Differences of alt-ast level and snail gene expression between patients with metastasis and non-metastasis colorectal cancer

Chrisna Budi Satriyo¹, Hendra Susanto², Adeodatus Yuda Handaya*³, Nurcahya Setiawan³, Imam Sofii³, Agus Barmawi³

¹Digestive Surgery Division Trainee RSUP Dr Sardjito Yogyakarta, Sleman, Indonesia
²Department of Biology, Faculty of Mathematics and Science, Universitas Negeri Malang, Malang, Indonesia
³Digestive Surgery Division Lecturer RSUP Dr Sardjito Yogyakarta, Sleman, Indonesia

Original Article

ABSTRACT

Background: Colorectal cancer (CRC) is a malignant epithelial tumour in the colon and rectum, and its main cause is distant metastasis. The main process of distant metastasis is the epithelial-mesenchymal transition (EMT) that may cause mesenchymal phenotype and malignant capabilities of tumour cells. Then it is regulated by one of transcription factors, the Snail.

Objective: This study aims to determine differences between Snail gene expression in CRC that has not metastasized and Snail gene expression in CRC that has metastasized.

Methods: The subjects of this study consisted of two groups of patients. The first group consisted of 20 patients with CRC (tumour), and the second group consisted of 12 patients with CRC with metastasis (metastasis tumour). The Snail gene expression was measured in both groups by using the RT-qPCR method and supported by laboratory examination to measure ALT and AST. Then all obtained data were analysed by using a statistical software analysis.

Results: This study indicated that there were differences of the Snail gene expression between both the groups, and the highest expression was in the metastatic tumour group with a significance level of 0.002 (p<0.05). This was also supported by AST and ALT which were significantly correlated between both the groups. The correlation value on the AST was 0.506 with a significance level of 0.003, and the ALT correlation value was 0.532 with a significance level of 0.002.

Conclusion: This study suggested that the Snail was a potential marker of metastasis in the CRC. Therefore, further research is needed to determine the role of Snail regulation in the CRC metastasis.


Tujuan: Penelitian bertujuan untuk mengetahui perbedaan ekspresi gen Snail pada kanker kolorektal yang belum bermetastasis dengan kanker kolorektal yang telah bermetastasis.

Metode: Subyek penelitian ini adalah pasien kanker kolorektal dengan dan tanpa metastasis, yang telah diperiksa dalam menegakkan diagnosis di RS Dr Sardjito terbagi menjadi 2 kelompok. Kelompok pertama...
terdiri 20 pasien kanker kolorektal tanpa metastasis (kelompok tumor) dan kelompok kedua terdiri 12 pasien kanker kolorektal dengan metastasis (kelompok tumor metastasis). Ekspresi gen Snail diukur menggunakan metode RT-qPCR dan didukung pemeriksaan laboratorium mengukur kadar ALT dan AST. Data dianalisis menggunakan analisis Software Statistik.

Hasil: Penelitian ini menunjukkan perbedaan ekspresi gen Snail pada kedua kelompok dengan ekspresi tertinggi didapatkan pada kelompok tumor metastasis, dengan taraf signifikansi 0,002 (p<0,05). Ini juga didukung oleh AST dan ALT yang berkorelasi signifikan diantara kedua kelompok. Nilai korelasi pada AST sebesar 0,506 dengan taraf signifikansi 0,003 sedangkan nilai korelasi pada ALT sebesar 0,532 dengan taraf signifikansi 0,002.

Kesimpulan: Snail merupakan marker potensial pada metastasis kanker kolorektal. Oleh karena itu, diperlukan penelitian lebih lanjut untuk mengetahui peran regulasi Snail dalam metastasis kanker kolorektal.

INTRODUCTION

Colorectal cancer (CRC) is a malignant epithelial tumour in the colon and rectum. Its occurrence varies widely throughout the world, but it is more often found in developed countries with a westernized lifestyle. Obesity, a sedentary lifestyle, consumption of red meat, alcohol and cigarettes are factors that trigger the growth of CRC cases. According to GLOBOCAN data in 2020, CRC is the third most common cancer in men (10.6%) and the second in women (9.4%) based on worldwide cases. CRC is in the second rank as the most common cause of death caused by cancer. The CRC continues to be a leading cause of cancer associated with significant morbidity and mortality in the United States (US) and worldwide.

