RESEARCH ARTICLE

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Differences in Expression of Epithelial-Mesenchymal Transition (EMT) Induction Genes *TGF-B* Pathway Transition in Colorectal Cancer Patients Non-Metastatic and Metastatic

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Abstract

Objective: This research focuses on molecular screening of mRNA by targeting EMT regulator genes in the TGF-β/SMAD pathway to determine the difference in EMT mechanisms between non-metastatic and metastatic primary tumor cells. **Methods:** The method uses Real time/quantitative Polymerase Chain Reaction (RT-qPCR) to measure the expression levels of target genes in colon tissue samples from non-metastatic and metastatic patient groups. Differences in target gene expression between the two groups were analyzed using t-tests. **Results:** The results of this study show significance differences in the expression of EMT-inducing genes on the *TGF-β/Smad* pathway between non-metastatic colorectal cancer groups and metastases. *TGF-β1* (p-value : 0.041), *Smad2* (p-value : 0.020), *Snail1* (p-value : 0.028), *Twist1* (p-value : 0.036), and *ZEB1* (p-value : 0.045) gene expression was higher in the metastatic tumor group. In contrast to these genes, the expression of the *Smad4* (p-value : 0.022), *E-cadherin* (p-value : 0.036), and *vimentin* (p-value : 0.048) genes was lower in the metastatic tumor group. **Conclusion:** The observed alterations in gene expression related to EMT within the *TGF-β/Smad* pathway in metastatic colorectal cancer are likely associated with the partial processes of EMT and MET. These alterations may contribute to further metastatic potential and increase the malignancy of the cancer.

Keywords: Colorectal Cancer- Epithelial Mesenchymal Transition- Metastasis- TGF-β/SMAD- tumor

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Introduction

Colorectal cancer is one of the malignant cancers that ranks 4th highest incidence and fifth for highest mortality based on late WHO's Global Burden Cancer data in 2022 [1]. CRC occurs as a result of disorders in the colon or rectum and is caused by abnormal proliferation of glandular epithelial cells in the colon or rectum [2]. Colorectal cancer is generally asymptomatic and symptomatic, such as bleeding, anemia, and abdominal pain appearing when the patient is already in an advanced stage. At this stage, the cancer has been aggressive, malignant, and metastasizes [3]. The global prevalence of colorectal cancer has been reported to have increased in recent years. Increased incidence of colorectal cancer associated with increased exposure to risk factors resulting from lifestyle and dietary shifts toward Westernization [4].

The leading cause of death in colorectal cancer patients is metastasized. The most common colorectal cancer metastasis site is the liver, which is present in 70% of patients. Compared to the lungs, lymph nodes, and peritoneal [5]. Another study showed that 60% of colorectal cancer patients staged IV develop liver metastases; the liver is the most important place common for the spread of colorectal cancer metastases [6]. Reported only 20% of patients have metastatic colorectal cancer that persists more than 5 years after diagnosis [7]. The presence of metastases increases the aggressiveness of tumor cells and decreases the survival rate and prognosis of patients [8].

The main contributor to the development of CRC is the tumor microenvironment including fibroblasts, non-mutant cells, and the extracellular matrix (ECM) [9]. One of the characteristics of the tumor is extracellular matrix deposition and remodeling; this triggers fibrosis, stiffening the stroma and promoting tumor severity. However, chronic fibrosis is a risk factor for cancer, and tumors are defined as "fibrotic wounds that do not

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heal."[10]. One of the main factors in the pathophysiology of fibrosis is epithelial-mesenchymal transition (EMT), a process that converts epithelial cells into mesenchymal cells over time [11]. Significantly, it has been determined that pathogenic EMT production largely depends on the chronic inflammatory microenvironment. Research also shows that excessive *TGF-β* expression leads to EMT, ECM deposition, and formation of cancer-associated fibroblasts, which can trigger fibrosis and cancer. *TGF-β* and its downstream molecules play an important role in the progression of fibrosis and cancer; therefore, targeting TGFB signaling as therapeutic is a promising strategy [12].

