RESEARCH ARTICLE

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Snail Expression as a Prognostic Factor in Colorectal Adenocarcinoma

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Abstract

Objective: The aim of this study was to analyze the expression of *Snail* in the colorectal adenocarcinoma. **Methods:** This study used a cross-sectional design. Seventy four paraffin embedded block of Colorectal Adenocarcinoma were assessed using *Snail* rabbit polyclonal antibody and their expression were performed using Olympus CX-43 light microscope. The relationship between *Snail* expression with histopathological grading, tumor budding grading, lymphovascular invasion and metastases of colorectal adenocarcinoma ability were statistically analyzed by Mann Whitney tests and presented in tables using SPSS 27. **Result:** From 74 samples examined, in samples with low grade tumor budding (n=11), there were 9 samples (81.8%) with weak expression, while those with strong expression were 2 samples (18.2%). In samples with intermediate grade tumor budding (n=28), there were 17 samples (60.7%) with weak expression, while those with strong expression were 11 samples (39.3%). In samples with high grade tumor budding (n=35), there were 13 samples (37.1%) with weak expression, while those with strong expression were 22 samples (62.9%). In samples with lymphovascular invasion (n=14), there were 10 samples (71.4%) with strong expression, while those with weak expression were 4 samples (28.6%). In samples with metastases (n=23), there were 16 samples (69.6%) with strong expression, while those with weak expression were 7 samples (30.4%). There was a significant relationship between the expression of *Snail* with tumor budding grade (p=0.003), lymphovascular invasion and metastases (p=<0.001), but there was no significant relationship with histopathological grade (p=0.942). **Conclusion:** The *Snail* expression can be used as a prognostic factor in colorectal adenocarcinoma.

Keywords: Colorectal adenocarcinoma- Snail- histopathological- tumor budding

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Introduction

Colorectal carcinoma is a malignant epithelial tumor of the colon and/or rectum. Adenocarcinoma is the most common, accounting for more than 90% of all colorectal carcinoma cases [1]. Colorectal carcinoma affects both men and women, and is one of the most common cancers in both developed and developing countries. Colorectal carcinoma is also one of the most common causes of cancer deaths worldwide. The incidence of colorectal carcinoma in Indonesia also receives special attention because new cases and death rates continue to increase every year. According to GLOBOCAN data in 2020, the incidence of new colorectal cancer in Indonesia was 33,427 cases or around 8.4% of the total 396,914 cancer cases.

Currently, early detection and prompt treatment have been developing well, but some cases of the advanced stage still can be found. Thus, colorectal carcinoma remains the main cause of cancer death [2]. Various prognostic factors have been studied in predicting the outcome of colorectal carcinoma patients, including the role of various clinicopathological factors. The histopathological parameters that are routinely assessed in the evaluation of histopathological preparations for colorectal cancer are the histopathological grade of the tumor, lymphovascular invasion (LVSI) status, degree of inflammation, margin status, nodal status, and Tumor Node and Metastasis (TNM) stage [3]. The histopathological grade assessment of colorectal carcinoma is based on gland formation, low grade and high grade [4]. Currently, specific morphological features are starting to be researched, namely the presence of tumor budding which is associated with tumor aggressiveness [5]. Tumor budding is defined as the presence of single tumor cells or small groups of up to four cells in the peritumoral stroma, whose tumor growth has been linked to Epithelial Mesenchymal Transition (EMT) [6].

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Epithelial Mesenchymal Transition is a biological process that allows polarized cells to interact with the basement membrane and then to assume a mesenchymal phenotype characterized by increased migratory capacity, invasiveness, increased resistance to apoptosis and increased production of extracellular matrix (ECM) components [7]. During EMT, carcinoma cells become more motile and invasive by acquiring characteristics similar to embryonic mesenchymal cells. Therefore, carcinoma cell penetration of the stroma surrounding the initial neoplastic focus can be occured. The transcription factor which is a repressor of E-cadherin is *Snail* [8].

