구강편평세포암종 환자에서 N-cadherin과 Twist 발현 양상과 임상적 중요성 연구

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〈Abstract〉

Expression of N-cadherin and Twist and Their Clinical Significance in Oral Squamous Cell Carcinoma

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Metastasis consists of complex cascades and a lot of factors are involved in each step of metastasis. In recent studies, the role of epithelial-mesenchymal transition (EMT) in metastasis is suggested. EMT has a feature of epithelial cells conversing into mesenchymal cells morphologically and phenotypically, is a characteristic of malignant and metastatic cells in most cancer. The mesenchymal cells usually show more malignant phenotype, including invasion and metastasis. EMT can play an important role in metastasis of oral squamous cell carcinoma (OSCC). Although the role of Snail, slug, other transcriptional factors and E-cadherin are well known in human cancers, there are a few studies on N-cadherin and Twist expression in OSCC. The present study was aimed to analyze the expression of N-cadherin and Twist protein in OSCC from Korean patients. The immunohistochemical stain was performed using 58 primary OSCCs and 6 metastatic OSCCs, and the correlation between the expression of these proteins and clinicopathological parameters of OSCC patients was analyzed.

The expression rate of high expression of N-cadherin was observed in 70.4% and Twist in 87.3% of OSCC. The expression of N-cadherin in metastatic OSCC increased than in corresponding primary OSCC ($p<0.05$). The spearman correlation coefficiency between N-cadherin and Twist was calculated as 0.228. The clinical factors such as lymph node metastasis and survival showed statistically significant correlation between N-cadherin expression. The expression of Twist was correlated with recurrence. In conclusion, the authors suggest that N-cadherin may play an important role in malignant behaviour of OSCC and can be considered as prognostic indicator of OSCC.

Key words : Oral squamous cell carcinoma, N-cadherin, Twist, EMT, Metastasis, Prognosis

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Ⅰ. Introduction

Oral squamous cell carcinoma (OSCC), is one of the common cancers of the head and neck region[1]. The annual incidence of oral cancer is estimated at around 275,000 with OSCCs representing 〉90% of these cases[2]. In spite of rapid advances in surgical and radiation

therapies for oral cancer, the 5-year survival rate and prognosis of OSCC patients remains relatively poor[3,4]. In addition, lymph node metastasis is an important determinant in the prognosis of OSCC[4]. Thus, it is very important to identify the factors that were related with the metastatic progression of OSCC in order to restrain the progression and improve the treatment strategies for oral cancer.

Recent researches suggested that epithelial-mesenchymal transition (EMT) might play an important role in the carcinoma progression and aggressiveness[5,6]. EMT describes a process in which epithelial cells lose their polarity and cell-cell junction and the properties of mesenchymal cell express through the cell changes of phenotype[7]. The hallmark of EMT is a cadherin switching, means that the normal expression of E-cadherin is replaced by the abnormal expression of N-cadherin or E-cadherin expression does not change but the expression of N-cadherin increases[7,8].

N-cadherin was first identified in the brain of mouse as cadherin-2[8]. It is normally expressed in neuroectodermal tissue, mesodermal-derived tissue such as nervous tissue, vascular endothelial cells, and skeletal and cardiac muscle cells but not normally in epithelial cells[8,9]. Throughout embryogenesis, N-cadherin is involved in the process of cell differentiation, migration, invasion and signal transduction[9]. In contrast, during tumor EMT process, a cadherin switch from E-cadherin to N-cadherin expression may enhance tumor aggressiveness[10]. Recent studies indicated that N-cadherin expression in tumor cells was associated with an increased tumor cell migration, invasiveness, metastasis, and drug sensitivities[11]. However, in OSCC, the expression of N-cadherin and the relation between the transcritptional factor involved in Cadherin switching is not elucidated.

Twist, a highly conserved basic helix-loop-helix (bHLH) transcriptional factor, which is involved in the regulation of Cadherin, may play an important role in EMT-related metastasis through the regulation of cadherin expression[12]. During EMT process, there was change of several transcriptional factors expression such as Twist, Snail, and Slug and Cadherins[13]. Furthermore, Twist- enhanced cancer metastasis in breast, gastric, and hepatocellular carcinoma was reported[14].