The causes and pathogenesis of CRC have a relationship with genetic and environmental factors. The mechanism of CRC has various stages of carcinogenesis. Adenocarcinoma which is the most common type of CRC originates from sequential changes involving mutations, oncogenes and loss of tumour suppressor genes. These mutations involve KRAS and the inactivation of the p53 tumour suppressor gene that leads to the pathogenesis of adenomas to adenocarcinomas. In addition, signalling pathways such as RAS-RAF-MEK-MAPK are interrelated in regulating the transcription of genes that regulate cancer cell proliferation, resistance, migration and angiogenesis that trigger CRC progression and promote metastasis.

Metastasis in CRC and other types of cancer is generally initiated by molecular regulation of the transition from normal cells to invasive cells. In general, the main process of distant metastasis is the epithelial-mesenchymal transition or the so-called epithelial mesenchymal transition (EMT). The EMT is basically a process during embryogenesis. As cancer progresses, the phenotypic shift from epithelial to mesenchymal is the result of modification of intercellular adhesion molecules so that cells can migrate and invade distant tissues. The EMT mechanism is limited to changes not only in cell phenotype but also in cell markers. In addition to involving the tumor microenvironment, growth factors, kinases, transcription factors, and miRNAs, the loss of epithelial cell markers such as E-cadherin is replaced by mesenchymal markers such as vimentin and alpha smooth muscle actin (-SMA). At the transcriptional level, EMT is induced by transcription factors including the Snail protein, Slug, zing finger (Zeb1/2) and the bHLH protein family (Twist1/2), which is bound to the downstream promoter region of the target gene; in addition, the EMT transcription factor can also decrease the expression of the gene encoding the epithelial cell marker (E-Cadherin) and increase the expression of the gene encoding the mesenchymal cell marker (vimentin).

Snails are activators at the transcriptional stage by interacting with gene coactivator complexes. Snail stability and activity are determined by posttranslational modifications and depend on extracellular signals and the tumour microenvironment. The Snail plays an important role in the development of
malignancies in humans, namely malignancies originating from the gastrointestinal tract, one of which is CRC. Snail as a transcription factor also plays an important role in cell movement during cancer development and metastasis.

Liver Function Tests (LFTs) are an initial screening for liver disease that use blood for analysis. Several types of LFTs are Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST). In this study, laboratory parameter such as AST and ALT were included as an early indicator of liver damage to diagnose organ and physical health of a patient. The physiological roles of AST and ALT are diverse and has long been identified as a biomarker of liver injury. AST and ALT levels that exceeds normal limits is a sign of impaired liver function.

Based on the discussion above, the authors assume that if there is CRC that has developed into distant metastasis, it will increase the Snail expression and activate the epithelial-mesenchymal transition process. Therefore, this study aims to investigate differences between Snail gene expression in CRC that has not metastasized and Snail gene expression in CRC that has metastasized. These genes are chosen with consideration of their important roles in the process of epithelial-mesenchymal transition and metastasis.

METHODS

Research Sample

This study was an analytic observation study with a quantitative approach and using human sample (respondents). Its samples were obtained from clinical data collection from September 2021 to February 2022, and all testing protocols were conducted with established procedures. The inclusion criteria of this study were CRC patients with and without metastasis who had gone through the stages of making a diagnosis at Dr Sardjito General Hospital Yogyakarta, had become candidates for surgery, and also had complete medical records. The exclusion criteria of this study were patients who refused to participate in this study. There were 32 patients diagnosed with CRC who were subjects of this study, consisting of 20 patients with CRC without metastasis which the authors referred to as the tumour group, and 12 CRC patients with metastasis whom the authors called the metastatic tumour group. In the CRC group with metastasis, all metastasis to the liver were found. Samples from both groups were obtained by cutting surgical specimens from the primary tumour which were then inserted into Eppendorf tubes. The ethical clearance of this study was registered with the Ethics Committee and was approved/permited by the institutional review board of Gajah Mada University and Dr. Sardjito General Hospital, Yogyakarta-Indonesia with the number KE/FK/0938/EC/2021. In addition, all included patients were asked to fill out a written informed consent.