Snail proteins and their families are transcriptional repressor factors. *Snail* is a repressor transcription factor for the E-Cadherin protein which is a factor in the formation of EMT. The *Snail* family of proteins plays a key role in regulating the EMT process, acting as transcription factors to control the expression of genes whose products determine the EMT phenotype and ultimately the development of a neoplasm. *Snail* protein can be identified in the stage of carcinogenesis, invasion, and metastasis. *Snail* expression on tumor cells can characterize the degree of malignancy and serve as a prognostic marker of the disease [9].

Therefore, this study assessed whether *Snail* expression correlates with histopathological grading, tumor budding grading, lymphovascular invasion and metastases of colorectal adenocarcinoma, so that it can be one of the candidate prognostic biomarkers of colorectal adenocarcinoma.

Materials and Methods

From January 2020 to Juny 2023, we obtained 74 paraffin block samples from patients who had been diagnosed with colorectal adenocarcinoma at the Anatomical Pathology Laboratory Dr. Wahidin Sudirohusodo, Hasanuddin University, and Makassar Pathology Diagnostic Center for this study. *Snail* rabbit polyclonal antibody immunohistochemical staining was carried out on slides that had not been stained. The paraffin blocks were used to create slides, which were subsequently cut using a 3 µm thick microtome. A poly-L-lysinie slide was used to take the cut in the water bath, and it was later deparaffinized using *Snail* rabbit polyclonal antibody for immunohistochemical staining.

Using a 400x light microscope, the expression of *Snail* was examined on the nuclear or cytoplasm of tumor cells. Assessment performed by two pathologists who were blinded of clinical data and results. The intensity and proportion of stained tumor cells were used to score *Snail* expression in a semiquantitative manner, and the total immunostaining score (TIS) was used to determine the overall score. The *Snail* expression score is the total immunostaining score (0-9) obtained by multiplying the proportion score of the tumor area stained positively (0-3) with the *Snail* staining intensity score (0-3). The proportion score 0: None; 1: Stained <10%; 2: Stained 10-50%; 3: >50%. The intensity of Snail: uncolored: 0/negative; weak: +1; moderate: +2; strong: +3. Furthermore, *Snail* expression was declared strong if TIS ≥6 and weak if TIS <6 [10].

Statistical Program for Social Science (SPSS) 27 for Windows was used to process the data for this investigation. To evaluate the correlation between categorical variables, the Mann Whitney test was applied.

Results

Table 1 shows the distribution of the 74 samples of colorectal adenocarcinoma by age, gender, tumor location, histopathological grading, tumor budding grading, lymphovascular invasion, metastases and *Snail* expression (Figure 1).

Based on Table 1, it can be seen that this study used a total of 74 samples, which the mean of age was 54.89 years old with a standard deviation of 10.98 years old. Samples with the age category <50 years were 20 samples (27,0%) and the age category >50 years were 54 samples (73,0%). There were 39 samples of male (52,7%) and 35 samples of female (47,3%). Based on the location of the tumor, the location of tumor in the proximal colon were 31 samples (41,9%), in the distal colon were 31 samples (41,9%), and in the rectum were 12 samples (16,2%). The low-grade colorectal adenocarcinoma group were consisted of 64 samples (86,5%) and 10 samples (13.5%) of the high-grade. Samples with low grade tumor budding were 11 samples (14,9%), intermediate grade tumor budding were 28 samples (37,8%) and high grade tumor budding were 35 samples (47,3%). Samples with positive lymphovascular invasion were 14 samples (18.9%) and 60 samples (81.1%) were negative. Samples with metastases were 23 samples (31.1%), while those without metastases were 51 samples (68.9%). *Snail* expression with strong expression were 35 samples (47.3%), while those with weak expression were 39 samples (52.7%). *Snail* immunohistochemical examination results were assessed using a semi-quantitative scoring system based on proportion and color intensity. Tumor cells nuclear or cytoplasms both displayed varying amounts and intensities of *Snail* expression. An example of *Snail* expression assessment for each color intensity is shown in Figure 2 below.

Table 2 shows that from 74 samples of colorectal adenocarcinoma, in high grade group there were 6 samples (11.3%) with strong expression and 4 samples with weak expression (19.0%). Meanwhile, in low grade group there were 47 samples (88.7%) had a strong expression and 17 samples (81.0%) had a weak expression. In samples with high grade tumor budding there were 22 samples (62.9%) with strong expression, while those with weak expression were 13 samples (37.1%). For samples with intermediate grade tumor budding there were 11 samples (39.3%) with strong expression, while those with weak expression were 17 samples (60.7%). And for samples with low grade tumor budding there were 2 samples (18.2%) with strong expression, while those with weak expression were 9 samples (81.8%). In samples with lymphovascular invasion there were 10 samples (71.4%) with strong expression, while those with weak expression were 4 samples (28.6%). For samples without lymphovascular invasion, there were 25 samples (41.7%)

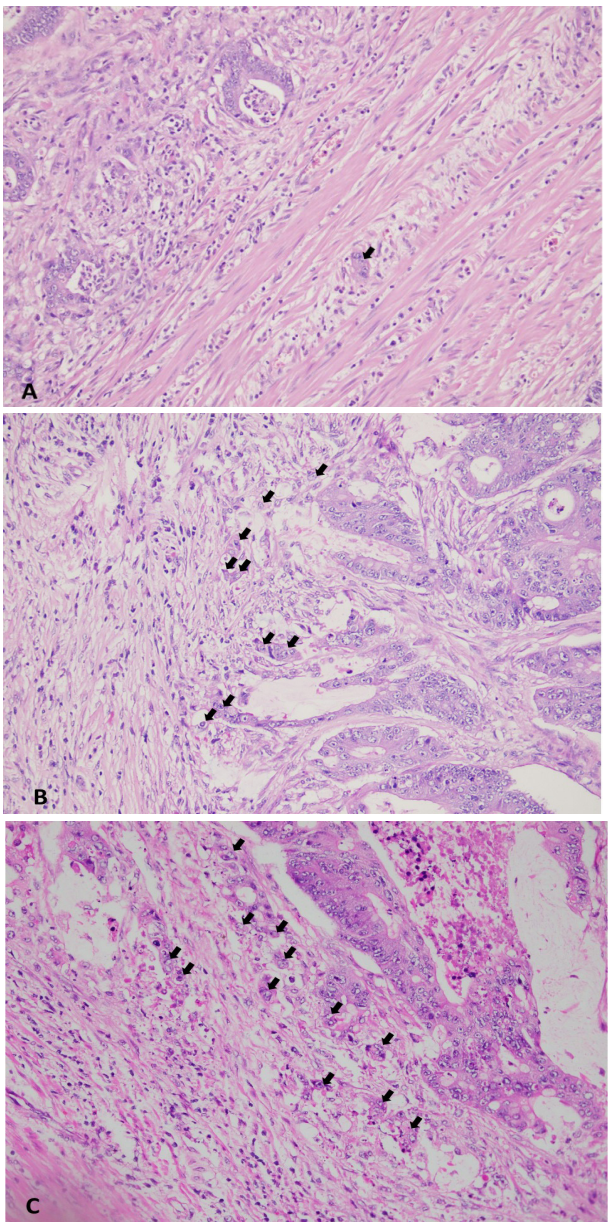

Figure 1. Tumor Budding in Adenocarcinoma Colorectal. A, low grade; B, Intermediate grade; C, High grade (200x Magnification).

Table 1. Characteristics of the Sample

Characteristics	n (%)
Age (yrs)	
<50	20 (27.0)
≥50	54 (73.0)
mean ± SD= 54,89 ± 10,98	
Gender	
Male	39 (52.7)
Female	35 (47.3)
Tumor location	
Proximal	31 (41.9)
Distal	31 (41.9)
Rectum	12 (16.2)
Histopathological grade	
Low grade	64 (86.5)
High grade	10 (13.5)
Tumor budding	
Low grade	11 (14.9)
Intermediate grade	28 (37.8)
High grade	35 (47.3)
LVSI	
Positive	14 (18.9)
Negative	60 (81.1)
Metastases	
Positive	23 (31.1)
Negative	51 (68.9)
Snail Expression	
Weak	39 (52.7)
Strong	35 (47.3)

with strong expression and 35 samples (58.3%) with weak expresion. Regarding to the metastases, there were 16 samples with metastases and strong expression of *Snail* (69.6%), while 7 samples with weak expression (30.4%). For samples without metastases, there were 19 samples (37.3%) with strong expression and 32 samples (62.7%) with weak expression. Based on statistical analysis using Mann Whitney test, it shows that there were a significant relationship between *Snail* expression with tumor budding grading (p=0.003), lymphovascular invasion and metastases (p=<0.001), but there were no relationship between *Snail* expression with histopathological grading (p=0.942).

Discussion

One of the transcription factors that plays a role in epithelial to mesenchymal transition is Snail. *Snail* is a transcriptional repressor containing a highly conserved C-terminal region including four and five zinc fingers and is involved in protein binding to target gene promoters containing E-box sequences and an N-terminal region containing the evolutionary SNAG domain required for transcriptional repression and is capable of binds methyltransferases and histone deacetylases [9]. The *Snail* family includes Snail-1 (Snail), Snail-2 (Slug) and Snail-3 (Smuc), and has been shown to be over-expressed in a wide variety of human malignancies including oral, breast, hepatocellular, gastric, colon and skin carcinomas. Snail is typically localized in the nucleus. However, the subcellular localization and stability are sensitive to Ser/Thr phosphorylation, when after being phosphorylated, Snail is translocated to the cytosol, where it is not active and is subsequently degraded [11]. In malignant tumor cells, Snail activates the TGF-β and Wnt pathways thus promoting tumor cell growth [12]. In some types of tumors, especially in colorectal cancer, excessive expression of Snail may associated with cancer progression and poorer prognosis [13].

In our study, there was no association between Snail expression and histopathological grade (Table 2). This finding is correlate with the report [14] which showed

Table 2. Relationship of *Snail* Expression with Histopathological and Tumor Budding Grade

Characteristics	No. of Patients	Snail positive No. (%)	P value*	Percent of Snail stained cells (Mean ± SE)	P value
Age (yrs)					
<50	20	10 (50)	0.983	5,10 ± 0,62	0,384***
≥50	54	25 (46,3)		4,44 ± 0,43	
Gender					
Male	39	18 (46,2)	1,000	4,64 ± 0,49	0,987***
Female	35	17 (48,6)		4,60 ± 0,53	
Tumor location					
Proximal	31	12 (38,7)	0.285	4,00 ± 0,51	0,371**
Distal	31	18 (58,1)		5,23 ± 0,55	
Rectum	12	5 (41,7)		4,67 ± 0,36	
Histopathological grade					
Low	64	47 (73,4)	0.456	4,50 ± 1,02	0,942***
High	10	6 (60)		4,64 ± 0,38	
Tumor budding grade					
Low	11	2 (18,2)	0,020	1,09 ± 0,89	0,003**
Intermediate	28	11 (39,3)		1,43 ± 0,57	
High	35	22 (62,9)		1,97 ± 0,46	
LVSI					
Positive	60	25 (41,7)	0.087	1,22 ± 0,41	< 0,001***
Negative	14	10 (71,4)		3,43 ± 0,62	
Metastases					
Positive	51	19 (37,3)	0,020	1,08 ± 0,46	< 0,001***
Negative	23	16 (69,6)		2,87 ± 0,44	

*, Chi-square test; **, Kruskal Wallis test; ***, Mann Whitney test

there was no relationship between Snail expression and various clinicopathological parameters including age, sex and histopathological grade in colorectal carcinoma. Apart from colorectal carcinoma, Snail expression was also found to be unrelated to histopathological grade in breast carcinoma and hepatocellular carcinoma [15, 16]. Due to the complex multistep molecular etiology of CRC, which includes many genetic and epigenetic changes, there are differences in Snail expression [13].

Epithelial-mesenchymal transition (EMT) is a biological process of polarized epithelial cells. The polarized cells are interacting with the basement membrane through their basal surface, undergoing several biochemical changes that allow them to assume the phenotype of mesenchymal cells, which include increased migratory capacity, invasive properties, increased resistance to apoptosis, and increased production of ECM components. EMT is characterized by degradation of the underlying basement membrane and the formation of mesenchymal cells that can migrate away from the epithelial layer from which they originate [17]. In tumorgenesis, EMT is an important process that gives tumor cells the ability to migrate out of the primary tumor and metastasize to distant sites. This is associated with a worse prognosis for cancer patients [18]. Furthermore, EMT can induce a cancer stem cell (CSC)-like phenotype in a number of tumor types [19]. After the tumor cells metastasize and reach their destination, the Mesenchymal-epithelial transition (MET) process plays a role. MET is the reverse process of epithelial-mesenchymal transition and has been shown to occur in normal development, inducing reprogramming, cancer metastasis and wound healing [17, 20]. Although relatively little is known about the role of MET in cancer when compared to the extensive research of EMT in tumor metastasis, MET is believed to participate in the formation and stabilization of distant metastases by allowing cancer cells to gain regain epithelial properties and integrate into distant organs. Between these two states, the cell is in an 'intermediate state', or what is called partial EMT.

Several transcription factors involved in controlling EMT. There are Snail, Twist, ZEB1/2, SIP1, and E12/E47. *Snail* was first identified in Drosophila as having an important role in mesenchyme formation [21]. *Snail* is a transcription factor that contains a helix-loop-helix structure, and is reported to have the ability to suppress E-cadherin transcription, induce the expression of matrix metalloproteniase-2, thereby degrading the extracellular matrix [16]. *Snail* is a transcription factor that mediates EMT in a number of tumor types, including colorectal cancer (CRC). *Snail* plays a role in the pathogenesis of several malignant neoplasms, especially by promoting invasion and metastasis [19]. The *Snail* family, including Snail-1 (Snail), Snail-2 (Slug) and Snail-3 (Smuc), has

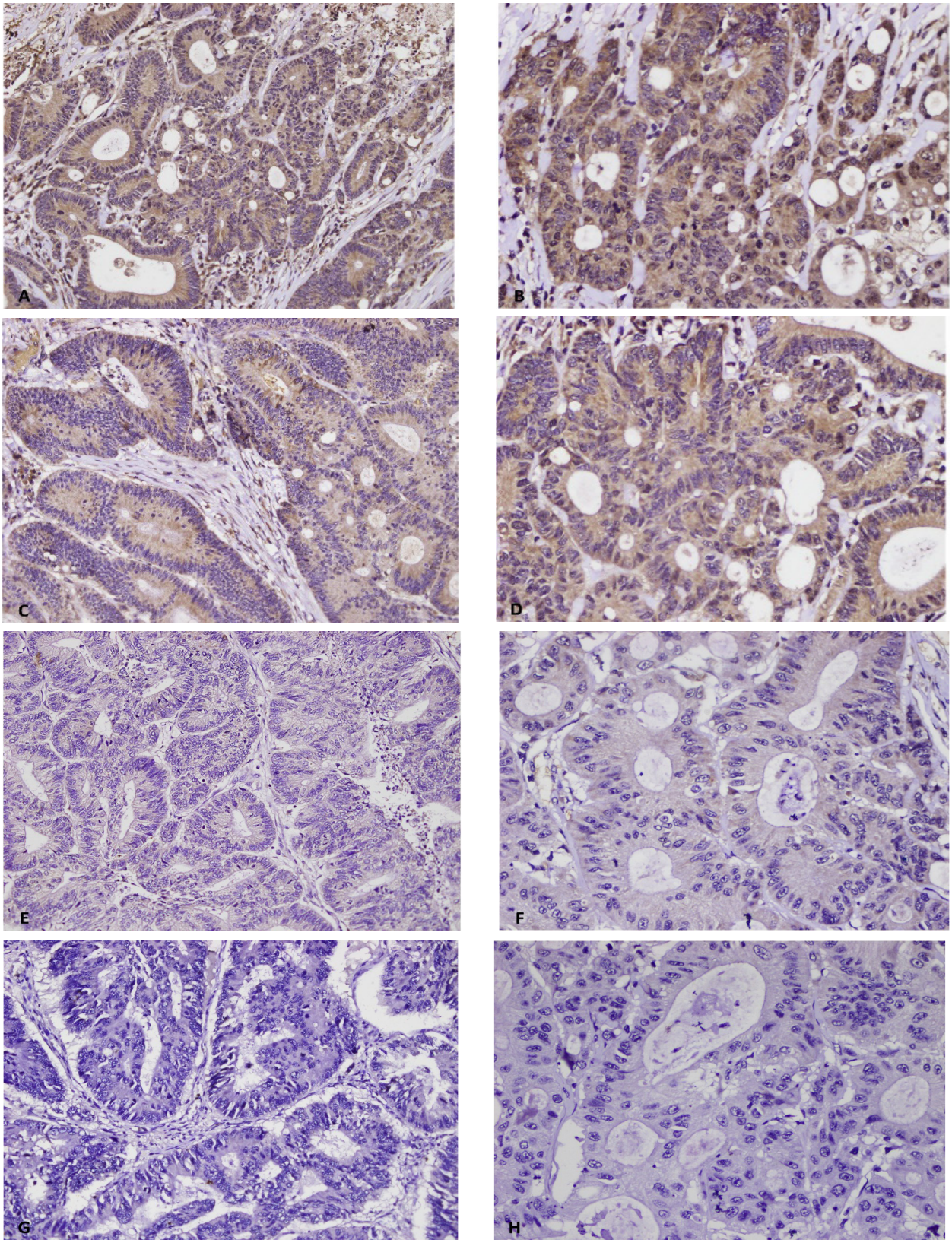

Figure 2. *Snail* Expression in Colorectal Adenocarcinoma. A-B, Strong; C-D, Moderate; E-F, Weak; G-H, Negative (200x and 400x Magnification).

been shown to be highly expressed in a variety of human malignancies including oral, breast, hepatocellular, gastric, colon and skin carcinomas [22]. *Snail* protein is expressed at high levels in the nucleus and cytoplasm of tumor cell adenomas and carcinomas. *Snail* is activated by several signaling pathways, including Wnt/β-catenin, TGF-β (transforming growth factor β), TNF-α (tumor necrosis factor α), RAS, ILK (integrin-linked kinase), NF-κB (nuclear factor κ-light chain enhancer of activated B cells), HIF (hypoxia-inducible factor), AKT activation and EGFR (epidermal growth factor receptor) signaling [11].

We also assessed the association of *Snail* expression

with tumor budding grading, lymphovascular invasion and metastases (Table 2). This research data shows that *Snail* expression is higher in colorectal adenocarcinoma with high grade tumor budding compared with low grade and intermediate grade tumor budding. Statistically, it shows that there is a significant relationship between *Snail* expression and tumor budding grade. Tumor budding overall showed a loss of epithelial markers and an increase in mesenchymal markers compared to the primary tumor mass. Although the primary tumor mass consists primarily of pure epithelial cells, the tumor budding is very rich in pure mesenchymal cells and even hybrid epithelial-mesenchymal cells. EMT is controlled by a core regulatory circuit consisting of: two microRNAs, known as miR-34 and miR-200 respectively, and two transcription factors, referred to as SNAIL and ZEB1/2, which respectively stand for Zinc finger E-box-binding homeobox-1 or -2 [23]. In our study, statistically it shows that there is a significant relationship between *Snail* expression and low grade, intermediate grade and high grade tumor budding in colorectal adenocarcinoma. Other studies also reported that *Snail* expression was significantly correlated with the formation of tumor budding at the invasive front and the incidence of lymph node metastasis. Soluble TGF-β in the CRC microenvironment may participate in Snail-induced EMT and restoration of CSC-like properties, which may lead to metastasis in patients with stage II CRC [24].

Snail expression significantly correlated with vascular invasion in colorectal adenocarcinoma. Epithelial-mesenchymal transition is a fundamental process that regulates the nature of invasion. E-cadherin plays a major role in development, organogenesis and tissue formation, but also in tumor progression. *Snail* is a transcription factor described as a direct suppressor of E-cadherin during development and carcinogenesis. The expression of E-cadherin and *Snail* in colorectal adenocarcinoma reported that *Snail* showed statistically significant aspects associated with vascular invasion. The results obtained demonstrate the implication of *Snail* and E-cadherin in EMT of colorectal adenocarcinoma, which is a useful aspect in the evaluation of adenocarcinoma patients for specific therapeutic targets. *Snail* was overexpressed in the group with positive lymph nodes, whereas Twist was overexpressed in patients with other metastases. Expression of *Snail* and Twist correlated with reduced membrane expression of E-cadherin [25]. The study concluded that *Snail* overexpression was significantly correlated with lymph node metastasis in colorectal adenocarcinoma. *Snail* expression is a common sign of poor prognosis in metastatic cancer, and tumors with increased *Snail* expression are very difficult to treat with current therapeutic treatments [19]. The importance of *Snail* as a prognostic indicator, its involvement in the regulation of EMT and metastasis, and its role in drug resistance and immunity suggest that *Snail* is an attractive target for tumor growth inhibition and a target for sensitization to cytotoxic drugs [26].

The limitation of this study is that it only uses one type of prognostic marker with only one protein detection modality via immunohistochemistry, so it is still less accurate in analyzing the complexity of the relationship between *Snail* and various other molecules involved in regulating its expression, as well as its relationship to colorectal adenocarcinoma tumor budding, invasion and metastasis.

In conclusion, there is a significant relationship between *Snail* expression with tumor budding grading, lymphovascular invasion and metastases in colorectal adenocarcinoma. *Snail* expression in the high grade tumor budding group was higher than in the intermediate and low grade tumor budding groups. *Snail* expression in the lymphovascular invasion group are higher than in without lymphovascular invasion group. While, *Snail* expression in the metastatic group are higher than in the non-metastatic group. There is no relationship between *Snail* expression and histopathological grade. *Snail* expression affects tumor budding grading, invasion and metastasis of colorectal carcinoma but does not play a role in histopathological grading. So, the *Snail* expression can be used as a prognostic factor in colorectal adenocarcinoma.

Author Contribution Statement

All authors contributed equally in this study and approved the final manuscript.

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Study Approval

This work was permitted by the research committee of the Faculty of Medicine, Hasanuddin University.

Ethical approval

The Faculty of Medicine's Ethics Committee waived informed consent for this study (Protocol #UH23070526 – Registry No. 621/UN4.6.4.5.31/PP36/2023).

Availability of Data

On reasonable request, the associated author will release the datasets used in this work.

Conflict of Interest

All authors state that they have no conflicting interests in this research.

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