In the present study, the authors investigated the role of N-cadherin and Twist protein in primary and metastatic OSCC. Furthermore, we compared the relationships between immunohistochemical scores and clinicopathological parameters to examine the pathological role of N-cadherin and Twist expression in Korean OSCC patients.

II. MATERIALS AND METHODS

1. Patients and tissue samples

Fifty-eight cases of primary OSCC and 6 cases of metastatic OSCC with good preservation of paraffin tissue and H/E slides were obtained from the files of Department of Pathology, Medical College, Pusan National University and Department of oral pathology Yonsei University College of Dentistry from January 2001 to December 2007.

The authorization of using the tissues for research was obtained from the Institutional Review Board in Pusan National University Hospital.

2. The clinicopathological characteristics of OSCC patients

Clinical data of OSCC patients were gathered from surgical records of the patients. Clinicopathological characteristics of OSCC patients were summarized in Table 1.

3. H/E stain and immunohistochemical stain

The OSCC tissue were fixed in 10% neutral formalin

solution for 12 hours, washed for 20 minutes, dehydrated in ethyl alcohol, washed in xylene and embedded in paraffin blocks. Three-μm sections were obtained and submitted for routine hematoxylin-eosin staining. For immunohistochemical staining, another 3-μm tissue sections were prepared. Immunohistochemical staining was performed with anti-N-cadherin and Twist antibody, respectively. The characteristics of primary antibodies were summarized to Table 1.

Antigen retrieval for N-cadherin and Twist was performed by boiling the slides in the citrate buffer (pH 6.0, Invitrogen, CA, USA) in 45 min, and then cooling for 25 min at RT, according to the manufacturer's instructions.

After antigen retrieval, the slides were washed thoroughly in phosphate buffered saline (PBS, pH 7.4) for 5 min 3 times each and incubated with H_2O_2 solution and blocking solution for 20 min at room temperature in a humid chamber, respectively. And then, N-cadherin and Twist antibody were diluted with 3% bovine serum albumin solution, the slides were incubated overnight at 4℃ with primary antibody, respectively. Visualization of bound antibodies was performed by using a CSA II biotin free tyramide signal amplification system (Dako, Carpinteria, CA, USA), which contain fluorescyl-tyramide complex and poly HRP conjugate (Invitrogen), according to the manufacturer' s instructions.

The slides were visualized with 3, 3'-diamonobenzidine tetrahydrochloride (Vector laboratories, Burlingame, CA, USA), counterstained with Mayer's hematoxylin, dehydrated in ethanol, cleared in xylene and mounted using Malinol. And then, the slides were examined under light microscopy (Olympus, BH-2, Tokyo, Japan).

4. Evaluation of immunohistochemical stain

For each slide, 5 non-overlapping fields were randomly selected, and photographed using light microscopy with a digital camera (Olympus, BX51T, Tokyo, Japan, ×100). The expressions of N-cadherin and Twist antibody were evaluated in a semi-quantitative manner[15]. Levels of immunore-activity (LI) were graded into four easily reproducible subgroups: (a) No detectable expression (Grade 0), (b) positive expression in less than 30% of cells (Grade 1) (c) positive expression in 30-50% of cells, indicating immunopositive subpopulations (Grade 2), and (d) positive expression in greater than 50% of cells (Grade 3). The Twist expression appeared in the nucleus was considered positive.

5. Statistical Analysis

Statistical analysis was performed by chi-sqaure test to compare the expression of N-cadherin and Twist and analyze the differences between primary OSCC and metastatic OSCC. In addition, the correlations between N-cadherin and Twist and clinicopathologic parameters (age, gender, survival months, TNM stage, recurrence, lymph node metastasis) of OSCC patients were determined

Table 1. Primary antibodies for immunohistochemical studies

Antibody	Dilution ratio	Clonality	Source
N-cadhrein	1:200	Polyclonal	BD Biosciences [a]
Twist	1:150	Polyclonal	Abcam [b]

*[a]BD Biosciences, Bedford, MA, USA
*[b]Abcam, Cambridge, MA, UK

by Spearman Correlation Coefficient. The Kaplan-Meier method was used for survival analysis grouping with N-cadherin expression and metastasis. Statistical analysis was performed using the Window PASW (Predictive Analytics SoftWare) version 18.0 (SPSS Inc, NY, USA). A p-value $\leq$ 0.05 was considered statistically significant.

