RESEARCH ARTICLE

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MicroRNA-21 (miR-21), Mutant Tumor Protein *p53*, and Matrix Metalloproteinase-9 (MMP-9) as Risk Markers for High-Grade Tumor Budding (HGTB) in Cervical Carcinoma: A Case-Control Study in a Balinese Population

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Abstract

Objective: High-grade tumor budding (HGTB) is still a controversial parameter in determining the aggressiveness of cervical carcinoma, yet the molecular mechanism of TB is still not comprehensively understood. This study aimed to evaluate whether *miR-21*, mutant *p53*, and *MMP-9* expression are associated with HGTB in cervical carcinoma. **Methods:** This nested case-control study was conducted at the Anatomical Pathology Laboratory of Balimed Denpasar Hospital, Mangusada Badung Hospital, and the Biomolecular Laboratory, Faculty of Medicine and Health Sciences, Warmadewa University, from November 2022 to April 2023. The samples were paraffin blocks from a radical hysterectomy of patients with cervical carcinoma. The expression of *miR-21* was examined by real-time quantitative PCR (RT-qPCR), while *p53* and *MMP-9* were analyzed by immunohistochemistry (IHC). A total of 40 samples were selected using simple random sampling, consisting of 20 low-grade tumor budding (LGTB) samples (controls) and 20 HGTB samples (cases). Data analysis was performed using SPSS version 24. **Results:** This study found that the risk of HGTB significantly increased in *miR-21* overexpression (OR=13.222; 95%CI: 2.790-62.670; p-value<0.001) with sensitivity (SN) 70% and specificity (SP) 85%, high mutant *p53* expression (OR=44.333; 95%CI: 4.783-410.943; p-value<0.001) with SN=95% and SP=70%, and high *MMP-9* expression (OR=10.524; 95%CI: 2.271-48.757; p-value=0.001) with SN=65% and SP=85%. Multivariate analysis showed a significant independent association between mutant *p53* expression (aOR=53.676; 95%CI: 3.844-749.474; p-value = 0.003) and high *MMP-9* (aOR=13.355; 95%CI: 1.359-131.237; p-value = 0.026), yet *miR-21* did not show a significant result. **Conclusion:** These findings showed a significant relationship between *p53* and *MMP-9* expression as pro-oncogenic and pro-invasive molecular factors in HGTB cervical carcinoma.

Keywords: Cervical carcinoma, *miR-21*, *MMP-9*, *p53*, tumor budding

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Introduction

Cervical carcinoma is one of the most common types of malignancy in women, with a significant cause of death in women, especially in developing countries such as Indonesia. Additionally, the high prevalence of cervical carcinoma, especially in the productive age, often leads to it being diagnosed in an advanced stage [1, 2]. Globally, there were an estimated 570,000 new cases and 311,000 deaths related to cervical carcinoma in 2018, with the highest incidence in Asia, especially China and India [3, 4]. Moreover, a previous local report in Bali Province showed that the majority of cases occurred in women aged 40-49 years [5]. The high mortality rate in patients with cervical carcinoma is reported to be related to metastasis, therapy resistance, and recurrence [6]. Additionally, aggressive therapy without proper cancer characterization can reduce quality of life and even cause faster cancer progression [7]. Therefore, it is necessary to have more accurate prognostic markers to accelerate and adjust the selection of appropriate therapy early in the disease course.

Several parameters, including clinical stage, lymph node metastasis (LMN), and lymphovascular invasion (LVI), have been widely used to predict the progression and prognosis of cervical carcinoma and to guide therapy decisions. Yet, tumor size in the clinical stage is used as the

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primary reference for therapy [7, 8]. Growing evidence has found that tumor size does not always correlate positively with cancer aggressiveness. This raises concerns that using tumor size alone could lead to inappropriate therapy, which could have detrimental effects on patients [9–11]. Although histopathological grading based on the Modified Broder's system was previously considered necessary, it is now only recommended without mandatory prognostic value. Therefore, further evaluation of more representative prognostic parameters is necessary, one of which is tumor budding (TB).

Tumor budding is a phenomenon where clusters of malignant cells are found in the stromal tissue, which is generally found at the invasive front of the primary tumor and suspected to be a marker of more aggressive tumor characteristics, as represent the epithelial-mesenchymal transition (EMT) [12]. Previous studies in colorectal cancer have incorporated TB as an integral part of the pathology report of colorectal cancer [13, 14]. Yet, in the case of other malignancies, especially cervical carcinoma, despite growing evidence, the role of TB in cervical carcinoma is controversial due to inconsistent findings [7, 15–21], as well as because the underlying molecular mechanisms of TB in cervical carcinoma are not yet well understood [8, 15].