Studies show overexpression of the *TGF-β* gene is associated with the formation of neoplastic stem cells in the tumor stroma, decreased immune response, and triggers EMT that supports the formation of metastases [13]. Interference in the *TGF-β*/Smad signaling path is one of the factors associated with the development of colorectal cancer [14]. In the canonical signaling path, *TGF-β* induces Smad binding to promoters of various transcription factors of EMT regulators such as *Snail*, Slug, *Twist1*, and *ZEB1* [15]. Regulatory transcription factor EMT causes decreased expression of epithelial markers, namely *E-cadherin* and B catenin, and increased mesenchymal markers, such as N-cadherin and vimentin [16]. Studies show that the expression of genes that redundancy of various transcription factors of EMT regulators related to invasion, metastasis, and poor prognosis in colorectal cancer patients [17].

Metastasis in colorectal cancer is the toughest challenge for the success of treatment. The need to find biomarker candidates for metastasis and prognosis of colorectal cancer is increasingly important [18]. Measurement of EMT markers in primary tumors with identifying patients who have the potential to have metastases may improve 4 risk stratification and appropriate treatment selection [19]. Profile Expression of metastatic genes in the early stages of colorectal cancer is also indispensable to prevent the development of colorectal cancer and increase the rate of patient survival [20].

Materials and Methods

Ethics and Sample Preparation

Ethics are obtained from the Medical and Health Research Committee, Faculty of Medicine, Gadjah Mada University, RSUP dr. Sardjito Yogyakarta. The Ethics code numbers are KE/FK/0938/EC/2021. The patient was diagnosed with colorectal cancer based on the results of clinical examination and CT scan conducted by a team of doctors at RSUP dr Sardjito Yogyakarta Hospital. Colon samples and baseline characteristic patient data were obtained from a team of doctors at RSUP Dr. Sardjito. This study used as many as 10 colon tissue samples from metastatic colorectal cancer patients and 18 colon tissue samples from non-metastatic colorectal cancer patients.

RNA Extraction

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

Reverse Transcription quantitative PCR (RTqPCR)

The cDNA (Reverse Transcription) synthesis procedure is carried out based on the ReverTrace qPCR-RT Master Mix (TOYOBO) kit manufacturing protocol as follows using the RNA that has been obtained: RNA template 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 88 µL: 1.8 µL, prepared DNAse I cocktail (4X DN Master Mix = 2 µL, Template RNA = 2 µL, Nuclease Free water = 4 µL), incubated DNAse I cocktail at 37°C for 5 minutes, prepared cocktail reverse transcription (cocktail DNAse I = 8 µL, 5X RT Master Mix = 2 µL, inserted the cocktail into the GeneAmp® PCR System 9700 (Thermo Scientific) machine with an incubation program at 37°C for 5 minutes, stored cDNA results at -4°C. The Real-time PCR procedure is based on the SYBR Green Real-time PCR Reagents (Bioline) kit manufacturing protocol using the qtower3 G (Analytik Jena) qPCR tool. The procedure is performed as follows: Prepared mixture of 2x Syber Green (5 µL), RNA template (1 µL), forward gene primer (0.8 µL), reverse gene primer (0.8 µL), RNAse free water (2.4 µL) in PCR White strip tube, then programmed and run qPCR (Analytic Jena qtower3) with a cycle (Pre-denaturation = 2 minutes at 95°C, Denaturation = 5 seconds at 95°C, Annealing/extension = 30 seconds at 60°C) (Table 1).

Data Analysis

The relative expression of the target gene is obtained based on the calculation of 2ΔCt with ΔCt in the form of the difference in the Ct value of the target gene and housekeeping gene in relative quantification. Differences in target gene expression between the two groups were analyzed using t-tests. Significance is indicated by $p<0.05$. The data obtained is then visualized with graphs using GraphPad Prism software and then analyzed descriptively.

