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Original Article

MicroRNA-21 promotes perineural invasion and impacts survival in patients with oral carcinoma

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Abstract

Background: Perineural invasion is a pathological feature that may affect cancer cell progression and thus can result in prognostic impacts, especially in oral squamous cell carcinoma (OSCC). However, factors regulating perineural invasion during OSCC remain obscure.
Methods: Expression of *miR-21* and phosphatase and tensin homolog was checked in surgical specimens from cases of OSCC. The results were analyzed for histopathologic factors, including perineural invasion and clinical prognosis.
Results: One-hundred cases of OSCC patients were enrolled in this study. High expression of *miR-21* was related to perineural invasion and worse prognosis in OSCC patients.
Conclusion: *miR-21* was an independent factor of disease survival of OSCC. *miR-21*/phosphatase and tensin homolog disregulation was related to perineural invasion.

Keywords: miR-21; oral carcinoma; perineural invasion; prognosis

1. Introduction

Incidence of oral cancers accounts for ~3% of all malignancies. Oral squamous cell carcinoma (OSCC) accounts for >95% of all malignancies in the oral cavity,[1] and it is the fifth leading cause of cancer death in the male population in Taiwan.

OSCC is treated mainly with surgery. Postoperative adjuvant treatment, (radiotherapy or chemo-radiotherapy) is followed according to the pathological results. The pathological results include several clinicopathological factors: tumor size and nodal metastasis (the TNM system), lymphovascular permeation, perineural invasion, tumor invasion front, differentiation, and tumor depth.[2] This means that identifying the clinicopathologic factors is important to the survival of OSCC patients. Unfortunately, these data were collected at the tissue level, not the cellular level, and there was no reliable method to detect those factors in the surgical specimens except microscopic examination by a pathologist. That suggested to us that small cancer with aggressive behavior could be undertreated because such poor prognosis factors might not be detected in the early stages.

MicroRNAs (miRNA) are small noncoding RNAs that mediate the translational repression of target messenger RNAs.[3] miRNA disruption is known to play a number of important roles in the neoplastic process, including OSCC.[4] *miR-21*, which functions as an oncogene, is noticeably upregulated in most human malignancies, including OSCC.[5,6] *In vivo* and *in vitro* studies have also found that *miR-21* is

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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able to serve as a diagnostic/prognostic marker for cancer therapy.[7] In addition, recent studies have shown that *miR-21* was expressed in the stromal cells of OSCC that had arisen from the tongue and floor of the mouth.[8] Phosphatidylinositol-3-kinase (PI3K)/AKT has been shown to play important roles in the neoplastic process, especially in the regulation of proliferation and invasion by tumor cells.[9]

Only a limited number of studies have ever explored the correlation of miRNAs and the clinicopathological factors. In this study, we investigated the relationship between the expression of *miR-21* and clinicopathologic factors in OSCC and explored its possible mechanism. Our purpose was to find a more specific cellular level factor, not the clinicopathological factor, to indicate the prognosis of OSCC patient.

2. Methods

2.1. Ethics statement

This study was approved by the Institutional Review Board of the National Yang-Ming University Hospital (approval number: 2013B01) and adhered to the tenets of the Declaration of Helsinki. Written informed consent for the publication of case details was obtained from every patient.

2.2. Patients

OSCC cases between the years 2008 and 2012 in the National Yang-Ming University Hospital and Taipei Veterans General Hospital were retrospectively reviewed in this study. Inclusion criteria were: (1) all cases were an initial diagnosis of OSCC and had undergone no previous treatment; (2) all cases received surgery and then the surgical specimens from the primary lesions were fully evaluated in order to determine that there was no evidence of a positive resection margin; and (3) all cases were followed for at least 1 year. A total of 100 cases were included in this study. The clinical staging for presurgical diagnosis was made based on a physical examination, together with a head and neck computed tomographic scan, a chest x-ray or a chest computed tomographic scan, an abdomen sonography examination, and a Tecnicium-99 (Pharmalucence, Bedford, MA, USA) whole- body bone scan; these were able to eliminate any possibility of distant metastasis or secondary malignancy. A treatment modality was planned for each patient according to the pretreatment cTNM stage. All received primary surgery plus neck dissection (selective or modified neck dissection was determined by the preoperation examination). Adjuvant therapy, including radiotherapy or concurrent chemoradiotherapy, was carried out according to the National Comprehensive Cancer Network guidelines.