One network of conceptual mechanisms that may be related to TB is the complex molecular mechanisms between *miR-21*, *p53*, and *MMP-9*. Theoretically, *miR-21* contributes to tumor aggressiveness by repressing tumor suppressor genes and is associated with increased stage and metastasis by regulating the expression of MMPs [9, 22, 23]. Additionally, mutation or overexpression of *p53* is known to worsen progression and therapeutic response [24, 25]. *MMP-9*, as one of the MMPs, supports invasion and EMT, and is thought to be involved in TB formation.[14, 27, 28]. Biologically, *miR-21*, *p53*, and *MMP-9* act as a pro-invasive network on the invasive front rather than a single linear pathway, aas TB in cervical carcinoma is associated with an EMT-like phenotype of cancer stem cells, while *miR-21* itself has been shown to promote EMT, migration, and invasion of cervical cancer cells [29, 30]. Increased *miR-21* also suppresses the reversion-inducing cysteine-rich protein with kazal motifs (RECK), an inhibitor of invasion and negative regulator of MMPs, thus weakening control of proteolytic activity [30]. Additionally, the *p53* pathway is at the control point because normal *p53* inhibits cell survival and migration and suppresses *MMP-9* activation [32]. Matrix metalloproteinase-9 then acts as a downstream effector that degrades the extracellular matrix. Therefore, the combination of high *miR-21*, *p53* dysfunction, and high *MMP-9* is hypothesized to be a strong biological basis for the formation of HGTB [33]. Meanwhile, research that evaluates the consistency of these relationships in one population, especially in the Balinese population, is still limited. Therefore, this study aimed to evaluate the association of *miR-21, p53*, and *MMP-9* expression in the formation of high-grade tumor budding (HGTB) in cervical carcinoma.

Materials and Methods

Study Design

This study used a nested case-control design in cervical carcinoma patients, with the case group consisting of patients with HGTB and the control group consisting of patients with low-grade tumor budding (LGTB), without individual matching between the two groups. The control group was selected from the same source population as the case group, namely cervical carcinoma patients who met the inclusion and exclusion criteria during the study period, so that it still represented the distribution of exposure in the original study to assess TB, a practical approach was used in the form of a two-level classification with systematically determined cutoff points (LGTB if the number of budding buds <3 buds per high-power field (HPF) and HGTB if the number of budding buds ≥3 buds/HPF, based on the area with the highest budding activity). The expression of *miR-21* was analyzed using real-time quantitative PCR (RT-qPCR) on formalin-fixed paraffin-embedded (FFPE) paraffin block samples, while the expression of p53, both the wild-type and mutant, and *MMP-9* were evaluated using IHC on cervical carcinoma surgical tissue examined at the Anatomical Pathology Laboratory of BaliMed Hospital, Denpasar. Potential confounding due to non-matching was minimized by statistically comparing the baseline characteristics of both groups and considering relevant confounding variables in further analysis.

Study Population and Sample Collection

This study was conducted at the Anatomical Pathology Laboratory of BaliMed Denpasar Hospital, Mangusada Badung Hospital, and the Biomolecular Laboratory of the Faculty of Medicine and Health Science, Warmadewa University, with medical data and paraffin block collection carried out from November 2022 to April 2023. The study sample consisted of Formalin-Fixed Paraffin-Embedded (FFPE) cervical carcinoma tissue from radical hysterectomies that were examined histopathologically and accompanied by complete medical data in the period 2020-2022. All cervical carcinoma patients undergoing radical hysterectomy between 2020 and 2022 were first identified, with a total of 86 patients meeting initial eligibility. Eighteen patients were excluded due to neoadjuvant therapy, nine patients were excluded due to damaged FFPE blocks or tissue preparations that could not be evaluated, and seven patients were excluded due to incomplete clinicopathological data. This left 52 patients who met the final study criteria. Patients were then stratified into the HGTB and LGTB groups. Simple random sampling was performed from each stratum to obtain a total of 40 samples, consisting of 20 samples from the HGTB group and 20 samples from the LGTB group. Inclusion criteria included all paraffin blocks of cervical carcinoma with any histopathological type that were suitable for immunohistochemical examination. Exclusion criteria included samples from patients with a history of neoadjuvant chemotherapy and damaged or moldy FFPE blocks.

