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Potential Oncogenic Role and Prognostic Implication of MicroRNA-155-5p in Oral Squamous Cell Carcinoma

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Abstract. *Background: Altered microRNA expression is associated with cancer progression. This study investigated the prognostic significance of microRNA-155-5p (miR-155-5p), a well-known oncomiR, in oral squamous cell carcinoma (OSCC). Materials and Methods: miR-155-5p expression was assessed using quantitative reverse-transcription polymerase chain reaction in 68 formalin-fixed, paraffin-embedded OSCC specimens. E-Cadherin immunohistochemistry was conducted to correlate epithelial–mesenchymal transition (EMT) with miR-155-5p expression. Results: Elevated miR-155-5p was associated with higher pathological TNM stage (p=0.048) and relapse (p=0.029). High miR-155-5p expression, along with angiolymphatic invasion and advanced stage, was a statistically significant prognostic factor for poorer disease-free survival. In patients with stage I-II disease, high miR-155-5p was the only significant prognostic factor (p=0.033). A significant negative correlation was observed between miR-155-5p and E-cadherin expression (p=0.015), suggesting a possible role for miR-155-5p in EMT. Conclusion: miR-155-5p expression might contribute to EMT-associated OSCC progression and serve as a biomarker for predicting relapse, especially for patients with early-stage OSCC.*

Oral squamous cell carcinoma (OSCC) comprises a set of diseases that affect multiple anatomical organs of the oral cavity; it is the sixth most common cancer worldwide, accounting for approximately 5% of all malignant tumors (1). Despite surgical treatment combined with adjuvant chemoradiation, the direct cause of fatality from OSCC is uncontrollable local recurrence at the surgical site or distant metastasis. The current rate of local and locoregional recurrences is 10-30% in patients with OSCC, and the mean overall 5-year tumor-free survival rate does not exceed 50% (2). It remains difficult to control locoregional recurrence and distant metastasis after standard treatment, particularly in those with early-stage cancer. In addition, the lack of clinically proven biomarkers can lead to treatment decisions being made based only on clinicopathological factors. However, disease progression and outcomes for patients with the same clinicopathological features can vary substantially (3). For this reason, there is a growing need for biomarkers that can classify patients at high risk of recurrence in order to facilitate stricter management.

Recent studies have emphasized that microRNAs (miRNAs) play an essential role in the initiation and development of various human cancer types, including carcinoma of the oral cavity or head and neck, as well as physiological processes (4-7). MicroRNA-155-5p (*miR-155-5p*), regarded as a proto-oncogene, is encoded by the human B-cell integration cluster gene, identified as a common retroviral integration site in avian-leukosis virus-induced B-cell lymphomas (8-10). The oncogenic role of miR-155-5p was first suggested for hematopoietic tumors based on the observation that its transgenic expression in B-cells initiated a chain of events that led to high pre-B-cell accumulation and acute lymphoblastic leukemia/high-grade lymphoma (11, 12). *MiR-155-5p* is also up-regulated in many solid tumor types, including breast, liver, lung, pancreas, and cervical cancer (13-17). Furthermore, increased *miR-155-5p* expression is correlated with poor prognosis in lung and colorectal cancer (18, 19). Additionally, *miR-155-5p* is up-regulated in head and neck squamous carcinoma (HNSCC) including OSCC (6, 20-23). Thus, several reports have suggested the prognostic value of *miR-155-5p* expression in patients with OSCC (6, 22, 23).

In this study, we aimed to clarify the clinicopathologicaI significance of *miR-155-5p* in OSCC and investigate its

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Key Words: Epithelial–mesenchymal transition, microRNA, miR-155, oral cancer, relapse.

Table I. *Clinicopathological features of oral squamous cell carcinoma according to miR-155-5p expression level (n=68).*

Variable		High *miR-155-5p* (≥median)	Low *miR-155-5p* (<median)	Value	*p*-Value
Age, years	Mean (range)	59.6 (31-80)	55.8 (23-84)	57.7 (23-84)	0.307
Gender, n (%)	Female	13 (38%)	10 (29%)	23 (34%)	0.609
	Male	21 (62%)	24 (71%)	45 (66%)	
Primary site, n (%)	Oral tongue	17 (50%)	22 (65%)	39 (58%)	0.379
	Buccal mucosa	9 (26%)	4 (12%)	13 (19%)	
	Mouth floor	5 (15%)	3 (9%)	8 (12%)	
	Retromolar trigone	3 (9%)	4 (12%)	7 (10%)	
	Upper alveolar ridge	0 (0%)	1 (3%)	1 (1%)	
Tumor size, mm	Mean (range)	30.3 (12-85)	27.6 (15-65)	28.9 (12-85)	0.460
Angiolymphatic invasion, n (%)	Absent	22 (65%)	27 (79%)	49 (72%)	0.280
	Present	12 (35%)	7 (21%)	19 (28%)	
Perineural invasion, n (%)	Absent	25 (74%)	24 (71%)	49 (72%)	1.000
	Present	9 (26%)	10 (29%)	19 (28%)	
P16 expression, n (%)	Positive	2 (6%)	5 (15%)	7 (10%)	0.427
	Negative	32 (94%)	29 (85%)	61 (90%)	
Pathological T-stage, n (%)	pT1	8 (24%)	13 (38%)	21 (31%)	0.069
	pT2	12 (35%)	14 (41%)	26 (38%)	
	pT3	1 (3%)	3 (9%)	4 (6%)	
	pT4a	13 (38%)	4 (12%)	17 (25%)	
Pathological N-stage, n (%)	pN0	21 (62%)	21 (62%)	42 (62%)	0.139
	pN1	5 (15%)	10 (29%)	15 (22%)	
	pN2b	8 (24%)	3 (9%)	11 (16%)	
Pathological TNM stage, n (%)	I	7 (21%)	11 (32%)	18 (27%)	0.048
	II	8 (23%)	9 (26%)	17 (25%)	
	III	2 (6%)	7 (21%)	9 (13%)	
	IVA	17 (50%)	7 (21%)	24 (35%)	
Relapse, n (%)	No relapse	20 (59%)	29 (85%)	49 (72%)	0.029
	Relapse	14 (41%)	5 (15%)	19 (28%)	
Total		34 (100%)	34 (100%)	68 (100%)	

*Largest dimension.

prognostic implications. To this end, *miR-155-5p* expression levels were examined in resected OSCC samples and the relationship between *miR-155-5p* expression and disease-free survival (DFS) was analyzed. Finally, we aimed to establish a model for recurrence prediction, especially for early-stage OSCC.

Materials and Methods

Patients and samples. A total of 68 patients with OSCC who underwent surgical resection at Seoul National University Bundang Hospital between 2003 and 2011 were enrolled. Patients who received preoperative chemotherapy or radiotherapy were excluded. Clinicopathological information was obtained by reviewing the medical records and pathology reports. The pathological tumor stage was determined according to the guidelines in the Cancer Staging Manual of the American Joint Committee on Cancer (seventh edition) (24). This study protocol was approved by the Institutional Review Board of Seoul National University Bundang Hospital (B-1502-288-301).

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Reverse transcription and real-time PCR were performed as previously described (25). Total RNA was extracted from 10-μm-thick formalin-fixed, paraffin-embedded tissue sections using RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA, USA). After measuring the RNA concentration with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), the RNA was stored at −80˚C until further use. To measure the relative expression level of hsa-*miR-155-5p* (Applied Biosystems), reverse transcription and qPCR were performed using 10 ng of total RNA, Universal PCR Master Mix, and TaqMan microRNA Reverse Transcription kit (Applied Biosystems). The threshold cycle (Ct) value was normalized to that of *U6* snRNA, used as a reference (*i.e.* ΔCt (miR, cancer)=Ct (miR, cancer) − Ct (*U6*, cancer), and the value was adjusted based on the expression level of miRNA in reactive tonsil tissue [ΔCt (miR, normal)]. The relative expression level of *miR-155-5p* in OSCC was then calculated as $2^{-\Delta\Delta Ct}$, where ΔCt=Ct (*miR-155-5p*) − Ct (*U6*), and ΔΔCt=ΔCt (tumor) − ΔCt (normal).

Immunohistochemistry (IHC). IHC was conducted using the Ventana Benchmark XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA) on tissue microarray sections (4-μm) with a primary antibody against E-cadherin (1:150, clone SPM471; Thermo Fisher Scientific). E-Cadherin IHC was scored by

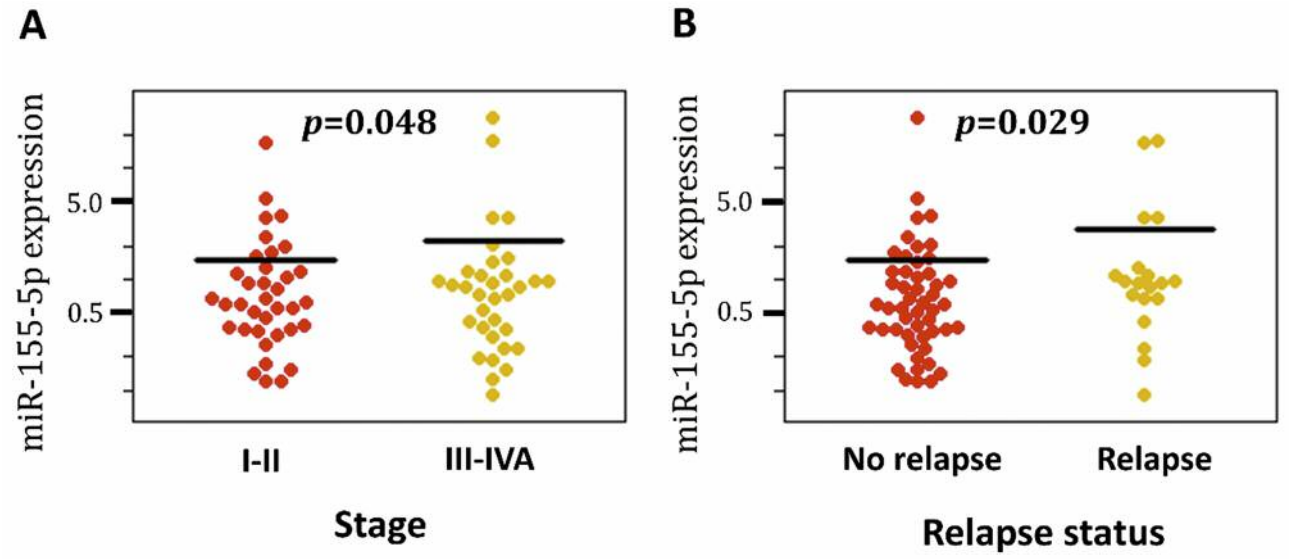

Figure 1. *Relative miR-155-5p expression level in oral squamous cell carcinoma according to stage (A) and relapse status (B). Horizontal lines indicate mean values. Vertical axes are plotted on logarithmic scale.*

multiplying the percentage of positively stained tumor cells (0-100%) by staining intensity (0, negative; 1, weak; 2, moderate; 3, strong) to generate a total score (H-score) ranging from 0-300 for each sample (26).

Statistical analysis. Spearman correlation, Chi-square test, and Fisher's exact test were used to evaluate the association between *miR-155-5p* expression levels and clinicopathological variables including E-cadherin expression in OSCC. Kaplan–Meier analysis, log-rank tests, and multivariate Cox proportional hazards regression were used for survival analysis. *p*-Values of less than 0.05 were considered statistically significant (two-tailed). All analyses were performed using SPSS version 21.0 (IBM, Armonk, NY, USA).

Results

Patient characteristics. The clinicopathological characteristics of the 68 patients are listed in Table I. Briefly, there were 23 women (34%) and 45 men (66%), with a mean age of 57.7 years (range=23-84 years). Primary sites were mainly identified in the tongue (n=39, 58%), followed in order by buccal mucosa, mouth floor, retromolar trigone, and upper alveolar ridge. The pathological TNM stage was I in 18 (27%) patients, II in 17 (25%) patients, III in nine (13%) patients, and IVA in 24 (35%) patients. Nineteen patients (28%) experienced relapse after curative surgery.

Associations between clinicopathological features and miR-155-5p expression. Patients were dichotomized into groups with high or low miR-155-5p expression based on the median expression value, and the association between miR-155-5p expression status and clinicopathological parameters was evaluated (Table I). The *miR-155-5p* level was positively associated with pathologic TNM stage (p=0.048; Figure 1A) and disease relapse (p=0.029; Figure 1B), whereas associations with sex, primary site, angiolymphatic invasion, perineural invasion, and p16 expression status were not observed.

Survival analyses of the entire cohort and the subgroup with early-stage disease. As shown in Table II, when considering the entire cohort, high *miR-155-5p* expression was a statistically significant prognostic factor for poor DFS (p=0.028; Figure 2A), along with the presence of angiolymphatic invasion (p=0.001) and advanced pathologicaI stage (III and IVA; p=0.019; Figure 2B). Multivariate analysis revealed that the presence of angiolymphatic invasion was an independent prognostic factor for poor DFS with marginal statistical significance (hazard ratio=2.656, p=0.050), whereas *miR-155-5p* level was not. Survival analysis of patients with early-stage (stage I and II) OSCC revealed that high miR-155-5p expression was the only prognostic factor for poor DFS (p=0.033; Figure 2C), while pT stage (p=0.339; Figure 2D) and other clinicopathological factors were not associated with DFS in this subgroup.

Association between miR-155-5p and E-cadherin expression. We next investigated the possible mechanism of high *miR-155-5p* expression and shorter DFS in early-stage OSCC. We hypothesized that epithelial–mesenchymal transition (EMT) might have a role in tumor aggressiveness and analyzed the association between *miR-155-5p* and expression of

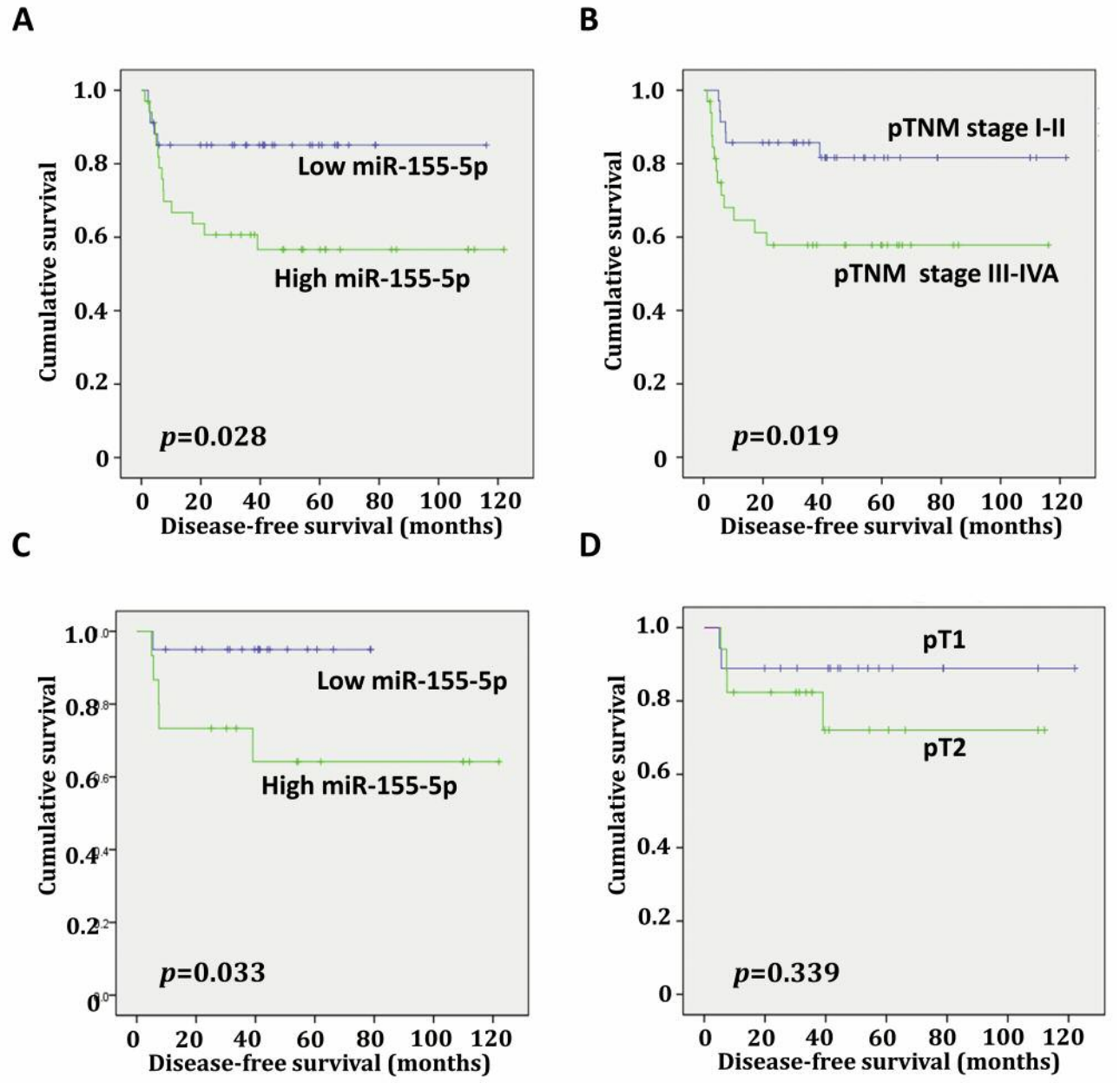

Figure 2. *Kaplan–Meier survival curves of disease-free survival in the full cohort (n=68) according to miR-155-5p expression (A) and pTNM stage (B), and in the early-stage subgroup (n=35) according to miR-155-5p expression (C) and T-stage (D). The median value of the full cohort was used to divide patients into low- and high-expression groups according to miR-155-5p expression.*

E-cadherin, a representative marker of EMT. As shown in Figure 2, *miR-155-5p* expression was negatively correlated with H-score of E-cadherin expression (Spearman's rho=−0.299, p=0.015; Figure 3A). Furthermore, 'loss' of E-cadherin expression, defined by an H-score of 0-100, was significantly correlated with high *miR-155-5p* expression (higher than median; p=0.015 by Fisher's exact test; Figure 3B), as shown in representative cases (Figure 3C and D).

Discussion

To date, reliable biomarkers have not been established to predict relapse and prognosis in early-stage OSCC. The best-known prognostic marker is T-stage, but it did not predict relapse successfully in the early-stage subset of our cohort. In the present study, based on the clinicopathological role of *miR-155-5p*, a well-known oncogenic miRNA in OSCC, we

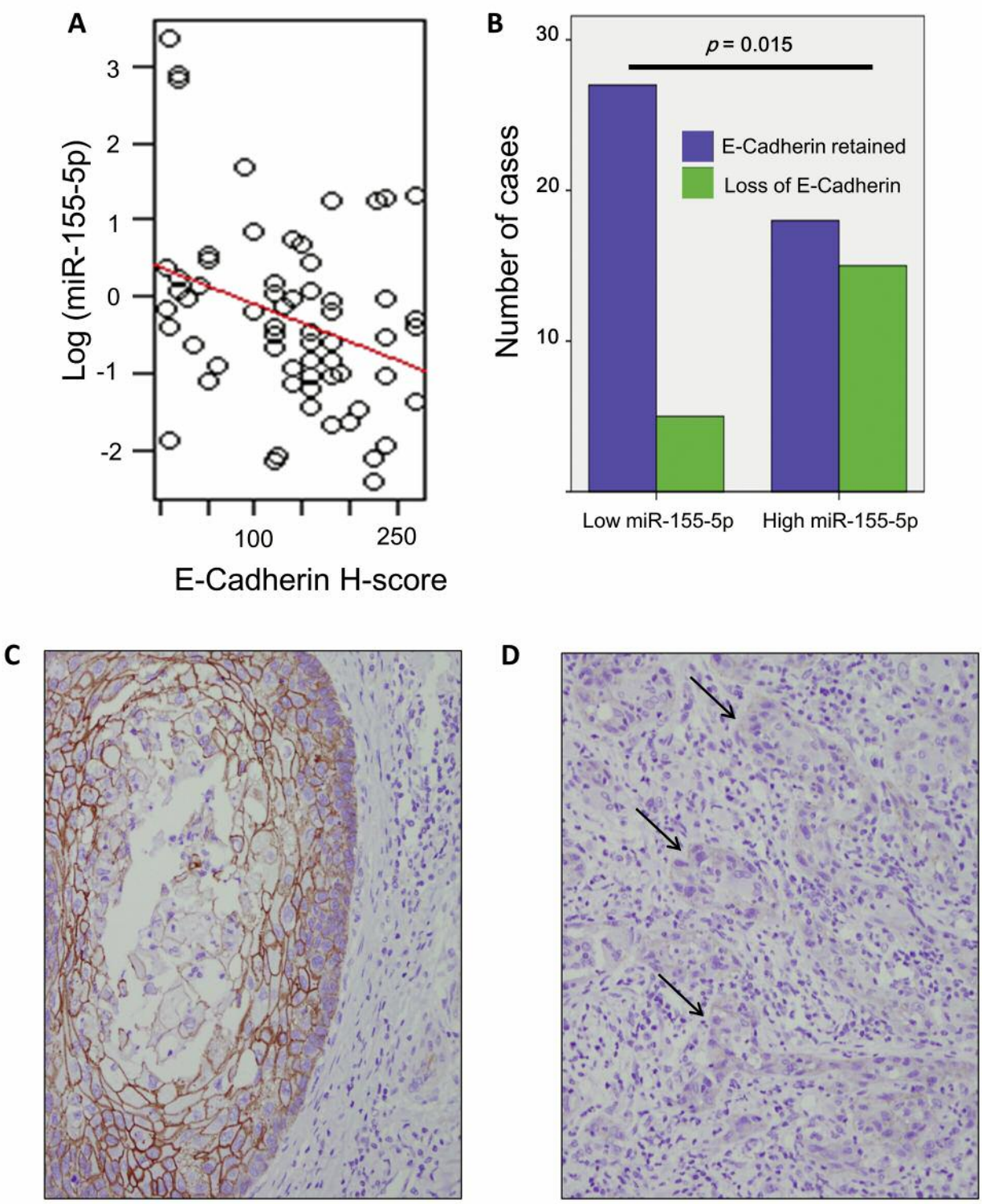

Figure 3. *Relationship between miR-155-5p and E-cadherin expression in oral squamous cell carcinoma samples. Dot plot (A) and bar graph (B) showing E-cadherin expression is inversely correlated with miR-155-5p. Representative cases are shown with immunohistochemical staining of E-cadherin. A case of well-differentiated squamous cell carcinoma with low miR-155-5p showing E-cadherin expression with distinct membranous staining and epithelial architecture (C), while a case of poorly differentiated squamous cell carcinoma with high miR-155-5p exhibits loss of E-cadherin expression and morphological resemblance of tumor cells to stromal cells, suggesting epithelial–mesenchymal transition (arrows) (D). (C) and (D) show immunohistochemical staining with anti-E-cadherin antibody counterstained with hematoxylin (×200 magnification).*

observed that: i) *miR-155-5p* was the only predictor of relapse in patients with early-stage OSCC, and ii) *miR-155-5p* expression level was inversely correlated with E-cadherin expression, suggesting that *miR-155-5p*-induced EMT might be an underlying mechanism of relapse in OSCC.

The oncogenic role of *miR-155-5p* has been studied in multiple tumor types (13, 27, 28). One of the mechanisms of *miR-155-5p*-induced tumor progression is EMT, the phenotypic change of epithelial cells into mesenchymal cells (29, 30). EMT is an early event in the multistep process of invasion and metastasis (31), and expression of E-cadherin, a key marker of the epithelial phenotype, is lost during this process (32). E-Cadherin anchors neighboring cells to each another to form adherens junctions, and loss of this marker is required for tumor cell migration (33). Furthermore, EMT is a key mechanism of cancer cell invasion. Cancer cells that have undergone EMT easily invade adjacent tissue *via* the action of matrix metalloproteinases (34). In addition, EMT is closely associated with cancer stem cells (35). In several types of carcinoma, relapse after surgical resection is correlated with a subpopulation of circulating tumor cells with an EMT phenotype (36-38). In this context, it is remarkable that *miR-155-5p* was associated with DFS and inversely correlated with E-cadherin expression in the present study. These findings suggested that OSCC relapse is mediated, at least in part, through *miR-155-5p*-induced EMT and associated invasiveness, especially in early-stage disease.

The mechanism of *miR-155-5p*-induced EMT in hepatocellular carcinoma tissue and cell lines is well known. *miR-155-5p* promotes EMT by inducing transforming growth factor β1 (29) or through the phosphoinositide 3-kinase/serum and glucocorticoid-regulated kinase 3/β-catenin signaling pathway (30). In OSCC, Baba *et al*. reported that both suppressor of cytokine signaling 1 (SOCS1) increased and signal transducer and activator of transcription 3 (STAT3) decreased in HSC-3 OSCC cells transfected with a *miR-155-5p* inhibitor, suggesting that the inhibition of *miR-155-5p* function down-regulates STAT3 by activating SOCS1 (23), thereby suppressing EMT (23). In another study, Zeng *et al*. noted that miR-155 down-regulated BCL6 expression and increased cyclin D2 expression, facilitating proliferation, migration, and invasion of CAL27 OSCC cells (39). It is known that BCL6 induces EMT by promoting the Zinc finger E-box binding homeobox 1 (ZEB1)-mediated transcriptional repression of E-cadherin in breast cancer cells (40). In accordance with these studies, our data from OSCC tissue samples, showing the quantitative association between *miR-155-5p* and E-cadherin expression, relapse, and DFS, indicate that *miR-155-5p* might act as a key modulator to determine aggressiveness and risk for relapse in OSCC.

During the treatment and management of patients with OSCC who undergo curative surgery, risk stratification for relapse, especially in those with early-stage disease, is critical for estimating survival. The current National Comprehensive Cancer Network guidelines recommend surgical resection for TNM stage I and II OSCC, and additional therapy is not recommended unless the disease progresses (41). In this study, we found that *miR-155-5p* has a predictive value for identifying relapse independently of clinicopathological indicators in early-stage OSCC; therefore, patients with stage I or II disease with elevated *miR-155-5p* expression might be suitable for earlier or more aggressive intervention. Thus, further studies are warranted and required to address this important area of research.

In conclusion, we found high *miR-155-5p* expression to be associated with shorter DFS, especially in patients with early-stage OSCC. Furthermore, we observed a strong, inverse relationship between *miR-155-5p* and E-cadherin expression, which suggests that OSCC relapse is mediated, at least in part, through miR-155-5p-induced EMT and EMT-mediated invasion, especially in early-stage disease. These data suggeste that increased *miR-155-5p* expression might be responsible for the aggressive nature and rapid relapse of early-stage OSCC. Therefore, *miR-155-5p* expression could potentially act as a biomarker predicting relapse for such patients.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

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