III. Result

1. The characteristics of OSCC patients

The OSCC patient age ranged from 31 to 78 years and most OSCC patients were distributed in the fifty and sixty (Table 2). The male:female ratio of OSCC patients was 3:1 (Table 3). Clinicopathological features of the OSCC patients were summarized in Table 4.

2. N-cadherin and Twist Expression in OSCC patients

The rate of high Twist expression was 87.3% and that of N-cadherin was 70.4% in OSCC. There was no difference between the N-cadherin and Twist expression of primary OSCC and metastatic OSCC. Conversely, the expression of N-cadherin in metastatic OSCC increased than in corresponding primary OSCC ($p<0.05$, Fig. 1, 2). The spearman correlation coefficiency between Twist and N-cadherin was calculated as 0.228 ($p<0.05$).

3. Correlation between N-cadherin expression and the clinicopathological parameters of OSCC patients

To analyze the association with the immunoreactivity of N-cadherin and Twist and the clinicopatholgical parameters of OSCC patients, Spearman's Correlation Coefficient was employed for correlation analysis. There was a significant correlation between the immunoreactivity of N-cadherin and regional lymph node metastasis and survival of OSCC patients (Table 5). In case of Twist, there was significant correlation between the Twist expression and gender and recurrence of OSCC patients.

4. Relationship between N-cadherin and Twist expression and survival months

In case of lower N-cadherin expression, survival months did not correlate with lymph node metastasis. In contrast, in case of higher N-cadherin expression, survival months were found to be associated with lymph node metastasis ($p<0.05$, Fig. 3).

IV. Discussion

EMT is characterized by loss of epithelial polarity and increasing cell motility in tumor cells[16]. It is thought that EMT plays critical role in invasion and metastasis in tumorigenesis[16]. During EMT process, the change of several transcriptional factors expression such as Twist, Snail, and SIP1, and Twist is reported to pivotal role in mesenchymal transition of epithelial cells[13,17]. Twist is involved in relatively early stage of tumorigenesis, and known as to activate EMT process, possibly through the regulation of cadherin expression[12,17]. Researches on EMT mechanism are now emerging as a new tool for identification the genes involved in tumor progression.

There were few studies on N-cadherin and Twist expression in OSCC. Mariotti reported that N-cadherin expression was found in membrane and cytoplasm of head and neck carcinoma cells[18]. In head and neck squamous cell carcinoma, high expression of N-cadherin was examined by immunohistochemical detection and significant correlated with invasion[9]. Thus, immunohistochemical

Table 2. Age distribution of OSCC patients

Age	OSCC cases	
	Primary	Metastatic
20-29 Years	1	
30-39 Years	3	
40-49 Years	8	1
50-59 Years	19	3
60-69 Years	15	1
70-79 Years	7	1
80-89 Years	1	
Unknown	4	
Total	58	6

Table 3. The gender distribution of OSCC patients

Gender	OSCC cases	
	Primary	Metastatic
M	40	5
F	14	1
Unknown	4	
Total	58	6

Table 4. The clinicopathological parameters of studied oral squamous cell carcinoma (OSCC) patients

		OSCC case	
		Primary	Metastatic
Survival	~12 months	7	3
	13~24 months	11	
	25~36 months	8	1
	37~48 months	5	1
	49~60 months	6	
	61~72 months	7	
	73~ months	8	1
	Unknown	6	
TNM stage	Stage 0	1	
	Stage Ⅰ	7	
	Stage Ⅱ	8	
	Stage Ⅲ	9	2
	Stage Ⅳ	27	4
	Unknown	6	
Recurrence	Recur (+)	2	1
	Recur (-)	51	5
	Unknown	5	

Table 5. The relationship between immuohistochemical results and clinicopathological parameters in OSCC patients

Clinical parameters		N-cadherin		Twist	
		Primary	Metastatic	Primary	Metastatic
Age	Spearman Co.	0.104	0.345	0.084	0.340
	p value	.089	.078	.169	.083
Gender	Spearman Co.	0.058	-.380	0.064	-0.438
	p value	.347	.051	.297	.022
Recurrence	Spearman Co.	-0.066	-0.014	0.135	-0.245
	p value	.287	.946	.028	.218
TNM stage	Spearman Co.	-0.058	-0.051	0.038	0.002
	p value	.353	.801	.543	.991
LN metastasis	Spearman Co.	-0.378		0.037	
	p value	.000		.531	
Survival	Spearman Co.	.085	-0.490	-0.064	0.062
	p value	.174	.010	.302	.760

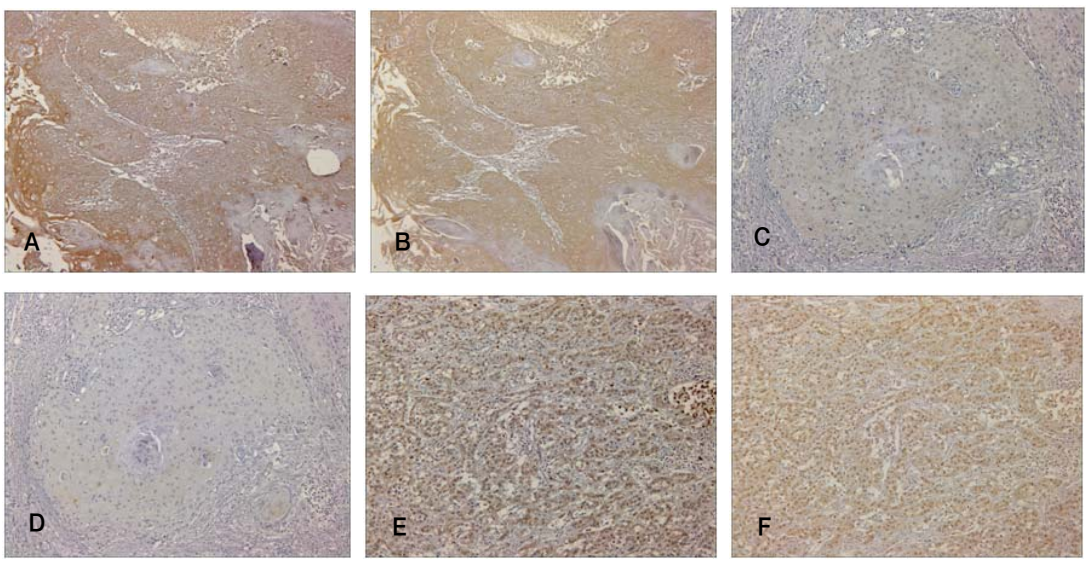

Fig. 1. N-cadherin and Twist immunohistochemical staining of primary OSCC (A, B, C and D) and metastatic OSCC (E and F). (X100)

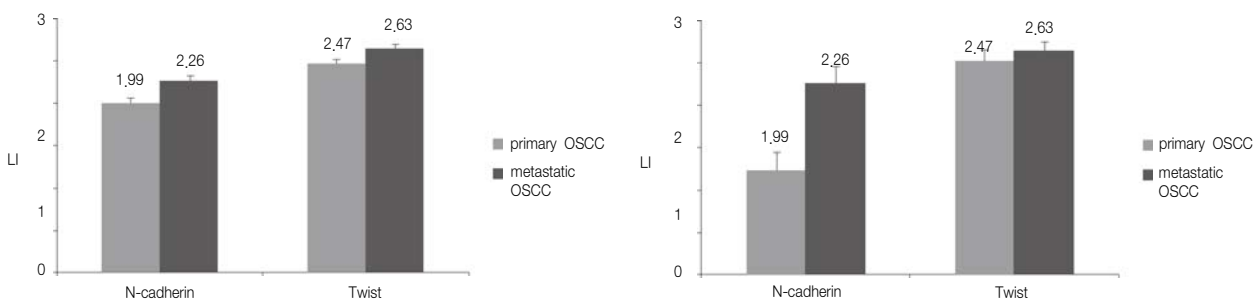

Fig. 2. The expression of N-cadherin and Twist protein in primary OSCC and metastatic OSCC (A: primary OSCC and metastatic OSCC, B: primary OSCC and corresponding metastatic OSCC).
* : $p<0.05$