Real-Time PCR Assessment for miR-21 Expression

The expression of *miR-21* was evaluated through an RT-qPCR procedure that began with the isolation of total RNA (including miRNA) from FFPE cervical carcinoma tissue using the RecoverAll™ Total Nucleic Acid Isolation Kit. The process included deparaffinization, protease digestion, RNA isolation with filter cartridges, and RNA elution in nuclease-free water. The purity and concentration of RNA were determined using a Nanodrop spectrophotometer.

Complementary DNA (cDNA) synthesis from miRNA was carried out using the TaqMan™ MicroRNA Reverse Transcription Kit with hsa-*miR-21*-specific primers and endogenous control U6, through a reverse transcription reaction containing dNTPs, reverse transcriptase, RNase inhibitor, and buffer. The incubation process using a thermal cycler included stages of 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes.

Real-time quantitative PCR was performed in singleplex duplex using TaqMan® Fast Advanced Master Mix and TaqMan MicroRNA Assay for *miR-21* and small RNA U6 as an internal control. Reactions were arranged in 40-well plates with a total volume of 20 µL and run on Applied Biosystems StepOne real-time PCR with cycles: 50°C (2 min), 95°C (20 s), followed by 40 cycles of 95°C (3 s) and 60°C (30 s). Relative expression analysis of *miR-21* was calculated using the comparative Ct (ΔCt) method, with U6 as an endogenous control and standard cervical samples as calibrators. The RQ (Relative Quantification) value indicates the fold change in *miR-21* expression in carcinoma tissue compared to normal tissue. Categorization of *miR-21* expression is grouped into 2, namely samples with *miR-21* overexpression (when the value is based on the cut-off value for overexpression if *miR-21* expression is > 6 times the reference sample), and normal.

Immunohistochemistry Procedure for Wild-Type p53, Mutant p53 and MMP-9 Expression

Examination of *p53* and *MMP-9* expression was performed through IHC staining of cervical carcinoma tissue specimens that had been fixed with 10% buffered formalin, processed, and sectioned using a microtome. The IHC procedure includes peroxidase blockade, application of primary antibodies or negative controls, application of labelled polymer, addition of substrate-chromogen (DAB), contrast staining with hematoxylin-Mayer, and mounting process using permanent media. The entire process was carried out at room temperature, and the humidity of the glass object was maintained to avoid non-specific staining. The expression of *p53* was examined using Monoclonal Antibody Clone PAb1620 ((wild-type, Merck, Germany) and R175H mutant antibody (GeneTex, USA). Examination was performed using an Olympus CX21 light microscope. Expression was assessed semi-quantitatively based on staining intensity (1–4) and percentage of positive cells (1–4), then calculated using the histoscore formula: Histoscore = intensity × percentage. Histoscore values >7 were categorized as high expression, while those ≤7 were classified as low [33]. Two independent anatomical pathologists performed the assessment. Meanwhile, *MMP-9* expression was analyzed using Monoclonal Rabbit Anti-Human *MMP-9* Antigen (Cell Marque, USA) with a similar method. Tumor cells are said to express *MMP-9* if brown staining is seen in the cytoplasm. High expression is determined if >10% of tumor cells show cytoplasmic staining, and low if <10% [34]. The evaluation was performed by two pathologists from the Warmadewa Faculty of Medicine and Health Sciences and Mangusada Hospital.

Statistical Analysis

All data were analyzed using SPSS version 24.0 for Windows. Descriptive analysis is presented in the form of frequency distributions and percentages for categorical variables such as tumor size, age, stage, histopathological type, tumor-infiltrating lymphocyte (TIL), and grade. The Chi-square test or Fisher's Exact test was used in bivariate analysis according to test requirements, with the size effects measured using the odds ratio (OR) and 95% confidence interval, and a significance value of $p \leq 0.05$. Variables with a p-value <0.25 in the bivariate analysis were included in multivariate analysis using binary logistic regression. In logistic regression, each regression coefficient (β) represents the change in the log-odds of the outcome event for each one-unit increase in the predictor variable, while the constant value (intercept) indicates the baseline log-odds of HGTB occurrence when all predictor variables are in the reference category. The constant and regression coefficients were obtained using the Maximum Likelihood Estimation (MLE) method. The regression coefficients were then transformed into odds ratios using an exponential function to facilitate clinical interpretation. The final model was considered significant if the p-value ≤ 0.05, and the probability of outcome occurrence was calculated using a logistic equation to determine the tendency of HGTB formation in each combination of predictor variables.

