SPANDIDOS PUBLICATIONS

An essential role for N-cadherin and ß-catenin for progression in tongue squamous cell carcinoma and their effect on invasion and metastasis of Tca8113 tongue cancer cells

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Abstract. It is well documented that adhesive molecules and catenins are closely associated with adhesion, invasion and metastasis in different types of tumors. Certain evidence indicates that another member of the cadherin family, N-cadherin, is expressed in highly invasive tumor cell lines that lacked E-cadherin expression. However, the role of N-cadherin in SCC of the tongue and relationship between N-cadherin and ß-catenin in SCC of the tongue remain unknown. Herein, the expressions of N/E-cadherin and ß-catenin were examined during the progression from normal tongue epithelium to invasive SCC of the tongue, the relationship between expressions of above proteins and clinicopathological characteristics in SCC of the tongue was analyzed. Further, we studied the effect of N-cadherin and ß-catenin on molecular mechanism of invasion and metastasis in SCC of the tongue. The results revealed that overexpression of N-cadherin and ß-catenin attributed to the progression of SCC of the tongue, whereas, E-cadherin showed a reverse result. In addition, the expressions of N-cadherin and ß-catenin were tightly associated with lymph node metastasis of SCC of the tongue ($P<0.01$). Furthermore, we transfected N-cadherin and ß-catenin siRNAs into Tca8113, a tongue SCC cell line, and analyzed the effects on cell migration, invasion, and metastasis *in vitro*. The results showed an obviously decreased invasion after transfection with N-cadherin or ß-catenin siRNAs compared to that of control and control siRNA, but after co-transfection with N-cadherin and ß-catenin siRNAs, a more profoundly decreased invasion was observed ($P<0.05$). In addition, after transfection with N-cadherin and ß-catenin siRNAs, down-regulation of N-cadherin and ß-catenin in Tca8113 cells gave rise to inhibition of invasion and metastasis of Tca8113 cells *in vitro*, probably associated with down-regulation of MMP-2 and MMP-9 proteins and up-regulation of the cell cycle inhibitor p21. Introduction of N-cadherin and ß-catenin siRNAs gave rise to proliferation suppression of the cells, accompanied with a cell cycle inhibition at the G0/G1 phase and cell apoptosis. These findings suggest that N-cadherin and ß-catenin are of vital importance in the invasion and metastasis of SCC of the tongue.

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Introduction

Squamous cell carcinoma (SCC) accounts for >90% of the head and neck tumors and is one of the six most frequent cancers worldwide. SCC of the tongue is a common oral malignancy, and despite advances in treatment, the worldwide trends in five-year survival for SCC of the tongue since the early 1970s have remained relatively constant at ~60% (1,2). However, the molecular mechanisms underlying its development and progression are still not fully understood. Therefore, a better understanding of the molecular mechanisms in development and progression of SCC of the tongue helps to improve the prognosis of patients with SCC of the tongue.

Adhesion molecules play an important role in the development and progression of human cancer (3,4). The large cadherin family includes calcium-dependent cell adhesion molecules responsible for cell-to-cell recognition and adhesion in solid tissues (5,6). Cadherins are single transmembrane proteins that form, especially with catenins, a calcium dependent cell-cell adhesion complex called adherent junction. The catenins bind to the intracellular domain of the cadherins and also to the actin filaments, connecting this adhesion complex to the cell cytoskeleton (7). Changes in the cadherin and catenin expression patterns have been observed and related with cellular invasion and metastatic disease in various types of cancer (8).

Cadherins are expressed in several types of tissues with some specificity; E-cadherin is mostly present in epithelial cells, and N-cadherin in the nervous system, smooth muscle cells, fibroblasts and endothelial cells, and VE-cadherin is specific for the endothelium (6,9). Although classic cadherins interact with similar intracellular partners, they can exert

specific activities. For instance, in epithelial cells, E-cadherin plays an important role in maintaining the epithelioid phenotype of the cells and in inhibiting density-dependent cell growth. In contrast N-cadherin exhibits the opposite activity, as it has been associated with a mesenchymal cell phenotype, increasing cell motility and invasion (10). Reduction in the expression of E-cadherin has been associated with lack of cohesiveness, higher malignant potential and invasiveness in epithelial neoplasms of the lung (11), breast (12), head and neck (13), and other sites. However, the expression of N-cadherin but not E-cadherin has been reported in human mesothelial cells and mesothelioma cell lines (14). A related adhesion molecule, N-cadherin, is up-regulated in invasive cancer cell lines (15,16) and tumors (17,18), and has effects on cellular behavior that are beyond a simple change in adhesive specificity. N-cadherin induced an invasive morphology in squamous tumor cells (19) and stimulated migration, invasion (20,21), and metastasis of breast cancer cells (20).

As a downstream linchpin of Wnt signaling pathway, nuclear ß-catenin functions as a transcriptional activator through complex formation with members of the Tcf/Lef family (22, 23). In addition to playing a critical role in the Wnt signaling pathway, ß-catenin also functions in cell adhesion (23). A human gastric cancer cell line has a homozygous deletion in the ß-catenin gene CTNNB1, which results in impaired cell-to-cell adhesion (24). Numerous studies have suggested that ß-catenin is a potent oncogene product, and its accumulation has been implicated in tumorigenesis in various cancers (25). In contrast, decreased expression of ß-catenin is found in esophageal, colon, gastric, and oral cancers (26-28). The rapidly increasing knowledge on the role of the adhesion molecules cadherins and catenins in cancer will hopefully provide useful molecular markers for tumor prognosis.

