Role of Twist in Head and Neck Carcinoma with Lymph Node Metastasis

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Abstract. *The transcription factor Twist protein has been found to be correlated with metastasis in various carcinomas, including hepatocellular, breast and prostate carcinomas. However, the role of Twist in head and neck squamous cell carcinomas (HNSCC) remains unknown. Head and neck cancer tissue microarrays (TMAs) of tumors from 50 patients with HNSCC were examined. Immunohistochemical (IHC) stain analysis showed that, out of the 50 patients, twenty (40%) showed Twist-positive staining in the tumor cells, and Twist expression was positively associated with differentiation status (p=0.027), lymph node metastasis (p=0.032) and disease progression (p=0.029). Further analysis revealed that the expression of Twist was positively correlated with CXCR4 (Spearman, r=0.408, p=0.003) and CCR7 (r=0.417, p=0.003). FindPatterns analysis suggested that the transcription factor Twist, as a basic helix-loop-helix (bHLH) protein, might regulate CXCR4 and CCR7 expression in squamous cell carcinomas, which in turn might be associated with lymph node metastasis.*

Head and neck squamous cell carcinomas (HNSCC) are epithelial malignancies originating from the oral cavity, oropharynx, sinonasal region, hypopharynx and larynx. It is one of the most common types of human cancer, with an annual incidence of more than 500,000 cases worldwide (1, 2). Many patients with HNSCC present with lymph node metastases at the time of diagnosis, which are associated with severe disease- and treatment-related morbidity and have a 5-year survival rate of approximately 50%. This rate has not improved in more than two decades (1, 3).

The Twist protein is a highly conserved transcription factor that belongs to the family of basic helix-loop-helix (bHLH) proteins (4). The bHLH molecules can form homo- or heterodimers with other bHLH molecules, and bind to conserved E-box regions (CANNTG) in the promoters of several genes to activate or inhibit transcription (5, 6). It has been reported that by using gene profiling analysis, up-regulation of Twist was found to be associated with malignant transformation of T-cells and melanocytes (7, 8). In addition, high expression of Twist has been found in many types of human carcinomas, such as breast cancer, prostate cancer, gastric cancer and rhabdomyosarcoma (9-12). Recently, Yang J *et al.* (11) reported that Twist is a key factor responsible for the metastasis of breast cancer by promoting the epithelial-to-mesenchymal transition (EMT), and found that down-regulation of Twist could suppress metastatic ability by inducing mesenchymal-to-epithelial transition (MET). In addition, Hosono *et al.* (13) also reported that increased Twist expression correlated with poor outcome and shorter survival in epithelial ovarian carcinoma patients. Therefore, Twist appears to be a novel oncogene that induces tumorigenesis in nonmalignant cells and promotes tumor progression in malignant cells (7, 10, 14).

The major purpose of the present study was to evaluate the significance of Twist expression in HNSCC. CH1 tissue microarrays (TMAs) composed of tumor samples from 50 patients with head and neck tumors were examined. Twist, as well as CXCR4, CXCR6 and CCR7, were evaluated by using immunohistochemical (IHC) stain analysis, and associations between the staining patterns and various clinicopathological features were studied.

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Patients and Methods

Immunohistochemical staining of tissue microarrays. CH1 tumor tissue arrays, purchased from SuperBioChips Laboratories, Seoul, Korea, were composed of up to 60 tumor specimens from 60 HNSCC patients. The TNM, stage classification and clinical parameters of these patients were provided by the manufacturers. The CH1 tumor tissue arrays were subjected to IHC study. Briefly, tissue sections were cleared of paraffin, rehydrated and blocked in hydrogen peroxide before antigen retrieval. After antigen retrieval by heating in a microwave for 5 min with citrate buffer (pH 6.0), these sections were reacted with anti-human Twist (1:100, sc-15393, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human CXCR4 (1:50, MAB171), anti-human CXCR6 (1:25, MAB699), or anti-human CCR7 (1:33, MAB197) using a standard indirect avidin–biotin–peroxidase method. All the antibodies to chemokine receptors were purchased from R&D Systems, Minneapolis, MN, USA. The color reaction was developed with diaminobenzidine (DAB) solution and the sections were counterstained with Mayer's hematoxylin solution. Breast cancer tissues expressing the corresponding proteins were used as positive controls. The specificity of immunostaining was confirmed by the use of serial sections with non-immune serum instead of the primary antibody as a negative control. The specimens were evaluated independently without prior knowledge of the clinicopathological information.