Results

Study Characteristics

A total of 40 samples were divided into two groups, each with 20 HGTB samples as cases and 20 LGTB samples as controls. The results of the analysis of subject characteristics showed no significant difference between the HGTB and LGTB groups in all variables studied ($p>0.05$). The majority of patients in both groups were aged <50 years (55.0% vs. 45.0%), had tumor size ≥2 cm (100% in HGTB and 90% in LGTB), and were in early cancer stage (75.0% in both groups). The histological type was dominated by squamous cell carcinoma (SCC) at 90% in both groups, and TIL ≤60% was found in 70.0% of the HGTB and 75.0% of the LGTB. No statistically significant differences were found in all of these variables ($p>0.05$) (Table 1).

Risk of HGTB in Cervical Carcinoma with miR-21 Overexpression

The mean relative expression value of *miR-21* in the HGTB group was 6.92 ± 2.15, significantly higher than the mean in the LGTB group (2.83 ± 2.21)

Table 1. Study Characteristics

Variable	Tumor Budding		p-value
	HGTB (n = 20)	LGTB (n = 20)	
Age; Mean ± SD	6.92 ± 2.15	2.83 ± 2.21	
<50 years	11 (55.0%)	9 (45.0%)	
≥50 years	9 (45.0%)	11 (55.0%)	
Tumor Size			0.487
<2 cm	0 (0.0%)	2 (10.0%)	
≥2 cm	20 (100.0%)	18 (90.0%)	
Cancer stage			1
Early	15 (75.0%)	15 (75.0%)	
Advanced	5 (25.0%)	5 (25.0%)	
Histologic Type			1
Non-SCC	2 (10.0%)	2 (10.0%)	
SCC	18 (90.0%)	18 (90.0%)	
TIL			0.723
>60%	6 (30.0%)	5 (25.0%)	
≤60%	14 (70.0%)	15 (75.0%)	

*Analysis was carried out using Pearson's Chi-Square Test. The result was considered significant if the p-value≤0.05.

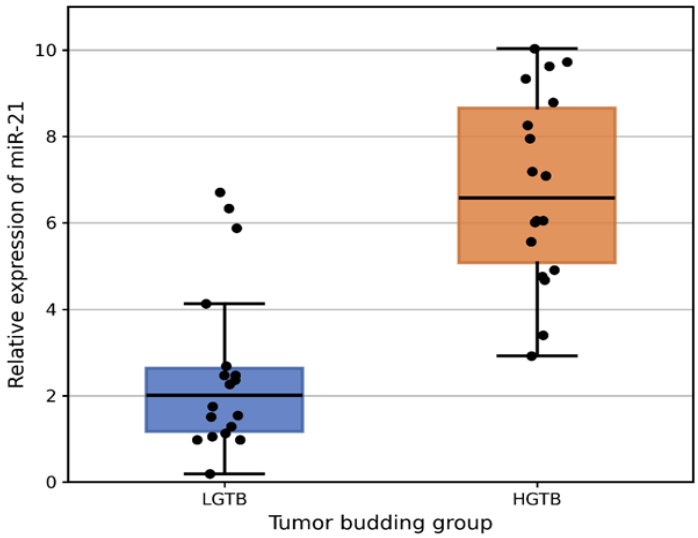

Figure 1. Comparison of the mean relative expression of *miR-21*. The mean relative expression of *miR-21* was higher in the case group (HGTB) compared to the control group (LGTB) (p-value ≤ 0.05).

(Figure 1). The results of statistical tests showed that *miR-21* overexpression was significantly more common in the HGTB group compared to the LGTB group (p-value < 0.001). The *miR-21* overexpression was found in 14 of 20 samples in the HGTB group (70.0%) and only in 3 of 20 samples in the LGTB group (15.0%), with an odds ratio (OR) of 13.222 (95%CI: 2.790-62.670), sensitivity 70%, and specificity 85% (Table 2).