Measurement of baseline characteristic parameters

All patient parameters/baseline characteristics were measured by standard clinical methods according to the basic anthropometric examination protocol. Blood pressure was measured by using a sphygmomanometer based on a standard protocol. Each patient sat in a chair and in a quiet environment for 5 minutes and was not allowed to consume caffeine, smoke and do physical activity for 30 minutes before taking blood pressure measurements, and during the procedure both the patient and staff did not speak. Ausculation during the measurement procedure was 2-3 cm above the elbow crease, while the squeezable bulb was aligned with the brachial artery. Then the patients’ BMI classification were divided into several categories, namely underweight (<18.5 kg/m²), normal (18.5-24.9 kg/m²), overweight (25-29.9 kg/m²), and obesity (≥30 kg/m²).

Laboratory Measurement

The measurement of laboratory tests applied the following methods, namely a spectrophotometer method for haemoglobin’s
calculation method haematocrit, a flow cytometry method for WBC and RBC, and a hexokinase method for glucose levels, a colorimetric assay method for albumin, an urease-GLDH method for BUN, a Jaffe method for Creatinine, an impedance method for PLT, an enzymatic colorimetric method for ALT and AST, and an ECLIA method for CEA.

Gene Expression Measurement

The quantitative gene expression test was performed by isolating total RNA from patient tumour samples and tested by Real Time quantitative PCR (RT-qPCR). The sample was a fresh tumour containing tumour tissue with a tumour cell count of more than 70%. The tumour was diagnosed as Colorectal Adenocarcinoma at Dr. Sardjito General Hospital Yogyakarta. 100 mg of fresh tumour samples were weighed and then were processed into the total RNA isolation.

Total RNA Isolation

The procedure for the total RNA isolation from fresh tumour samples was referred to the manual method in the QIAzol Lysis Reagent (Qiagen) kit. Small pieces weighing 100 mg of tumour tissue were homogenized in 500 L of Trizol solution by using a sonicator. The samples were then centrifuged at 12,000 x g for 10 min at 4°C. The aqueous phase was transferred slowly to a new Eppendorf tube, precipitated with 200 L of chloroform and then homogenized with a pipette. In the next step, all samples were incubated on ice for 10 min and centrifuged again at 12,000 x g for 10 min at 4°C. Colourless supernatant as much as ± 200 L was transferred to a tube containing 600 L isopropanol and then homogenized with a pipette and left at room temperature for 10 minutes. Next, it was centrifuged at 12,000 x g for 10 minutes, and the supernatant was then discarded, and 200 L of 70% ethanol was added without mixing. Subsequently, it was centrifuged at 7500 x g for 5 minutes, and the supernatant was discarded. The pellet was then air-dried for ± one hour in a sterile area and dissolved with 50 L of RNase Free Water. The RNA isolation results were then stored for RT-qPCR at -20 °C.

Reverse Transcription and Real time Quantitative PCR (RT-qPCR)

Conversion from RNA to cDNA was performed by using a reverse transcription kit (ReverTra Ace® qPCR RT Master Mix with gDNA Remover, TOYOBO, Japan). The premix preparation was by mixing 1 µL gDNA Remover: 50 µL 4x DN Master Mix° mixture. For RNA templates/samples, 6 µL was placed in a rotor tube, incubated at 65°C for 5 min and placed on ice for some time. DNase I reaction solution was prepared on ice with a formula: 2 µL of 4x DN Master Mix and 6 µL RNA template. The next step was to incubate the solution at 37°C for 5 minutes. Furthermore, the reverse transcription solution was prepared on ice with a formula: 8 L DNase I reaction and 2 µL 5x RT Master Mix II. The transcription solution was then incubated at 37°C for 15 minutes, 50°C for 5 minutes, and 98°C for 5 minutes. In the final stage, the solution was then stored at 4°C or -20°C.