Results

In this study, there were two groups, namely non-metastatic and metastatic tumor groups. This study utilized 10 colon tissue samples from colorectal cancer patients with metastasis and 18 samples from patients without metastasis. We acknowledge that the limited

Table 1. List of Primer Sequences Used

Genes	Sequences		References
	Forward (5'-3')	Reverse (5'-3')	
β-actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	21
TGF-β1	AAGTGGACATCAACGGGTTC	GTCCTTGCGGAAGTCAATGT	21
Smad2	TCATAGCTTGGATTTACAGCCAG	TTCTACCGTGGCATTTCGGTT	22
Smad4	AAGGCCTAGCACCACCTTAG	AGCCTTAAACTCTGACCTGT	23
Snail	ACTGCAACAAGGAATACCTCAG	GCACTGGTACTTCTTGACATCTG	24
Twist1	GTCCGCAGTCTTACGAGGAG	GCT TGA GGG TCT GAATCTTGCT	25
ZEB1	TTACACCTTTGCATACAGAACCC	TTTACGATTACACCCAGACTGC	26
E-cadherin	AAAGGCCCATTTCCTAAAAACCT	TGCGTTCTCTATCCAGAGGCT	21
Vimentin	AGTCCACTGAGTACCGGAGAC	CATTTCACGCATCTGGCGTTC	27

sample size represents a constraint of the study and may influence the robustness and generalizability of the findings. Although the sample size is limited, this study represents an important step toward understanding the underlying processes contributing to cancer severity in patients.

Patients in the non-metastatic tumor group are in stages I-III C, along with tumors that have not spread elsewhere (metastases). In the group of metastatic tumors, patients are entirely at stage IVB, which indicates that they have developed metastases. All patients in this group had the development of metastases in the liver. This study measured the expression of the target gene and also recorded baseline data such as age in the two research groups. In this study, there were 13 out of 18 patients in the non-metastatic tumor group aged between 50-70 years. Meanwhile, in that age range, there were 8 out of 10 patients in the metastatic tumor group. This aligns with the fact that older age is a major risk factor for CRC incidence. Individuals over 50 years old are generally at high risk, and 90% of colorectal cancer cases consist of individuals in that age group. Advanced age and aging are closely related to the risk of cancer. Aging causes a gradual loss of function or degeneration at the molecular, cellular, tissue, and bodily levels. One characteristic of aging is hyperplasia, which can develop into cancer.

Target gene expression in this study was obtained through a real-time PCR method using β-actin as a housekeeping gene. The *TGF-β1* target gene is upstream of the fibrogenesis and Epithelial-Mesenchymal Transition (EMT) regulatory pathway, while the *Smad2*, *Smad4*, *Snail*, *Twist1*, *ZEB1*, Vimentin, and *E-cadherin* genes are downstream of the *TGF-β1* pathway. *TGF-β* is widely recognized as the main mediator of fibrogenesis [28]. Based on the study's results, the expression of *TGF-β1*, *Smad2*, *Snail*, *Twist1*, and *ZEB1* genes in the metastatic tumor group was higher than in non-metastatic tumors (Figure 1). The expression of *Smad4*, *E-cadherin*, and