Statistical analysis. The statistical significance of the individual findings and their association indices were evaluated using the Chi-square test with Yates' correction. Probability (p) values less than 0.05 were considered significant. The Spearman correlation test was used to evaluate pairwise association between abnormal expressions of Twist, CXCR4 and CCR7 proteins. The calculations were performed using SPSS 12.0 statistical software for Windows (SPSS Inc, Chicago, IL, USA).

Results

Expression profiles of Twist, CXCR4, CXCR6 and CCR7 proteins in CH1 TMAs. The Twist protein was mostly found in the cytoplasm and nucleus, whereas CCR7, CXCR4 and CXCR6 were detected in the cytoplasm and at the membrane. Some cases with nuclear staining of CCR7 and CXCR4 were also found. Representative expression patterns for these proteins are shown in Figure 1. Although the data sheet stated that the array slide contained 60 tumor tissues from HNSCC, only 50 dots with tissue could be identified and interpreted. Out of the 50 cases of head and neck cancer, 20 (40.0%), 25 (50.0%), 25 (50.0%) and 20 (40.0%) showed positive staining in the tumor cells for Twist, CXCR4, CXCR6 and CCR7, respectively.

Twist, CXCR4, CXCR6 and CCR7 expression patterns in relation to clinicopathological factors. In order to investigate whether these tumor-associated proteins were clinically significant, these variables were correlated with various clinicopathological factors (Table I). The median age of these patients was 60 (range 28-82). Apart from some patients with insufficient information, Twist expression was significantly correlated with differentiation status ($p=0.027$), lymph node metastasis ($p=0.032$) and clinical stage ($p=0.029$). Furthermore, CXCR4 expression was significantly correlated with age ($p=0.023$) and clinical stage ($p=0.039$), and showed a trend that was correlated with lymph node metastasis ($p=0.056$); CCR7 expression was significantly associated with lymph node metastasis ($p=0.014$). The CXCR6 expression was not significantly associated with the variables examined. Twist was found to be positively correlated with CXCR4 (Spearman, r=0.408, $p=0.003$) and CCR7 (r=0.417, $p=0.003$) by using pairwise association analysis.

By using the FindPatterns nucleic acid pattern search in the SeqWeb v3.1.2 program (Accelrys Software Inc., San Diego, CA, USA), 18 and 10 E-box sequences located within the promoters and the exon 1 and intron 1 regions of the human CCR7 gene (GeneBank No. EF064758) and CXCR4 gene (GeneBank No. AF052572), respectively, were identified (Figure 2).

Discussion

It has been reported that overexpression of Twist is associated with tumor metastasis in breast cancer, prostate cancer and hepatocellular carcinoma (11, 12, 15). The present results were consistent with those reports and showed that Twist was significantly correlated with lymph node metastasis and clinical stage in head and neck cancer.

A complex network of chemokines and their receptors influences the development of primary tumors and metastases (16, 17). Recent studies have clearly demonstrated the importance of CCR7 and CXCR4 expression in metastasis to lymph nodes by breast cancer (18) and colorectal cancer (19). Other reports have shown that overexpression of CCR7 or CXCR4 were associated with metastasis and poor overall survival (20, 21). Our previous study also explored the heterogeneous expression of CXCR4, CCR7 and CXCR6 in primary tumor specimens of nasopharyngeal carcinoma (NPC) patients and found an abundant expression of these chemokine receptors in the metastatic NPC (22). In the present study, the head and neck carcinoma cells also expressed Twist, CCR7, CXCR4 and CXCR6 heterogeneously, and Twist, CCR7 and CXCR4 expression were associated with lymph node metastasis. One possibility suggested by this finding is that all three molecules are under the same regulatory control. Sosic *et al.* demonstrated that Twist expression could be regulated by TNF-α through nuclear factor-κB (NF-κB) activity in mouse fibroblasts (23). Additionally, latent membrane protein 1 (LMP1) could directly induce Twist expression *via* NF-κB in nasopharyngeal epithelial cells (24). Moreover, it has been reported that CXCR4 can be up-regulated through NF-κB activation in prostate and breast carcinomas (25, 26), and that CCR7 has the same signaling activation in Hodgkin's