The mix solution was prepared according to the following formula. One reaction volume consisted of 10 µL 2x SensiFAST SYBR® No-ROX Mix, 1 µL 10 µM forward primer, 1 µL 10 µM reverse primer, 1 µL cDNA template, and 7 µL sterile distilled water. The primers for Snail gene sequences used were forward (5’ ACTGCAACAAGGAATACCTCAG 3’) and reverse (5’ GCACTGTTACCTTGACATCTG 3’), while the primers for β-actin (housekeeping gene) were forward (5’CATGTACGTTGCTACCGAGG3’) and reverse (5’ TCCCTAAATGTCACGGCAG3’). The Real-time quantitative PCR program was then set to 2-step cycling as follows: 1 cycle at 95°C for 2 minutes for polymerase activation, 40 cycles consisting of 95°C in 10 seconds for the denaturation step, and the primary Tm temperature at 30 seconds for the annealing/extension step.

Data analysis

All data related to the expression of Snail
from this study were analysed by a statistical software analysis [JMP 6 Software (Chicago, IL; North Carolina, USA)]. The normality test was performed by the Kolmogorov–Smirnov test. Baseline characteristic differences between groups were analysed by unpaired t-test. Bivariate correlation analysis was performed by using the Spearman correlation method. Snail gene expression differences between groups were analysed by t-test. The level of significance in this study was 95% or p<0.05 (statistically significant). The obtained data were visualized by using GraphPad Prism 5.

RESULTS
All obtained data were tested for their normality by using the Kolmogorov-Smirnov. The results of the test obtained a normal data distribution, and then c unpaired T test analysis was implemented to determine the difference in mean ± standard error of mean (SEM) between the two groups, namely Tumour Non-Metastasis group and Tumour with Metastasis group. The results are presented in Table 1 below.

The profile of Clinicopathological characteristics in this study was presented in the table above (Table 1). The Table 1 indicated different clinical characteristics and laboratory result data from the non-metastasis and liver metastasis of the CRC patients. The most significant difference was observed in their BMI, blood glucose level, CEA and liver fibrosis marker (ALT and AST). The highest ALT and AST scores were found in the metastasis group since the rate of cancer invading the liver increased to almost 4 times in ALT and 2 times in AST. The same finding was also observed on the profoundly high results of the CEA test from the metastasis group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tumour Non-Metastasis (n = 20)</th>
<th>Tumour with Metastasis (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>54.50 ± 3.12</td>
<td>57.08 ± 3.00</td>
</tr>
<tr>
<td>Body mass index (kg/m$^2$)</td>
<td>23.41 ± 1.19</td>
<td>18.92 ± 0.49*</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>123.15 ± 2.43</td>
<td>123.33 ± 4.33</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>78.10 ± 1.99</td>
<td>79.33 ± 2.33</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>11.38 ± 0.47</td>
<td>11.63 ± 0.47</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>34.91 ± 1.37</td>
<td>35.37 ± 1.24</td>
</tr>
<tr>
<td>White Blood Cells (10$^3$/μl)</td>
<td>8.77 ± 0.85</td>
<td>10.70 ± 1.36</td>
</tr>
<tr>
<td>Red blood cells (10$^6$/μl)</td>
<td>4.39 ± 0.12</td>
<td>4.22 ± 0.14</td>
</tr>
<tr>
<td>Fasting glucose level (mg/dL)</td>
<td>120.80 ± 10.28</td>
<td>142.33 ± 10.55*</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>3.63 ± 0.16</td>
<td>3.14 ± 0.16</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dL)</td>
<td>12.69 ± 1.50</td>
<td>16.78 ± 2.32</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.91 ± 0.71</td>
<td>0.97 ± 0.14</td>
</tr>
<tr>
<td>Platelet count (10$^3$/μl)</td>
<td>284.10 ± 21.33</td>
<td>370.58 ± 31.56</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>11.65 ± 1.74</td>
<td>28.25 ± 5.13*</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>21.35 ± 4.51</td>
<td>47.92 ± 10.71*</td>
</tr>
<tr>
<td>Carcinoembryonic antigen (µg/L)</td>
<td>14.36 ± 5.51</td>
<td>307.53 ± 127.30*</td>
</tr>
</tbody>
</table>

Independent Samples t-test was used to compare the differences among the groups. Data are presented as mean ± SEM.
*Significant value of each parameter compared to non-metastasis group by Unpaired t test (p≤0.05).
group, signifying the spread of cancer from the colon to the liver (systemic disease).