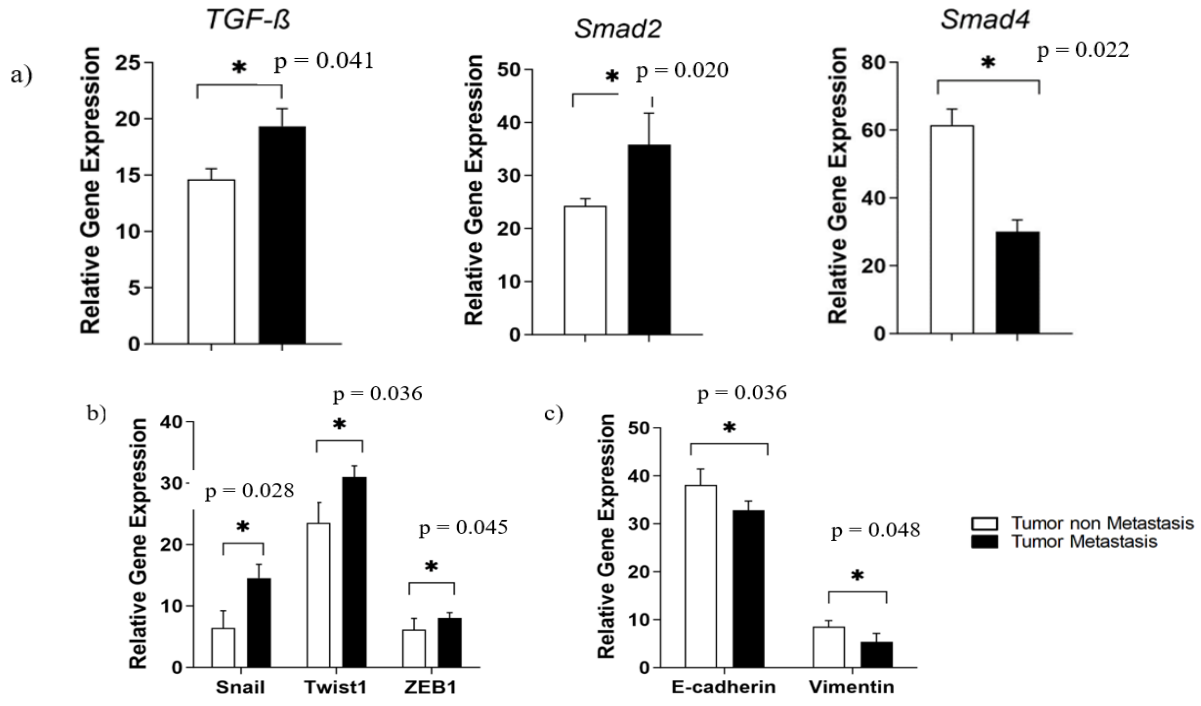

Figure 1. Expression of Genes in the Fibrogenesis Related Pathway (*TGF-β/SMAD*) in non-metastatic and metastatic tumor groups; a) EMT Regulator Upstream Genes; b) EMT Transcription Factor genes; c) Cell Adhesion Regulator Genes. *Significant result with $p \leq 0.05$.

vimentin genes in the metastatic tumor group was lower than in the non-metastatic tumor group (Figure 1).

Discussion

This study showed an increase in the expression of *TGF-β1*, *Smad2*, *Snail*, *Twist1*, and *ZEB1* genes in the metastatic colorectal cancer group compared to non-metastatic (Figure 1). *TGF-β* is upstream of Epithelial-Mesenchymal Transition (EMT) transcription regulation through the *TGF-β*/Smad (Smad dependent pathway) pathway. Increased expression of *TGF-β1* and its signaling through Smad indicates the activation of various processes that result in cancer metastasis, one of which is the EMT process. *TGF-β1* affects colorectal cancer metastases through normal colon transition to cancer, triggering EMT, formation of pre-metastatic niches, formation of fibrotic environment, suppression of immune function, angiogenesis, and tumor cell adaptation [29]. The increase in *TGF-β1* expression is also influenced by pro-oncogenic signaling of *TGF-β1* that does not go through Smad (Smad independent pathway). *TGF-β1* expression was reported to be significantly increased in metastatic tumor tissue of colorectal cancer [30].

In response to *TGF-β*, *Smad2*, and Smad3 became active and interacted with *Smad4*. The *Smad2*/Smad3/*Smad4* complex then accumulates in the nucleus to regulate the target gene of *TGF-β*, namely the EMT regulator gene [31]. Increased expression of *TGF-β1* as upstream triggers activation and increased expression of transcription factors of EMT regulators (*Snail*, *ZEB1*, *Twist1*). These three transcription factors are downstream targets of *TGF-β1* canonical signaling in the nucleus through *Smad2*, Smad3, and *Smad4*. Smad is an intracellular downstream effector of *TGF-β1*. The downstream factor of *TGF-β*, namely *Smad2*/Smad3, is an important mediator of *TGF-β* signaling in fibrosis and tumorigenesis [12]. Increased expression of *TGF-β1* in the metastatic tumor group affected the increased expression of *Smad2* as a downstream mediator of *TGF-β*. The main function of Smad is to control gene regulation and signal transduction that regulates Notch, ERK/MAPK, Hippo, JAK/STAT, and *TGF-β*/Smad signaling [32]. This study showed significantly increased expression of *Smad2* in the metastatic tumor group. The increase in *Smad2* expression was triggered by increased expression of *TGF-β1* upstream in the metastatic tumor group. Increased expression of *TGF-β1* via *Smad2* in late-stage tumors activates the tumorigenesis process by triggering the activation of the epithelial-to-mesenchymal transition (EMT). The epithelial-to-mesenchymal transition causes cells to have a phenotype capable of migrating and invading, thus supporting the process of metastasis in colorectal cancer [17]. Other studies have shown that increased expression of *Smad2* in colorectal cancer patients correlates with a higher tumor stage [33]. In contrast to the results of this study, other studies showed that low expression of *Smad2* correlated with clinical malignancies and affected immune regulation in tumor microenvironment [34].

In contrast to *Smad2*, the expression of *Smad4* in this study decreased in the metastatic tumor group. *Smad4* is an important downstream regulator in *TGF-β* signaling. The *TGF-β*-activated *Smad4* complex moves into the nucleus and regulates the transcription of genes associated with the downstream target of *TGF-β*. *Smad4* has a central role in *TGF-β* signaling by influencing tumorigenesis on various mechanisms such as EMT, apoptosis, immune regulation, induction of cell cycle stopping, and so on [35]. Mutation, inactive function, and loss of *Smad4* expression are commonly found in advanced colorectal cancer progression. The decrease in *Smad4* expression in the metastatic tumor group is due to a change in *TGF-β* signaling from tumor suppressor to tumor progression trigger [36]. *Smad4* is a tumor suppressor gene involved in *TGF-β* signaling. The decrease in *Smad4* expression in the metastatic tumor group is thought to be related to the role of Carcinoembryonic antigen (CEA). The results of this study showed that the serum level of CEA in the metastatic tumor group far exceeded the normal limit (>250 μg/L). CEA is known to play a role in suppressing downstream tumor suppressor signaling in *TGF-β*, namely *Smad4*, thereby triggering colorectal cancer metastasis [37].

Based on the results of this study, the increase in the expression of the three transcription factors (*Snail*, Twist, *ZEB1*) led to a decrease in the expression of the epithelial marker *E-cadherin* in the metastatic tumor group compared to non-metastatic tumors (Figure 1b). Increased expression of EMT regulator transcription factors (*Snail*, Twist, Zeb) is reported to trigger tumor invasion in cell lines and mouse xenograft models and is associated with a poor clinical prognosis in cancer [38]. The three groups of transcription factors, namely *Snail*, Twist, and *ZEB1*, are reported to play a role in activating EMT by directly or indirectly suppressing E-Cadherin expression and increasing mesenchymal marker expression [39]. In contrast to the characteristic of EMT, which is characterized by increased expression of mesenchymal markers, the results of this study show that the expression of *E-cadherin* in both research groups is much higher than the expression of mesenchymal markers, namely vimentin (1c). The results of this study also showed a decrease in the expression of *E-cadherin* and Vimentin in the metastatic tumor group compared to the non-metastatic tumor group. Vimentin in the metastatic tumor group compared to the non-metastatic tumor group. The increased expression of EMT regulatory transcription factors in metastatic tumors of this study did not trigger an increase in Vimentin expression. These results contradict EMT characterized by increased transcription factors (*Snail*, Twist, Zeb) that increase the expression of mesenchymal markers such as vimentin. The results of this study also showed that the expression of *E-cadherin* was higher than vimentin in both research groups. High expression of *E-cadherin* can be caused by *Snail* acetylation, which changes the function of the *Snail* which initially suppresses the transcription of junctional genes (E-Cadherin) into *E-cadherin* activators [40].

Increased expression of EMT regulator transcription factors (*Snail*, Twist, Zeb), high expression of epithelial markers, and low vimentin expression found in metastatic tumor groups are suspected to indicate a partial state or

Hybrid EMT/MET. The contradiction of the results of various studies has shown the limitations of applying the EMT theory to cancer metastasis. EMTs are reportedly involved in forming metastases, while METs contribute to metastases of cancer cells that have been disseminated. Cells that undergo partial EMT/MET have epithelial phenotypes (have adhesions between cells) and mesenchymal (capable of migration), thus causing cells to migrate collectively. Cancer cells are reported to undergo MET (opposite EMT) to form secondary tumors or macrometastases. The EMT/MET process causes tumor cells that are 90% epithelial characteristics to be able to disseminate and colonize in metastatic target organs [41].

EMT/MET is an intermediate state when the cell simultaneously expresses the spectrum of epithelial and mesenchymal markers. EMT and MET programs have been involved in balancing invasive and proliferative states and also in the acquisition of stem cell properties in cancer [42]. Cells that underwent partial EMT/MET had the highest plasticity and could evenly produce epithelial and mesenchymal subpopulations. Other results showed that cells that underwent hybrid EMT/MET showed metastatic potential that significantly exceeded the metastatic tendency of complete EMT or MET [43]. The presence of partial EMT/MET indicates that tumor cells may not lose *E-cadherin* expression completely. Although EMT plays a role in cancer development, most metastatic carcinomas have well-differentiated epithelial characteristics. Identifying cells that have undergone EMT in carcinoma tissue in vivo is difficult. Various recent studies have shown that cancer cells in primary tumors, cell lines, and circulating tumor cells have supported the concept of partial EMT [41, 44].

Although the study provides valuable insights into gene expression patterns in cancer patients, the small sample size restricts broader interpretation. Future studies with larger cohorts are needed to validate these findings and explore additional gene expression differences. The limitation of this study is its focus solely on measuring the expression levels of genes previously identified as being associated with metastatic progression. While this provides valuable insights into potential molecular drivers of metastasis, the study does not include functional analyses to confirm the biological relevance of these gene expression changes. Specifically, key characteristics of malignancy such as cell migration and invasion were not assessed using in vitro functional assays, such as migration and invasion assays. Although gene expression analysis offers insight into transcriptional activity, it is not sufficient on its own to evaluate protein function, pathway dynamics, cellular heterogeneity, or behavioral changes over time. As such, a comprehensive understanding of cancer cell behavior requires the integration of proteomic, single-cell, spatial, and functional analyses alongside gene expression data [45]. This study, however, is limited to gene expression analysis at the mRNA level. As a result, the actual contribution of the identified genes to the metastatic potential of cancer cells remains speculative within the scope of this study. Future research incorporating these assays would be necessary to establish a more direct correlation between gene expression and metastatic behavior. Gene expression analysis provides an overview of the transcriptional process, which does not directly reflect the level of protein expression, whereas this expression plays a role in cellular function. It is known that changes in mRNA levels do not always translate directly into changes in protein levels, due to various factors such as mRNA stability, translation efficiency, and post-translational modifications. Therefore, here is the limitation of our research which only measures the level of gene expression at the mRNA level, not reaching the protein level.

In conclusion, the results of this study show differences in the expression of EMT-inducing genes on the *TGF-β*/Smad pathway between non-metastatic colorectal cancer groups and metastases that are allegedly involved in supporting metastases by triggering aggressive phenotypes in cancer cells. *TGF-β1*, *Smad2*, *Snail*, Twist, and *ZEB1* gene expression were higher in the metastatic tumor group. In contrast to these genes, the expression of the *Smad4*, *E-cadherin*, and vimentin genes was lower in the metastatic tumor group. The increased expression of EMT regulator transcription factors (*Snail*, Twist, Zeb) and the higher expression of *E-cadherin* markers compared to vimentin expression in the metastatic tumor group showed a contradiction with EMT characterized by an increase in vimentin as a mesenchymal marker. Based on the results of this study, the increase and decrease in gene expression involved in EMT on the *TGF-β*/Smad pathway in metastatic colorectal cancer is allegedly related to the partial process of EMT/MET, which increases the potential of further metastases and malignancy of cancer.

Author Contribution Statement

AA, HS, SRL, MS, AYH, AL conceptualised and designed the study. AYH and AL conducted the sample collection. AA, HS, SRL, and MS analyzed the data and wrote the manuscript.

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Ethics approval

This research has received approval from the ethical committee of the Faculty of Medicine, Gadjah Mada University, Dr. Sardjito General Hospital in Yogyakarta, with the number: KE/FK/0938/EC/2021.

Conflict of interest

The authors declare that there are no conflicts of interest related to the publication of this study.

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