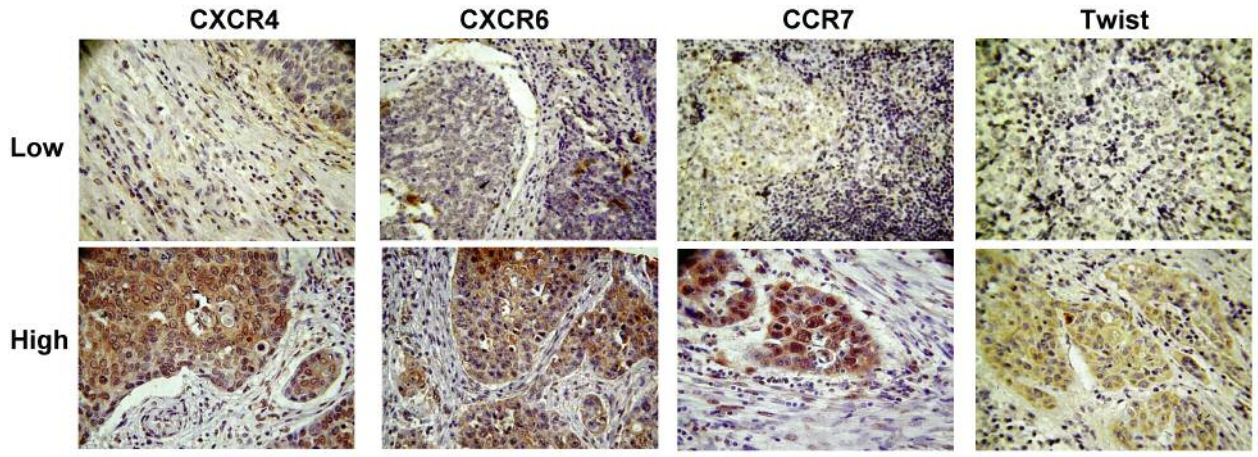

Figure 1. *Immunohistochemical staining of Twist, CXCR4, CXCR6 and CCR7, in CH1 tissue microarrays. Expression patterns of Twist, CXCR4, CXCR6 and CCR7 in examples of low- and high-grade CH1 TMA sections (x400).*

Table 1 Correlation between clinicopathological characteristics and examined variables in CH1 tissue array (n=50).

		CXCR4***		*P*-value	CXCR6***		*P*-value	CCR7***		*P*-value	Twist**		*P*-value
	n (%)	Pos	Neg		Pos	Neg		Pos	Neg		Pos	Neg	
Age (years, n=50)				0.023*			1.000			1.000			0.325
<60	23 (46.0)	7	16		11	12		9	14		7	16	
≥60	27 (54.0)	18	9		14	13		11	16		13	14	
Gender (n=50)				0.602			0.602			0.242			0.915
Female	4 (8.0)	1	3		1	3		0	4		1	3	
Male	46 (92.0)	24	22		24	22		20	26		19	27	
Tumor size (n=46)				0.740			1.000			1.000			0.976
T1 and T2	11 (23.9)	5	6		6	5		4	7		4	7	
T3 and T4	35 (76.1)	20	15		18	17		14	21		15	20	
Differentiation (n=42)				0.135			0.567			0.632			0.027*
Well	11 (25.6)	6	5		7	4		5	6		8	3	
Moderate	16 (37.2)	5	11		7	9		5	11		7	9	
Poor and undifferentiated	15 (34.9)	10	5		7	8		7	8		3	12	
Lymph node metastasis (n=47)				0.056			0.876			0.014*			0.032*
N0 and N1	25 (53.2)	9	16		12	13		6	19		6	19	
N2 and N3	22 (46.8)	15	7		12	10		14	8		13	9	
Stage (n=48)				0.039*			0.846			0.269			0.029*
I, II and III	15 (31.3)	4	11		7	8		4	11		2	13	
IV	33 (68.7)	21	12		18	15		16	17		17	16	
Location (n=50)				0.560			1.000			0.258			0.610
Larynx	31 (62.0)	14	17		15	16		10	21		14	17	
Others	19 (38.0)	11	8		10	9		10	9		6	12	