In the Table 2, from the available clinical and laboratory data, it was found that Snail gene expression significantly correlated with ALT and AST values in all participants from both groups as evidenced by the Spearman correlation test and Omnibus logistic regression test.

Table 2. Bivariate correlations with Snail in all participants

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Snail r</th>
<th>p-value (a)</th>
<th>p-value (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>0.139</td>
<td>0.447</td>
<td>0.108</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>-0.271</td>
<td>0.134</td>
<td>0.075</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>0.229</td>
<td>0.208</td>
<td>0.091</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>0.168</td>
<td>0.357</td>
<td>0.127</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>0.019</td>
<td>0.920</td>
<td>0.224</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>-0.039</td>
<td>0.833</td>
<td>0.201</td>
</tr>
<tr>
<td>White Blood Cells (10³/µl)</td>
<td>0.207</td>
<td>0.256</td>
<td>0.097</td>
</tr>
<tr>
<td>Red blood cells (10⁶/µl)</td>
<td>-0.222</td>
<td>0.221</td>
<td>0.111</td>
</tr>
<tr>
<td>Fasting glucose level (mg/dL)</td>
<td>0.332</td>
<td>0.063</td>
<td>0.051</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>-0.057</td>
<td>0.758</td>
<td>0.168</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dL)</td>
<td>0.127</td>
<td>0.487</td>
<td>0.076</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>-0.126</td>
<td>0.493</td>
<td>0.135</td>
</tr>
<tr>
<td>Platelet count (10⁵/µl)</td>
<td>-0.052</td>
<td>0.777</td>
<td>0.196</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>0.532</td>
<td>0.002*</td>
<td>0.021*</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>0.506</td>
<td>0.003*</td>
<td>0.013*</td>
</tr>
<tr>
<td>Carcinoembryonic antigen (µg/L)</td>
<td>0.079</td>
<td>0.688</td>
<td>0.099</td>
</tr>
</tbody>
</table>

*Significant with p≤0.05
r = Spearman correlation coefficient, p-value (a) = Spearman correlation test, p-value (b) = Omnibus test regression logistic.

Figure 1. Snail gene expression levels in CRC patients. Snail gene expression levels increase in patients with metastatic tumours, with p=0.002. *Significant with p ≤ 0.05 by t test.
The obtained Ct value was calculated to find the $2^{\Delta\Delta Ct}$, the relative gene expression of Snail (target gene) and β-actin as the housekeeping gene. The obtained data were visualized by using GraphPad Prism 5. In Figure 1, it was clear that the differences in each group are also statistically significant with p=0.002 (Significant with p≤0.05) by t test. The metastatic group had the highest expression level.

**DISCUSSION**

The results of the analysis showed significance in the tumour samples with metastasis in the parameters of Body Mass Index (BMI), Fasting Glucose Level, Alanine Aminotransferase (ALT), Aspartate Transferase (AST) and Carcinoembryonic Antigen (CEA) (Table 1). The average BMI of patients with metastatic tumour samples was classified as normal, namely 18.92±0.49. However, based on the results of the correlation test in (Table 2), it proved that the BMI parameter with p-value > 0.05 was 0.134 so that it could be said that the BMI did not correlate with the Snail target genes in all the patients. BMI as a risk factor of CRC was found relevant to the other studies. Adiposity processes can trigger metabolic and systemic changes that can lead to carcinogenesis mechanisms. Cytokines can be released from adipose tissue, and one of the cytokines associated with obesity is IL-6 (proinflammatory cytokine), and an increase in IL-6 expression is known to be involved in the stages of cancer development, from tumour initiation to metastasis. Obesity can also be associated with increased levels of leptin in the human body, and some studies found that leptin acts as a mitogenic factor in cell types.