Histopathological factors, including differentiation, lymphovascular permeation, invasion front, depth, and perineural invasion (PNI), were recorded. Regular follow-up was arranged for each patient. The follow-up outcomes, including local recurrence, regional/distant metastasis, and new occurrence of oral neoplasia, were also recorded on the patient's chart.

2.3. In situ hybridization

The miRCURY LNA *miR-21* probe labeled with digoxigenin (Catalogue #35065-01) and all associated reagents were purchased from Exiqon Ins. (Vedbaek, Denmark). After rehydration, the paraffin sections of tumors were digested using protease K and fixed with 4% paraformaldehyde. The sections were then prehybridized for 4 hours, followed by hybridization with a 10-μM *miR-21* probe at 45°C overnight.[10] Next, the slides were washed and incubated with an anti-digoxigenin antibody at room temperature for 3 hours. Finally, 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium reagent was used to detect the signal in the samples. Scramble assays were carried out using the same protocol and with a scrambled probe (Catalogue #300514-15; Exiqon) as hybridization controls.

The intensity of the *miR-21* staining was determined based on the percentage of positive cells in at least five randomly selected high-power fields from each specimen. Furthermore, a scoring system was also used to classify the intensity of each sample, which were graded as follows: low (score = 1): 0−33% positive cells; medium (score = 2): 34−66% positive cells; high (score = 3): 64−100% positive cells.[8] Sample scores of low or medium were defined as having lower *miR-21* expression.

2.4. Immunohistochemistry

Tissue sections were processed and incubated with primary antibody at 4°C overnight in a humid chamber. Antibodies against phosphatase and tensin homolog (PTEN; Catalogue #MA5-12278; Thermo Fisher Scientific, Waltham, MA, USA) at 1:75 dilution and phosphor(ylated)-S6 (Catalogue #2211S; Cell Signaling Technology, Danvers, MA, USA) at 1:100 dilution were used for the immunohistochemistry (IHC).[11] The immunoreactions were performed using a LSAB2 streptavidin−biotin complex system (Dako, Carpinteria, CA, USA). Preimmunized mouse immunoglobulin-G was used as a negative control. The epithelial tumor cells present in the OSCC samples were examined with microscopy to detect immunoreactivity. Tumors exhibiting PTEN and phosphor-S6 immunoreactivity were scored by the same scoring system that was used to analyze *miR-21* expression.

2.5. Statistical analysis

Kaplan-Meier analysis was used to assess disease-free survival, which was defined as the time from study entry to tumor recurrence, regional/distant metastasis, or the occurrence of a new primary tumor. Differences were assessed by Log-rank test. Cox's proportional hazards regression analysis was performed to determine potential prognostic factors for survival, the hazard ratios and 95% confidence intervals. Fisher's exact test was used to analyze binary data. Mann−Whitney test was used to compare the differences in *miR-21* between subsets. Correlation was tested by Spearman's coefficient of rank correlation. Two-way analysis of variance test was used to evaluate differences in the cell migration data.

Differences were considered statistically significant when $p < 0.05$. Statistical analysis was performed with SPSS 17.0 (SPSS, Inc. Chicago, IL, USA) and GraphPad Grism 5 (GraphPad Software, Inc. La Jolla, CA, USA).

3. Results

3.1. Characteristics of the patients and their clinical outcomes

The characteristics of the patients are presented in Table 1. In total, 100 patients were selected from a cohort of 195 patients enrolled from 2008 to 2012 using the criteria described in Materials and methods. The male:female ratio was about 11.5:1, and the average age was 55.3 years. Seventy patients had an advanced tumor size (T3 or T4). Among patients who had neck lymph node metastasis ($n = 40$, 40%), 22 had ipsilateral one-node metastasis, 12 had ipsilateral multiple-node metastasis, and six had bilateral or contralateral node metastasis. After surgery, 32 patients received adjuvant radiotherapy and 22 patients had adjuvant concurrent radiochemical therapy.

Table 1
Clinicopathological characteristics of oral squamous cell carcinoma patients and the relationship with *miR-21*.

	n	*p* Univariate analysis[a]	*p* Multivariate analysis[b]
Sex			
Male	92	0.327	
Female	8		
Age (y)			
≦55	56	0.670	
>55	44		
T classification			
T1, 2	30	0.681	
T3, 4	70		
N classification			
N0	60	0.001	0.456
N+	40		
Stage			
I and II	23	0.722	
III and IV	77		
Location			
Buccal mucosa	37	0.521	
Tongue	35		
Mouth floor	12		
Others	16		
Differentiation (major)			
Well	47	0.351	
Moderate or poor	53		
Perineural invasion			
Negative	72	<0.0001	< 0.0001
Positive	28		
Lymphovascular permeation			
Negative	57	0.051	0.751
Positive	43		
Invasion front			
Pushing	41	0.112	
Infiltration	59		
Depth of invasion (mm)			
≤4	22	0.080	
>4	78		

[a] Mann-Whitney test.
[b] Logistic regression.