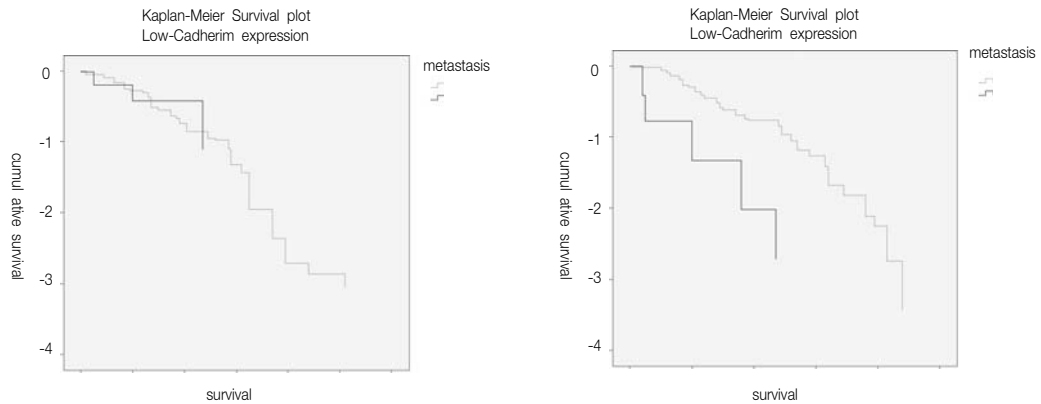

Fig. 3. Kaplan-Meier overall survival curves according to the expression of N-cadherin and lymph node metastasis (A: low N-cadherin expression, B: high N-cadherin expression).

detection of these proteins can be a useful tool in the EMT research.

In the present study, there wasn't difference between the N-cadherin and Twist expression of primary OSCC and metastatic OSCC. Furthermore, no significant correlation between Twist and patient's age, TNM stage, lymph node metastasis and survival were found. Instead, a weak positive correlation between Twist and recurrence was detected. Thus, it was thought that Twist-induced EMT didn't show any association with OSCC. Gasparotto suggested that Twist1 was signi- ficantly overexpressed in head and neck cancer, but failed to correlate with clinicopathological parameters[5]. Additionally, Nakashima reported that Twist expression varied among human lung cancer cell lines[12], thus, it is thought to show variable Twist expression depending on the type of tumor. However, further studies are now needed which attempt to analyze the exact role of Twist-induced EMT in OSCC with larger samples.

Interestingly, the N-cadherin expression increased in metastatic OSCC, as compared to corresponding primary tumor. Wang reported that E-cadherin was suppressed in oral tongue squamous cell carcinoma[19]. Cadherin switching is known to reflect EMT[20]. In laryngeal carcinoma, alteration of Twist had an effect on the expression of N-cadherin[21]. Although there was a significant correlation between N-cadherin and twist expression, whether Twist regulate N-cadherin in OSCC is not clear.

Tumor metastasis is very complex process, and various factors involve in each step of the process of metastasis[22]. Nguyen suggested that N- cadherin might play an important role in metastasis of spindle cell carcinoma of head and neck region[20]. Additionally, N-cadherin expressing MCF-7 cell induced the metastatic potential[11]. The gain of N-cadherin may trigger interaction with surrounding fibroblasts and facilitate metastasis[23]. Thus, it is thought that increased N-cadherin expression may play important role in the metastasis of OSCC. However our result demonstrated that there was a negative correlation between N-cadherin expression and lymph node metastasis. Therefore, in our cases, it is thought that N-cadherin induced EMT doesn't have a significant influence on the metastasis of OSCC.

A notable finding of our research is the poor prognosis in OSCC cases showing high expression of N-cadherin. In OSCC, up-regulated N-cadherin had a signi- ficant correlation with worst prognosis[24]. Furthermore, in bladder cancer, high expression of N-cadherin was

suggested to be a prognostic factor[25]. It is reported that N-cadherin itself may promote invasion of head and neck carcinoma cells[9]. N-cadherin interact with various signaling pathways, such as epidermal growth factor, platelet- derived growth factor and fibroblast growth factor[9,26]. It is needed to carry out further researches on the detailed mechanism of N-cadherin regulation as well as exact role in oral progression. Taken together, we suggest that N- cadherin can be a new candidate of prognostic marker of OSCC.

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