Risk of HGTB in Cervical Carcinoma with High p53 Expression

This study evaluated the expression of wild-type and mutant *p53* proteins to confirm which *p53* protein was overexpressed. The analysis showed that none of the study subjects overexpressed wild-type *p53* in both groups (Figure 2), while mutant *p53* expression was significantly more common in the HGTB group compared to the LGTB group (p-value<0.001). Therefore, wild-type *p53* was not included in further analyses due to uniformly low expression. Nineteen samples (95.0%) in the HGTB group showed high mutant p53 expression, while only 6 out of 20 samples (30.0%) in the LGTB group (OR = 44.333; 95% CI: 4.783-410.943), with a sensitivity of 95.0% and a specificity of 70.0% (Table 3). Figure 3A showed higher mutant p53 expression in HGTB tissues, compared with lower expression in LGTB (Figure 3B).

Risk of HGTB in Cervical Carcinoma with High MMP-9 Expression

High *MMP-9* expression was significantly more common in the HGTB group compared to the LGTB group (p-value = 0.001). As shown in Table 3, 13 out of

Table 2. Overexpression of *miR-21* in the Case and Control Groups

Variable	Tumor Budding		OR 95% CI	SN	SP	p-value
	HGTB (n = 20)	LGTB (n = 20)				
miR-21 overexpression			13.222	70%	85%	<0.001*
Yes	14 (70.0%)	3 (15.0%)	(2.790-62.670)			
No	6 (30.0%)	17 (85.0%)				

*Analysis was carried out using Pearson's Chi-Square Test. SD: Standard deviation; SN: sensitivity; SP: specificity. The result was considered significant if the p-value≤0.05.

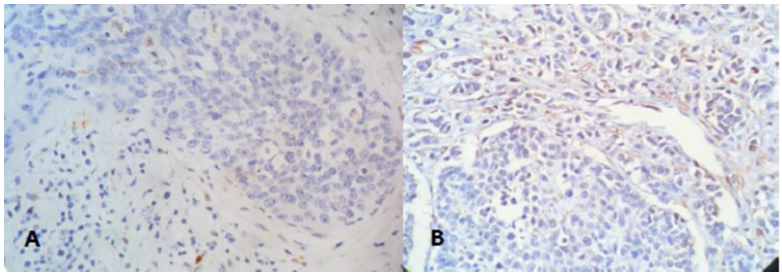

Figure 2. Wild-Type *p53* Expression in the Case and Control Groups. Low expression of wild-type p53 staining in both the case and control groups, resulting in no further wild-type *p53* activity in cervical carcinoma. Caption: Wild-type *p53* staining. Figure (A) score 1 for HGTB (*p53* wild-type, 400x); Figure (B) score 1 for LGTB (*p53* wild-type, 400x).

20 samples (65.0%) in the HGTB group showed high *MMP-9* expression, while only 3 out of 20 samples (15.0%) in the LGTB group. High *MMP-9* expression was associated with an increased risk of HGTB by 10.524 times (OR = 10.524; 95% CI: 2.271-48.757), with a sensitivity of 65.0% and a specificity of 85.0%. Figure 3 shows the visualization of *MMP-9* expression, where positive staining >10% is clearly visible in HGTB tissue (Figure 4A). In comparison, low expression (<10%) is more dominant in LGTB (Figure 4B).

Multivariate Analysis of miR-21, mutant p53, and MMP-9 Expression on the Risk of HGTB in Cervical Carcinoma

Multivariate analysis was conducted on the relationship

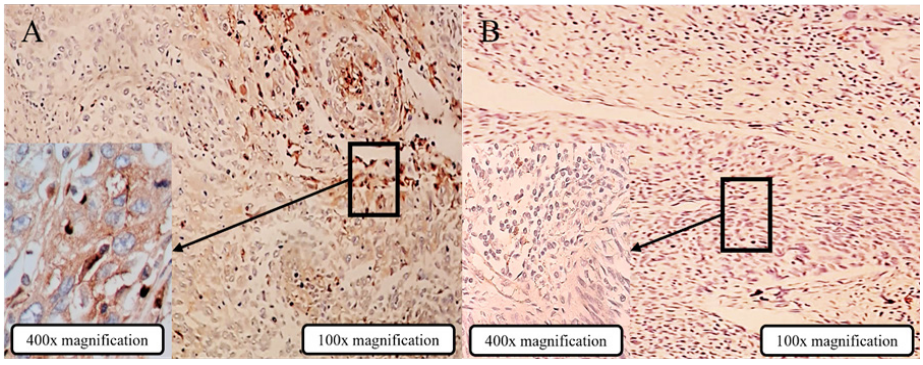

Figure 3. Mutant *p53* expression in HGTB and LGTB. Mutant *p53* expression appears higher in HGTB than in LGTB. Panel (A) shows mutant *p53* expression with a score of 12 in HGTB, with the main image at 100x magnification and the inset showing the same area at 400x magnification. Panel (B) shows mutant *p53* expression with a score of 4 in LGTB, with the main image at 100x magnification and the inset showing the same area at 400x magnification.