*$p<0.05$; **Twist was considered as positive when more than 30% of tissue cells were stained in the nucleus and cytoplasm ***CXCR4, CXCR6 and CCR7 were considered as positive when more than 50% of tissue cells were stained in the cytoplasm and at the membrane.

disease (27).Thus, Twist, CXCR4 and CCR7 might simultaneously be regulated through NF-κB signaling.

Nevertheless, Twist, as a transcription factor, could regulate CXCR4 and CCR7 expression. In the pairwise association analysis in the present study Twist was positively correlated with CXCR4 and CCR7 in the head and neck carcinomas. Furthermore, screening for E-box (CANNTG) motifs, specific sequences for Twist binding (6, 28), located within the promoter, exon 1 and intron 1 regions in the CXCR4 and CCR7 genes, and showed data to support this

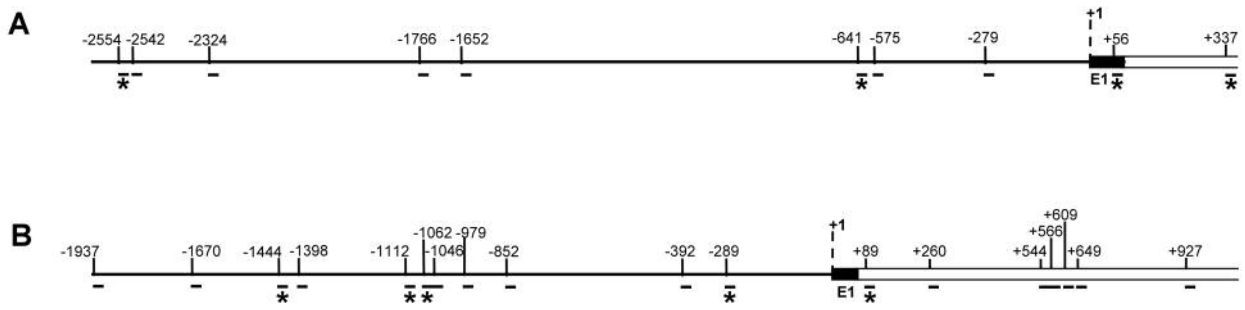

Figure 2. *Predicted twist-binding sites within the promoter regions in the CXCR4 and CCR7 genes. Schematic showing ten potential E-box (CANNTG) sequences (marked –) located within the promoter, exon 1 (E1) and intron 1 regions (3000 bp, from -2642 to 358) of the human CXCR4 gene (A), and 18 E-box sequences (marked –) located within the promoter, exon 1 (E1) and intron 1 regions (3000 bp, from -1939 to 1061) of the human CCR7 gene (B). The transcriptional initiation site is numbered as +1. The * stands for strong or moderate binding sites for Twist (CATATG, CATGTG, CATCTG or CACGTG).*

possibility. According to the gel-shift assay conducted by Lee *et al.* (29), Twist could strongly bind to the E-box sequences CATATG or CATGTG and could moderately bind to the E-box sequences CATCTG or CACGTG. Consequently, there might be 4 and 5 significant E-box sequences out of 10 and 18, respectively, located in the CXCR4 and CCR7 genes. However, the distinct E-box regulatory elements for Twist binding in head and neck carcinomas need further exploration.

In summary, this is the first report taking Twist, CXCR4 and CCR7 into simultaneous consideration. The data indicate that Twist is a pivotal transcription factor that may positively regulate the gene expression of CXCR4 and CCR7 during lymph node metastasis. The precise mechanism of this regulation warrants further investigation.

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