The fasting glucose parameter of the non-metastatic sample was significantly different from that of the metastatic tumour samples (Table 1), with values of respectively 120.80 ± 10.28 and 142.33 ± 10.55. Fasting glucose levels are important in controlling glycaemic in individuals with diabetes. Based on the Atlas International Diabetes Federation (IDF), a person's standard rate is considered diabetes if fasting blood glucose is ≥ 7.0 mmol/L (126 mg/dL) with a fasting time of 8 hours; therefore, based on this reference value, patients with metastatic tumours is considered diabetes. The mechanism of cancer can be associated with comorbidities such as diabetes mellitus (DM), but the relationship between DM and CRC is still not widely found. High glucose can trigger cancer through microRNA modification, activate the mammalian target of rapamycin (mTOR) in excess which can promote the growth of tumour cells, and metastasize to other healthy tissues. Most of the tissues experiencing tumour malignancy have increased absorption of fludeoxyglucose which is related to the increased rate of glycolysis and glucose transport. The increased rate of glycolysis plays a role in cell proliferation through intermediate products in glycolysis, thereby triggering de novo biosynthetic metabolic pathways that produce nucleotides, lipids, amino acids and glycoconjugates. High blood glucose can trigger a variety of direct and indirect mechanisms that work together in the initiation of cancer cell proliferation, and high blood glucose in this study occurred in patients suffering from metastatic tumours which are in line with the findings that there are an increase in proliferation in 4 lineages of colorectal cancer cells, namely SW1116 in high-glucose treatment.

The results of this study showed that the ALT and AST parameters in the metastatic tumour group were significantly different from the
non-metastatic tumour group (Table 1), due to the development of CRC metastasis to the liver which caused liver damage. Distant metastasis are the most important independent risk factor for poor prognosis and the leading cause of death in CRC patients. CRC patients generally have liver metastasis because of their anatomy related to the portal circulation. More than 50% of CRC patients develop metastasis to the liver.24 Liver enzymes such as ALT and AST are generally elevated in patients with liver disease, indicating liver damage.25 Serum ALT and AST are released by damaged hepatocytes into the blood, resulting in an increase in serum levels. ALT and AST levels are known to be effective tools for detecting the liver disease. This is supported by studies showing that ALT and AST correlate with liver disease and hepatocellular carcinoma (HCC).26

The results of the analysis on the parameters of the carcinoembryonic antigen (CEA) in (Table 1) showed a significance in the non-metastatic tumour group and the metastatic tumour group with values of 14.36 ± 5.51 and 307.53 ± 127.30, respectively. CEA is a prognostic indicator of CRC.27 In most patients diagnosed with CRC, blood CEA increases and tends to decrease after surgery and treatment.28 A previous study found that patients with positive CEA showed significantly different T, N, M stages and tumour differentiation.29 Normal CEA levels are < 5.0 (µg/L).30 CEA levels in Table 1 in the non-metastatic tumour group and metastatic tumours far exceeded normal limits, but patients in the metastatic tumour group had significantly higher CEA levels than patients in the non-metastatic group, so in this case CEA levels were associated with tumour development and metastasis.

Baseline data on the CRC patients were tested for their correlation by using linear correlation statistical tests, their results are presented in Table 2. The results of the bivariate correlation test (p ≤ 0.05) of this study showed that ALT and AST were significantly correlated with the relative expression of the Snail gene (Table 2). This is related to the role of the Snail in promoting metastasis with a higher tendency in the CRC patients to have liver metastasis. The presence of tumours in the liver causes damage to liver cells so that there is an increase in levels of liver enzymes, namely ALT and AST. The Snail plays an important role in cell movement during tumour progression and metastasis by acting as a regulator or a transcription factor of EMT. The Snail is involved in the epithelial mesenchymal transition (EMT) mechanism during cancer progression, which is an important mechanism leading to invasion and metastasis of neoplasms at high stages.31 EMT is a reversible process involved in cancer cell invasion, migration, apoptosis, cell cycle and cancer cell resistance to therapy.32 During EMT, cells undergo tight junction dissolution, disruption of basal-apical polarity and cytoskeletal reorganization causing cells to develop an invasive phenotype.33 EMT is abnormally regulated by a variety of extracellular stimuli originating from the tumour microenvironment including growth factors, inflammatory cytokines and physical stress. EMT causes tumour cells to adapt to changes in the tumour microenvironment so that tumour cells can successfully metastasize. Through molecular changes, tumour cells activate EMT to remove epithelial properties and acquire mesenchymal properties to increase cell motility and to promote invasion and metastasis. These changes allow tumour cells to pass through the extracellular matrix and colonize in lymph vessels or blood vessels, thus starting the first step in the metastatic stage.34