The median follow-up time for patients was 38.5 months. At the time of analysis, 60 (60%) patients were alive without disease, and 31 (33%) were deceased (30 due to the primary cancer, and one due to noncancer causes). Furthermore, among the patients, 19 had developed local recurrence, 12 had developed neck recurrence, four had developed local and neck recurrence, and five had developed distant metastasis.

3.2. High miR-21 expression had reverse correlation with PTEN expression in the tumor samples and with worse prognosis in OSCC patients

miR-21 staining was mainly found in the cytoplasm, but was occasionally present in nuclei of tumor cells (Fig. 1). Among the various tumor samples, 38 tumors showed low expression (including 12 cases with no *miR-21* expression; Fig. 1A); 32 tumors showed medium expression (Fig. 1B); and 30 tumors had high expression (Fig. 1C).

Positive cytoplasmic immunoreactivity against PTEN was detected in 75 (75%) tumors. However, most of the tumors ($n = 59$, 59%) had low expression (Fig. 2C), and only 16 (16%) had medium expression (Fig. 2D). Notably, no tumor exhibited a high PTEN expression in this cohort. In order to investigate the correlation between the expression of *miR-21* and the expression of PTEN, Spearman's test was performed. A significant reverse correlation was found between *miR-21* staining and PTEN immunoreactivity (Spearman's rho $= -0.48$, $p < 0.0001$, 95% confidence interval $= -0.63$ to -0.30).

The phosphor-S6 immunoreactivity was generally highly intense, and no tumor in this cohort had negative or low positive phoshor-S6 immunoreactivity (not shown). The correlation relationship between *miR-21* expression and phosphor-S6 immunoreactivity or between PTEN expression and phosphor-S6 immunoreactivity was not statistically significant ($p = 0.81$ and $p = 0.73$, respectively).

The disease-free survival was significantly different between the high, medium, and low *miR-21* groups. Kaplan–Meier analysis showed poor prognosis in the higher *miR-21* group ($p = 0.033$; Fig. 1D).

3.3. Association between miR-21 and clinicopathological factors

The Mann–Whitney test was used to evaluate the association between the expression scores for *miR-21* and clinicopathological factors (Table 1). Only nodal status and PNI showed statistically significant association with *miR-21* intensity. Lymphovascular permeation showed borderline significant ($p = 0.051$). Other factors, including tumor size, stage, differentiation, invasion front, and invasion depth were not found to be associated with either *miR-21* expression.

Logistic regression was used as multivariate analysis. The result showed that PNI was the only independent factor for higher *miR-21* expression.

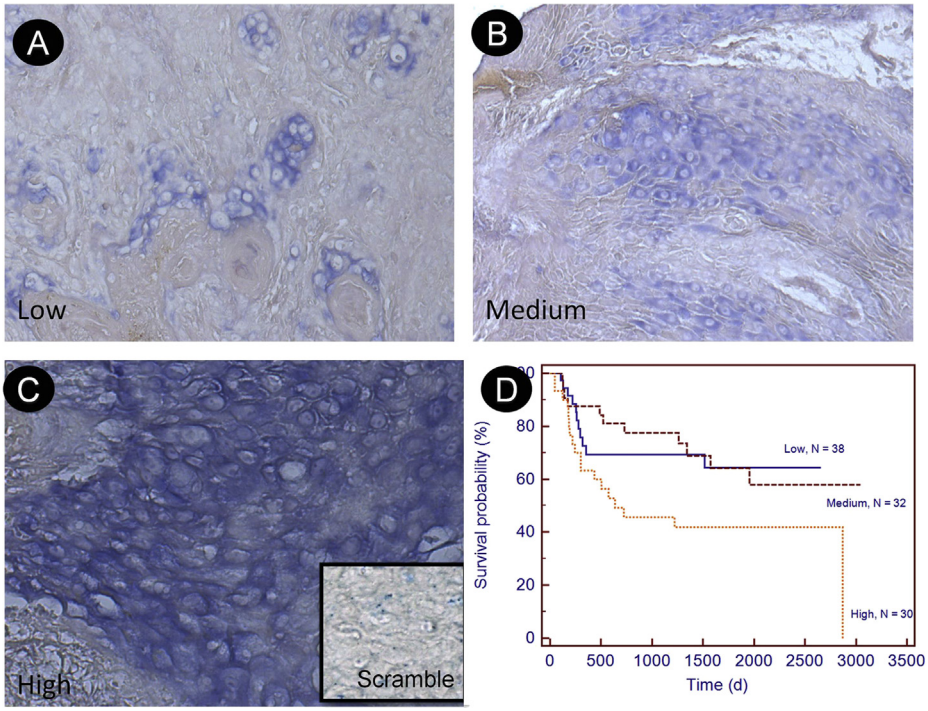

Fig. 1. *In situ* hybridization analysis of *miR-21* staining in tumor cells. (A) Low expression of *miR-21*; (B) medium expression; and (C) high expression. Lower right small figure in C revealed a representative staining of tumor using scramble probe. (D) Disease-free survival determined by *miR-21* expression. Low and medium expression of *mir-21* had similar curves. Significantly worse survival was shown in the group of high *mir-21* expression. $p = 0.033$, Kaplan–Meier analysis.

3.3. Factors related to disease-free survival

The factors related to disease-free survival were tested by Kaplan–Meier analysis (Table 2). Tumor size, nodal status, clinical stage, PNI, lymphovascular permeation, and higher *miR-21* staining were significantly related to disease-free survival. Multivariate analysis further showed that tumor size, nodal status, clinical stage, and higher *miR-21* were independent survival predictors in this study.

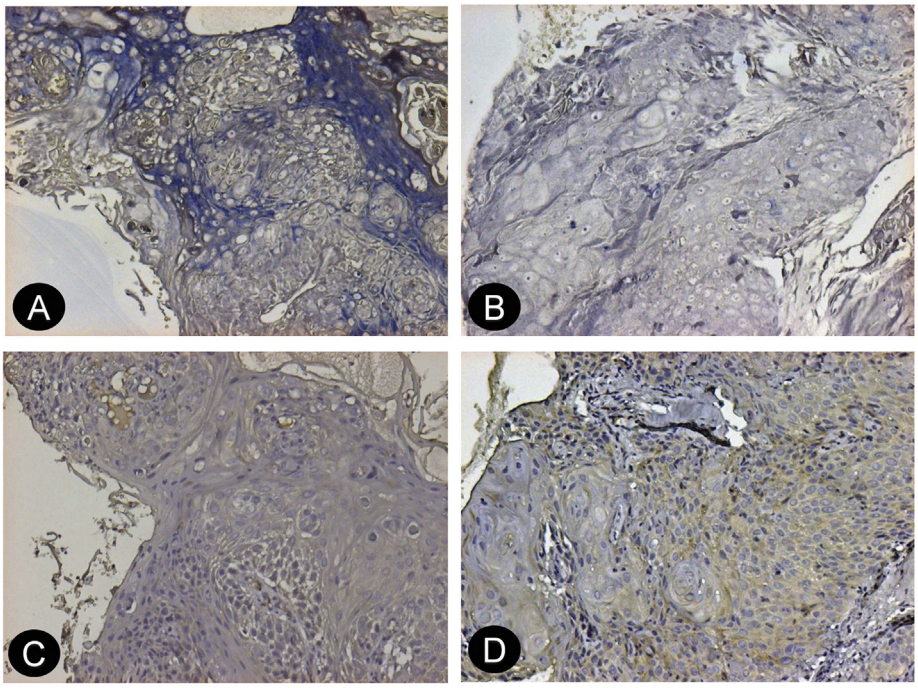

Fig. 2. *miR-21* staining and phosphatase and tensin homolog (PTEN) immunoreactivity in oral squamous cell carcinoma. (A, B) *miR-21* staining. (C, D) PTEN immunoreactivity. (A, C) were from the same patient. The *miR-21* staining score was high, while the PTEN immunohistochemistry score was low. (B, D) were from another patient. The *miR-21* staining score was low, while the PTEN immunohistochemistry score was medium.

Table 2
Factors related to disease-free survival.

	p^a	p^b	Adjusted hazard ratio	95% confidence interval
T classification	0.014	0.021	1.47	1.04–2.08
N classification	0.0001	0.010	1.33	1.06–1.67
Stage	0.011	0.002	2.27	1.34–3.80
Differentiation	0.332			
Perineural invasion	0.012	0.260	1.65	0.68–3.97
Lymphovascular permeation	0.015	0.302	1.42	0.51–4.21
Invasion front	0.421			
Depth of invasion	0.255			
miR-21 staining	0.003	0.004	1.87	1.21–2.87

[a] Kaplan–Meier analysis.
[b] Cox proportional-hazards regression.

4. Discussion

Our data supported that *miR-21* was an independent factor of disease-free survival in OSCC patients. Our results led us to identify the relationship between *miR-21* and PNI in OSCC. *miR-21* could promote cancer cells to invade the nerve bundle and spread out. The *miR-21*/PNI pathway was likely to decrease the expression of PTEN, but its mechanism was still not clear.

PNI is a situation and process whereby nerve bundles are invaded by cancer cells. PNI is neither an extension of lymphatic metastasis nor simply tumor cell migration through a low-resistance plane[12] but is instead a distinctive pathological or programmed response to nerve attraction that would seem to be independent from lymphatic or vascular invasion. PNI has been recognized as a prognostic indicator of poor survival in many types of malignancies.[13,14] It has also been considered to be an indicator of a poor prognosis in OSCC in several studies.[15,16] However, the molecular mechanisms of PNI have been unclear. Since adjuvant radiotherapy does not appear to reduce the incidence of disease recurrence in PNI-positive patients,[17] understanding the mechanism of PNI in order to help the development of an appropriate target agent is critical. In the future, the blockage of *miR-21* and of other factors related to the facilitation of PNI need to be validated as adjuvant therapeutic approaches that might help to counteract perineural tumor spreading; such an approach should help to improve patient prognosis.[16]

Previous reports have shown that *miR-21* is overexpressed in many solid tumors; furthermore, this overexpression has been shown to be related to tumor progression in hepatocellular carcinomas,[18] breast cancers,[19] and colon carcinoma.[20] Therefore, *miR-21* seems to be a useful prognostic factor for various kinds of malignancies. Studies also showed the high expression levels of *miR-21* in OSCC are associated with a poorer prognosis for patients.[8] In our study, we have also shown that cases with high *miR-21* expression have a significantly worse disease progression. This implies that cases with high *miR-21* are suitable for aggressive treatment option, for example, selective neck dissection or adjuvant therapy. Analysis of *miR-21* expression may be particularly useful when carrying out a prognostic evaluation of this patient subset.

Previous studies have demonstrated localization of *miR-21* staining in stromal cells of OSCC associated with the tongue and mouth floor[8,21]—this might be a useful prognostic predictor.[8] We also observed *miR-21* staining in the stromal cells of tumors. However, since multiple lines of evidence indicate the involvement of *miR-21* in the aggressiveness of OSCC,[18,22,23] our approach has rather focused on studying the epithelial tumor cells. We have been able to show that there is modest *miR-21* staining in the tumor cell component of OSCC, and that this activity is involved in modulating cell migration in OSCC cells. It seems likely that the discrepancies among oral sites, in the clinical stage of the tumor and in the race of the patient, may underlie the differences in stromal *miR-21* staining that occur across different study subsets.

Decreased expression of PTEN is an indicator of a poor prognosis in many cancers, including colorectal cancer,[24] prostate cancer,[25] and breast cancer.[26] A deficiency of PTEN expression is also a poorly prognostic factor for OSCC.[27] As *miR-21* targets multiple oncogenic events, including PTEN, during pathogenesis, and because PTEN is an inhibitor of the activator PI3K/AKT/S6 pathway,[18,22,23] our study was able to show a reverse association between *miR-21* expression and PTEN expression. Nevertheless, our analysis also showed high immunoreactivity for phosphor-S6 across all OSCC samples in this cohort, even in tumors with medium PTEN immunoreactivity. Similar ambiguity is found in other studies.[28,29] Therefore, this study is unable to link PNI to the PI3K/AKT pathway. Our results seem to imply that, although *miR-21*-PTEN cascade may reinforce OSCC cells with a PNI propensity, the PI3K/AKT/S6 pathway does not seem to be the single factor involved in causing PNI in OSCC. These findings are in agreement with a previous investigation of PNI associated with pancreatic carcinoma.[30]

In conclusion, this study provides a new line of evidence demonstrating that there is an association between *miR-21*-PTEN disregulation and PNI in OSCC. *miR-21* is an independent factor associated with disease-free survival in OSCC. High *miR-21* expression is able to predict worse prognosis among OSCC patients. Our data also hint that miR-21 expression may be an indicator of PNI-positive and more aggressive treatment may be of benefit to the OSCC patient without PNI in the surgical specimen.

Acknowledgments

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