Table 3. Expression of *p53* in the Case and Control Groups

Variable	Tumor Budding		OR 95%CI	SN	SP	p-value
	HGTB (n = 20)	LGTB (n = 20)				
P53 wt			-	-	-	1
Yes	0 (0.0%)	0 (0.0%)				
No	20 (100.0%)	20 (100.0%)				
p53 mut			44.333	95.00%	70.00%	<0.001*
High	19 (95.0%)	6 (30.0%)	(4.783-410.943)			
Normal	1 (5.0%)	14 (70.0%)				

*Analysis was carried out using Pearson's Chi-Square Test. SD, Standard deviation; SN, sensitivity; SP, specificity. The result was considered significant if the p-value≤0.05.

Table 4. Multivariate Analysis of High Expression of *miR-21*, mutant *p53*, and *MMP-9* on the Formation of HGTB in Cervical Carcinoma

	Variable	Adjusted OR	95%CI	p-value
Initial Model	High *miR-21* expression	1.833	0.243-13.793	0.556
	High mutant *p53* expression	41.503	2.653-649.143	0.008*
	High *MMP-9* expression	10.529	0.939-118.036	0.056
Final Model	High mutant *p53* expression	53.676	3.844-749.474	0.003*
	High *MMP-9* expression	13.355	1.359-131.237	0.026*
	y = -9.409 + 3.983 *p53*mut + 2.592 *MMP-9*			

*Analysis was carried out using a logistic regression test. The result was considered significant if the p-value≤0.05.

between several independent variables and the formation of HGTB. The independent variables included in the model were *miR-21*, mutant *p53*, and *MMP-9* (Table 4). Based on multivariate analysis (Table 5), in the initial model, not all p-values of the variables were significant (p>0.05). This indicates that not all variables are associated with HGTB. After continuing with the second stage of multivariate analysis, the best model was obtained, namely the final model, which shows that, statistically, only the mutant *p53* and *MMP-9* variables are simultaneously strongly related to predicting HGTB. The strongest independent predictors of HGTB formation were, in order, mutant p53 expression (adjusted OR = 53.696; 95%CI 3.844-749.474) and MMP-9 expression (adjusted OR = 13.355; 95%CI 1.359-131.237). The results of the multivariate analysis also produced a calculation of the risk of HGTB (y) based on the log OR of mutant *p53* and *MMP-9* expression. The ratio of mutant *p53* increases the risk of HGTB by 3.983, and the ratio of *MMP-9* increases the risk of HGTB by 2.592. Therefore, high mutant *p53* and high *MMP-9* remained independently associated with HGTB.

Discussion

This study shows that *p53* and *MMP-9* are independently associated with HGTB in cervical cancer. Overexpression of *miR-21* was also significantly associated with an increased risk of HGTB. However, after adjusting for mutant *p53* and *MMP-9* in multivariate analysis, the effect of *miR-21* was attenuated, suggesting that some of the effects of *miR-21* on tumor budding may be mediated or influenced by other molecular pathways. These findings are consistent with previous studies in colon and pancreatic cancer that also reported an association between *miR-21* overexpression and HGTB [23, 35]. Overexpression of *miR-21* is hypothesized to facilitate EMT, which increases the motility and invasiveness of cancer cells, thereby accelerating budding, lymphovascular invasion, and metastasis [36, 37]. The EMT process activates the TGF-β and WNT/β-catenin pathways, which increase the transcription factors SNAIL, ZEB, and TWIST, then decrease E-cadherin and