This study showed that the relative expression of the Snail gene increased with the severity of CRC. The highest relative expression of the Snail gene was found in the metastatic tumour group with a mean value of 14,017.300 and in the tumour group with a mean value of 11,049.100; the differences in each group were also statistically significant with p=0.002 (p ≤ 0.05) by t test (Figure 1). This is in line with studies showing that increased
Snail expression was associated with a more aggressive phenotype, poor clinical outcome and more frequent distant metastasis in colorectal cancer.\textsuperscript{35} This is also in line with a study by Roy et al that demonstrated an increased Snail frequency in colon tumours with metastasis compared to colon tumours without metastasis. This is also supported by other studies in various types of cancer indicating that Snail gene expression was associated with invasion, secondary metastasis, and poor prognosis in patients.\textsuperscript{36} The increased expression of Snail is triggered by its role in the pathogenesis of CRC by triggering EMT which can enhance the invasive and metastatic properties of cancer cells.

Several signaling pathways, including TGF-β, Wnt, and Notch, work together to initiate EMT progression and activate Snail expression.\textsuperscript{37} A study demonstrated that Snail upregulation could be regulated by increased Wnt signalling. Crosstalk between Wnt and Snail signalling oscillates in a positive feedback loop as Snail overexpression increases the Wnt target gene expression.\textsuperscript{38} Snail is also known to be induced in response to TGF-β which induces EMT in cells via the Smad pathway.\textsuperscript{39} Snail triggers EMT by suppressing the expression of E-cadherin, an epithelial marker inducing the expression of various mesenchymal genes. In addition, Snail and Slug coordinate with various pathways required to activate the expression of mesenchymal genes such as vimentin, N-cadherin and fibronectin.\textsuperscript{40} Fan et al pointed out that increased Snail expression could induce EMT and Cancer Stem Cell (CSC) phenotypes that could increase the invasion and chemoresistance of cancer cells; in this case, Snail was thought to be a potential therapeutic target for CRC metastasis.

Based on the results of this study, there was an increase in the Snail expression along with the severity of the tumour, and the highest Snail expression was from the metastatic tumour group. This study implies that Snail is thought to be a potential marker of metastasis in CRC, but this study has limitations in terms of the samples obtained and the method that only measured the expression of the Snail gene at the mRNA level. Other methods such as Western Blot or ELISA may also be considered to measure the Snail gene expression in the future. Also, this study is a single centre study. Therefore, further multicentre research is needed to determine the role of Snail regulation in the CRC metastasis.

**CONCLUSION**

There was a difference between the Snail expression in non-metastatic tumours and the Snail expression in metastatic tumours; the increase in Snail expression occurred in patients with metastatic tumour supported by clinical parameters, the AST and ALT. These parameter were correlated with samples of patients with liver damage characterized by elevated AST and ALT enzymes in the metastatic group. The Snail expression in CRC requires further research with the development of other methods to get better results.

**CONFLICT OF INTEREST**

There is no conflict of interest in this study.

**ACKNOWLEDGEMENT**

The authors thank to all lecturers at the Department of Digestive Surgery, RSUP Dr. Sardjito Yogyakarta for their assistance in writing this scientific paper. The authors also thank to Mr. Hendra Susanto, S.Pd., M.Kes., Ph.D. (Universitas Negeri Malang) and the team who have assisted in the molecular analysis process of RT-qPCR.

**REFERENCES**

2. Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal
20. Leontieva O V, Demidenko ZN, Blagosklonny M V. Rapamycin reverses insulin resistance (IR) in high-glucose medi-


37. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal