer through anti-inflammatory activity [21]. The nuclear estrogen receptors β (Erβ) exert anti-tumor effects through selective activation of pro-apoptotic signaling, increased DNA repair, inhibition of oncogene expression, regulation of cell cycle progression, and alteration of miRNA pool and DNA methylation [22]. Gender differences in colorectal cancer have aspects of sexual dimorphism (differences in hormones and genes) and gender differences (differences in attitudes and behavior) [23].

Patients in both CRC groups in this study were generally over 50. This aligns with research showing that around 90% of CRC cases occur in individuals over 50 years [24]. Preliminary studies of CRC in Indonesia during 2008-2012 showed that colorectal cancer was higher in men (54%) than in women (46%) and most cases found at the age of 50-54 years [25–26]. Another study stated that 47.2% of metastatic colorectal cancer patients aged 50-69 years were included in the middle age group [27]. Old age and aging are closely related to cancer risk. Aging causes gradual degeneration at the molecular, cellular, tissue, and body levels. One of the characteristics of aging is hyperplasia which can develop into cancer. Apart from being over 50 years old, there are CRC patients who are under 50 years old. This shows an increased incidence of colorectal cancer in patients under the age of 50 years [28]. A significant trend in the epidemiology of CRC among Indonesian younger patients was also found [29].

The BMI category in this study was based on WHO criteria, BMI under 18.5 was considered underweight, BMI in the range of 18.5-24.9 was considered normal, and BMI above 24.9 was considered overweight. Non-metastatic colorectal cancer patients were generally in the normal BMI range, then in the overweight BMI and underweight BMI. Metastatic colorectal cancer patients are generally in the normal BMI range, and three people are followed in the underweight BMI range. Research shows that overweight and obese

individuals have a 40% higher risk of developing colorectal adenoma, the main precursor of colorectal cancer [30]. In addition, obesity and type 2 diabetes (T2DM) have been linked with an increased risk of developing colorectal cancer, liver cancer, pancreatic cancer, kidney cancer, and other cancers [31]. Normal and underweight BMI in metastatic colorectal cancer patients indicates weight loss due to cachexia associated with the aggressiveness of metastatic disease [32]. Cachexia in CRC sufferer is closely related to White adipose browning, which increases energy expenditure and causes wasting syndrome [33]. It is reported that weight loss in CRC patients occurs at a prediagnostic time estimated three to 6 years before colorectal cancer occurs [34]. Colorectal cancer patients with a consistent decrease in BMI and low BMI are more susceptible to treatment side effects and even death due to nutritional deficiencies or loss of muscle mass [35].

The staging of CRC patients in this study was based on tumor node metastases (TNM) measurements from The American Joint Committee on Cancer (AJCC) eighth edition. Non-metastatic tumor patients in this study were at stage I-III C along with tumors that had not spread, while metastatic tumor patients were all at stage IV B. Stage IV B indicates patients have metastases in other organs or various lymph nodes. All patients in the metastatic group developed liver metastases. Early-stage diagnosis of colorectal cancer increases the potential for cure and treatment in patients. However, survival is poor when patients are diagnosed with end-stage CRC [36]. The prognosis of colorectal cancer patients is highly dependent on the stage, and a poor prognosis is closely related to a higher stage.

TGFB was identified as the main metastatic pathway in numerous cancer cases and it was revealed that it involves MTA3, Snail, and E-cadherin via TGFBR2[37]. One of the mechanisms by TGF-β to support migration, invasion, and metastasis is through Epithelial-Mesenchymal Transition (EMT). Based on the results of this study, the expression of TGF-β1 and Smad2 in the metastatic CRC group was higher than non-metastatic CRC (Figure 1). Cancer cells frequently increase the amount of active TGF- β produce, which not only causes EMT and makes the cells invasive but also promotes angiogenesis in the tumor microenvironment, giving migratory mesenchymal cells a way to escape [38].

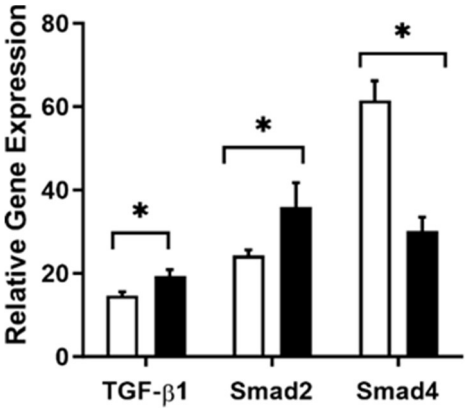

Fig 1. Expression of Genes in the TGF-β, Smad2, and Smad4 in Non-Metastatic and Metastatic Tumor Groups. * Significant by t-test ($p \leq 0.05$)

Higher TGF-β1 expression in the metastatic group aligns with studies showing that TGF-β expression is significantly increased in primary metastatic tumors and colorectal cancer metastases [39]. The study reported that TGF-β expressed by metastatic colorectal cancer cells has a pro-tumorigenic function by influencing crosstalk between cancer and endothelial cells, increasing angiogenesis [39]. TGF-β triggers tumor growth, invasion, and metastasis in advanced colorectal cancer [40] Colorectal cancer with higher TGF-β activity has a poor

clinical prognosis and a highermetastatic rate. It has been demonstrated that TGF-β also plays a role in the development of liver disease at every step, starting with the first liver injury [41]**.** In addition, increased levels of TGF-β have a pathogenic function in increasing angiogenesis and inducing immunosuppression thereby worsening the prognosis of colorectal cancer [42]. TGF-β signaling via Smad2 in late-stage tumors triggers tumorigenesis, activating epithelial to mesenchymal transition (EMT) [43]. TGF-β1 is an upstream regulator of the EMT pathway, while Smad2 and Smad3 complex activation induced by TGF-β through direct C-terminal phosphorylation by TGF-βRI. Phosphorylated Smad2 and Smad3 join with Smad4 to form trimers,then translocate to the nucleus and cooperate to activate or repress target genes such as activate EMT regulator transcription factors. EMT regulatory network involves various pathways such as Wnt, Notch, PI3K-AKT, and TGF-β. EMT has been reported to be aberrantly active during fibrosis and required for the fibrotic response, and this is in line with the role of TGF-β as an EMT trigger and a major factor for fibrosis [44].

In this study, Smad2 expression was found to be higher in the metastatic colorectal cancer group than in the non-metastatic group. This is in line with research showing that increased Smad2 expression in colorectal cancer patients correlates with a higher tumor stage [45]. Studies indicate that overactive TGFβ-Smad2 signaling further contributes to the establishment of an EMT phenotype by maintaining the epigenetic silencing of key epithelial marker genes, such as E-cadherin, claudin-4, kallikrein-10, and cingulin. This appears to be mediated via Smad2-dependent regulation of DNA methyltransferase 1 (DNMT1) binding activity and DNA methylation of the corresponding gene promoter regions. Smads indirectly regulate gene expression by maintaining promoter DNA methylation, which is very important in silencing epithelial gene expression in cells that have undergone EMT [46]. In contrast to Smad2, Smad4 gene expression was found to be higher in the metastatic group than in the non-metastatic group. Mutations, inactive function, and loss of Smad4 expression are commonly found in the progression of advanced colorectal cancer triggered by changes in the signaling effect of TGF-β from tumor suppressor to tumor development trigger[47]. Studies showing that low Smad4 expression correlates with greater potential for invasion, metastasis formation, treatment resistance, a poor response to 5-fluorouracil-based chemotherapy, and a poor prognosis in colorectal cancer patients. The mentioned potential is a characteristic feature of metastatic tumors [48]. The loss of Smad4 results in a poor prognosis, and Smad4 expression is positively correlated with survival and metastasis colon cancer. Colorectal cancer patients with normal Smad4 expression has benefit to chemotherapy based on the drug 5-fluorouracil than those with Smad4 deletion. According to previous research, Smad4 depletion may indicate a poor prognosis for 5-fluorouracil therapy in colorectal cancer patients.

TGF-β as an upstream, plays a role in regulating the activation of EMT regulatory transcription factors. Epithelial cell transition to mesenchyme (EMT) is triggered by transcription factors that regulate intercellular adhesion, cell polarity, and motility. The acquisition of the EMT phenotype results in cells with reduced adhesive capacity that are highly migratory and invasive due to increased secretion of extracellular proteases. Therefore, EMT is the basis for the development of metastases that result in primary tumor cells being able to migrate. The acquisition of mesenchymal properties through EMT causes cancer cells to escape from the extracellular matrix and enter the blood vessels (extravasation) to become circulating tumor cells, then form micrometastases in distant sites[49]. When cancer cells disseminate and settle, cancer cells are thought to acquire epithelial properties, which are a reversible process of EMT called MET. It is suspected that cancer cells gain the ability to invade other tissues through EMT and gain the ability to survive in new places with MET. EMT mobilizes cells in the primary tumor, but MET terminates the migration process resulting in cancer cell colonization. Studies have shown that partial EMT is involved in tumor invasion, and partial MET is involved in lymph node

metastases and liver metastases [12]. EMT is a reversible process that often occurs in metastatic cancer.

The EMT process is related to the malignant nature of colorectal cancer, namely tumor budding, circulating tumor cells, and resistance to treatment [50]. When EMT occurs, colorectal cancer cells become more aggressive and resistant to chemotherapy treatments such as oxaliplatin and 5-FU [51]. Colorectal cancer metastases show the severity of the disease, which is difficult to treat with surgery, radiotherapy, and chemotherapy methods. Screening in peopleat high risk of colorectal cancer is highly recommended. The goal of screening is to find CRC at an early stage, making it more likely to be treated because it can find and remove colorectal polyps before they develop into cancer [52]. Therefore, in reducing colorectal cancer incidence and mortality, further exploration of potential biomarkers is urgently needed to strengthen therapeutic efficacy [53]. Although further analysis is needed to understand the initiation and developmental process of metastases in colorectal cancer, this study demonstrated the expression of candidate metastatic biomarkers in colorectal cancer, such as the EMT regulatory gene in the TGF-β/Smad pathway. However, this study has limitations regarding the number of samples used, and the method used only measures expression at theRNA level.

4 Summary

Increased expression of TGF-β1 and Smad2 genes was found in the metastatic CRC group. This is thought to be due to the pro-metastatic role of TGFB via the Smad2 pathway in increasing EMT activity and malignancy potential in metastatic CRC patients. Decreased expression of the Smad4 gene was also found in the metastatic CRC group. The decrease in Smad4 expression is thought to be caused by the inactive function of Smad4 expression, which leads to cancer malignancy and is often found in advanced colorectal cancer. Based on the results, further research is needed to determine the correlation and regulation of these genes in colorectal cancer metastatic organs in driving the incidence of subsequent metastases and their implications for cancer severity.

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