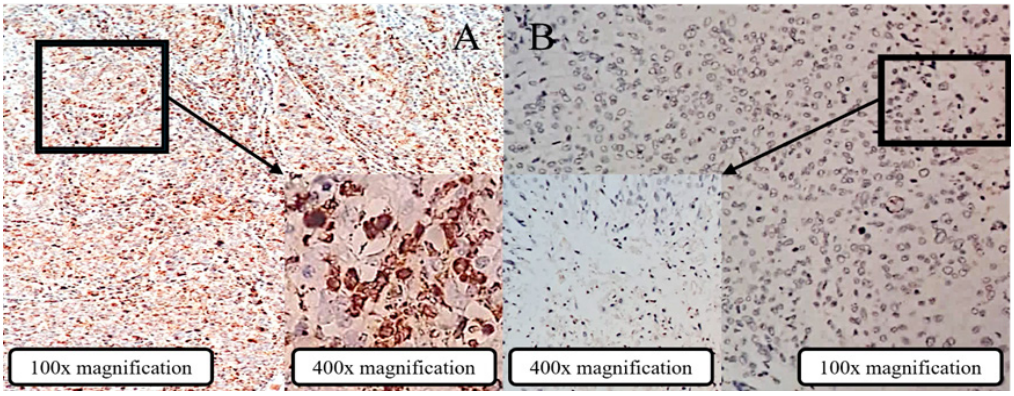

Figure 4. *MMP-9* Expression in HGTB and LGTB Groups. (A) HGTB showing high *MMP-9* expression, with positive staining in >10% of tumor cells (*MMP-9*, 100x and corresponding 400x magnification). (B) LGTB showing low *MMP-9* expression, with positive staining in <10% of tumor cells (*MMP-9*, 100x and corresponding 400x magnification).

Table 5. Expression of *MMP-9* in the Case and Control Groups

Variable	Tumor Budding		OR 95% CI	SN	SP	p-value
	HGTB (n = 20)	LGTB (n = 20)				
MMP-9			10.524	65.00%	85.00%	0.001*
High	13 (65.0%)	3 (15.0%)	(2.271-48.757)			
Normal	7 (35.0%)	17 (85.0%)				

*Analysis was carried out using Pearson's Chi-Square Test. SD, Standard deviation; SN, sensitivity; SP, specificity. The result was considered significant if the p-value≤0.05.

increase vimentin and N-cadherin. *miR-21* strengthens the TGF-β-SMAD cascade and suppresses *PTEN*, thus activating *PI3K/AKT,* which accelerates tumor budding, migration, and invasion of cervical cancer cells [37, 38].

This study shows that high expression of mutant *p53* increases the risk of HGTB in cervical carcinoma, while wild-type *p53* expression remains low in both groups and does not show significant results towards TB. These findings support that mutant *p53*, rather than decreased wild-type *p53*, might play a key role in this process. Molecularly, *TP53* mutations activate the JNK/c-JUN, SRC/FAK, and SRC/ERK pathways, triggering proliferation, stromal remodeling, EMT, and increasing the secretion of *miR-21*-carrying exosomes [25, 39–41]. Once established, budding cells affected by mutant *p53* produce pro-tumor factors such as TGF-β, VEGF, IL-6, CXCR4, and MMPs that reinforce the invasive environment. Mutant *p53* also suppresses the function of wild-type *p53*, activates SLUG, TWIST, RhoA/ROCK, and Toca-1, and, together with stromal cells, enhances metastatic potential through various growth mediators [24, 25, 39, 42].

This study found that high *MMP-9* expression significantly increases the risk of HGTB in cervical carcinoma, supporting its role as an independent risk factor for TB. No previous studies have specifically assessed this association in cervical carcinoma, but studies in colorectal and breast cancer have shown similar results, although González et al. reported different results in invasive ductal carcinoma of the breast. Biologically, *MMP-9* degrades the extracellular matrix, particularly collagen IV, and an imbalance between MMP and TIMPs promotes invasion and facilitates EMT [43–46]. Matrix metalloproteinase-9 is produced by budding cells, stromal cells, and inflammatory cells surrounding tumors. The interaction of uPA and cathepsin B increases *MMP-9* production, activates the MAPK pathway, inhibits DNA repair, and promotes proliferation and progression of TB in cervical carcinoma [26, 45].

These three factors likely represent distinct mechanisms, namely epigenetics by *miR-21*, intracellular genetics by mutant *p53*, and extracellular proteins by *MMP-9*. This study demonstrated that all three are associated with an increased risk of HGTB in cervical carcinoma, but in multivariate analysis, only mutant *p53* and *MMP-9* remained independently associated. Mutant *p53* had the strongest effect, followed by *MMP-9*, through loss of tumor suppressor function and gain-of-function, which promotes cancer cell proliferation and migration [25, 47]. Mutant *p53* is also known to disrupt the activity of wild-type *p53* by forming nonfunctional tetramers, and facilitates EMT through activation of SLUG and TWIST that repress E-cadherin and anti-invasive genes such as CCN-S/WISP2 [24, 39]. Additionally, *MMP-9*, as gelatinase B, plays a role in collagen IV degradation and ECM remodeling, and enhances cell migration through modulation of EMT [26], and is also increased in hypoxic conditions due to tumor proliferation, and is influenced by an imbalance with TIMPs [46]. Although not independently significant, *miR-21* is still suspected to contribute to the formation of HGTB through the suppression of various tumor suppressor genes, which can indirectly increase the expression of *MMP-9* and the activity of mutant *p53* [48, 49]. *miR-21* is also known to enhance the activation of the TGF-β/SMAD pathway, which decreases *E-cadherin* and increases the expression of *ZEB, SNAIL*, and *TWIST* [37, 41]. This study found that the formation of HGTB in cervical carcinoma follows two main stages of EMT, which are supported by the complex interaction of *miR-21*, mutant *p53*, and *MMP-9* [10, 14].

This study showed no significant differences between the case and control groups in age, tumor size, clinical stage, histopathological type, TIL, or grade. Although there are reports of associations between tumor size and stage with TB, in this study, neither affected the analysis of *miR-21*, *p53*, and *MMP-9* expression in HGTB [7, 17, 18, 19, 50]. Additionally, the histopathological type of SCC dominated both groups without significant differences, in line with the WHO report (2020) [51], and was not consistently shown to affect the degree of TB according to the studies of Huang et al. and Park et al. [7, 18] Low TIL grade also dominated both groups, but was not significantly different and was considered to have been controlled in this study. Although previous studies have stated the relationship between low TIL and increased metastasis and TB [52, 53], this study did not assess the independent relationship of TIL, but rather assumed that the role of the body's immune system against tumors was controlled through lymphocyte homogeneity. Therefore, the expression of *miR-21*, *p53*, and *MMP-9* can be analyzed more objectively as factors that influence HGTB.

The main strength of this study is the successful development of an HGTB risk index based on multivariate regression analysis. However, the retrospective case-control design with a small sample size limits the generalizability of the findings and likely contributes to the wide confidence intervals. The use of archival paraffin blocks may also impact the quality of biomarkers, particularly *miR-21*, which is susceptible to RNA degradation. This study did not assess other factors, such as HPV status and other EMT biomarkers. Because it is observational, a causal relationship cannot be established, and larger prospective studies are needed.

This study found that mutant *p53* and high *MMP-9* expression were independently associated with the risk of HGTB in cervical carcinoma. These findings add to the body of evidence supporting TB as a biologically meaningful marker of tumour aggressiveness in cervical carcinoma and encourage further evaluation of its role in prognostic grading systems. However, because *miR-21* did not show an independent association, further studies of other microRNAs are needed. Yet, *miR-21* may still be relevant as part of a broader microRNA signature.

Author Contribution Statement

NWA was responsible for concepting the idea and method. NWA and DPOL contribute to the study procedures. NWA and IGWWW were responsible for the statistical analysis. NWA, DPOL, and IGWWW contributed to the manuscript writing. NWA, DPOL, and IGWWW contributed to manuscript inspection and result

evaluation.

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Approval

This study has received approval for the feasibility of the Research Ethics Commission of the Faculty of Medicine, Udayana University, in accordance with the Research Permit from BaliMed Hospital, Denpasar, and Mangusada Regional Hospital, Badung Regency.

Ethical Declaration

This study has received approval for the feasibility of the Research Ethics Commission of the Faculty of Medicine, Udayana University, in accordance with the Research Permit Letter No: 1143/TU/RSBM/EXT/XII/2022 from BaliMed Hospital, Denpasar, and No: 445/7506/RSDM/2023 from Mangusada Regional Hospital, Badung Regency.

Data Availability

The data supporting the findings of this study are available in the supplementary file and upon request from the corresponding author.

Study Registration

Research Permit Letter No: 1143/TU/RSBM/EXT/XII/2022 from BaliMed Hospital, Denpasar, and No: 445/7506/RSDM/2023 from Mangusada Regional Hospital, Badung Regency.

Conflict of Interest

None declared.

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