To our knowledge, a comprehensive analysis of the role of N-cadherin and ß-catenin in SCC of the tongue has not yet been investigated. Therefore, the purpose of this study was to analyze the expression of the adhesion molecules N-/E-cadherins and ß-catenin by immunohistochemical method and their clinical implications in the SCC of the tongue, and subsequently, to investigate the effect of N-cadherin and ß-catenin siRNAs on invasion, metastasis, cell cycle and apoptosis in oral tongue SCC cell line Tca8113 *in vitro*.

Materials and methods

Tissue samples. Fifty-three tissue samples of SCC of the tongue were obtained from The First Affiliated Hospital of Zhengzhou University. Tissues were fixed in 10% buffered-formalin and embedded in paraffin. The patients did not receive any form of treatment prior to surgery. Cases were selected based on containing both the pathological lesion, either dysplasia (n=53) or invasive carcinoma (n=53) and normal adjacent lateral tongue epithelium (n=53). The study population consisted of 32 men and 21 women. The mean age of patients was 54.6 years.

Immunohistochemistry and evaluation of immunoreactivity. For immunohistochemical detection of N-/E-cadherins and ß-catenin proteins, the standard avidin-biotin-peroxidase complex technique was carried out according to the manufacturer's protocol. In brief, deparaffinized and rehydrated sections were put in a 10^{-3} mol/l sodium citrate buffer (pH 6.0) after bringing the solution to a boil in a pressure cooker and boiled for 20 min while maintaining the pressure. The non-specific binding was blocked by incubating the sections for 30 min with diluted normal horse serum.

For the detection of specific immunoreactivity, the sections were incubated with a 1:80 dilution of the primary antibodies (N-/E cadherins and ß-catenin, Santa Cruz Company, CA) overnight at 4˚C. After this, the sections were incubated with biotinylated secondary anti-mouse antibody (Transduction Laboratories, Lexington, KY, USA) followed by incubation with an avidin-biotin-complex (ABC Elite kit, Vector, Burlingame, CA, USA) for 30 min at room temperature. Finally, the slides were developed for 2 min with the enzyme substrate 3,3'-diaminobenzidine and hydrogen peroxide, and were counter-stained with haematoxylin for 30 sec. Negative control slides processed without primary antibody were included for each staining. The scoring methods for N-/E-cadherins and ß-catenin were: the mean percentage of positive tumor cells was determined in at least five areas at 200-fold magnification and assigned one of the following five categories: 0, <5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; and 4, >75%. The intensities of N-/E-cadherins and ß-catenin immunostaining were scored as follows: 1+, weak; 2+, moderate; and 3+, intense. The scores indicating percentage of positive tumor cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores <1 were defined as negative, and cases were otherwise defined as positive.

Cell culture and siRNA transfection. Tca8113 cells were maintained in RPMI-1640 medium under 5% CO_2 at 37˚C. N-cadherin siRNA, ß-catenin siRNA and control siRNA were purchased from Santa Cruz Company. siRNAs were transfected with Lipofectamine™ 2000 (Invitrogen) into Tca8113 cells at 10 nmol/l following manufacturer's protocol. After 48 h of transfection, cells were harvested and subjected to *in vitro* invasion assay and Western blotting, real-time PCR, cell cycle and cell apoptosis analysis as described below. In addition, at different time points (24, 48, 72, 96, and 120 h) after transfection, cells were also harvested for cell proliferation analysis.

Real-time quantitative PCR. Quantitative PCR was performed using SYBR-Green real-time PCR method (29). Total RNA was extracted from tissues and Tca8113 cells, respectively using TRIzol reagent (Invitrogen, USA). Quantitative real-time PCR was performed on an ABI 7300 PCR instrument (Applied Biosystems, Foster City, CA) using three-stage program parameters provided by the manufacturer as follows: 2 min at 50˚C, 10 min at 95˚C, and then 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. All real-time PCR reactions were done in a 25 μl mixture containing 1X reaction buffer (0.01 nmol of each primer, 0.25X SYBR-Green I, 200 μmol/l deoxynucleotide triphosphate, 2 mmol/l $MgCl_2$, and 1.5 units of TaqDNA polymerase). Specificity of the produced amplification product was confirmed by examination of dissociation

plots. A distinct single peak indicated that a single uence was amplified during PCR. Each sample was tested in triplicate using quantitative real-time PCR, and samples obtained from three independent experiments were used for analysis of relative gene expression using the $2^{-\Delta\Delta Ct}$ method (30).

The following primers for real-time PCR were designed using Primer Express software (Applied Biosystems): N-cadherin (F: 5-CCGGAGAACAGTCTCCAACTC-3, R: 5-CCCACAAAGAGCAGCAGTC-3); E-cadherin (F: 5-TGCCCAGAAAATGAAAAAGG-3, R: 5-GTGTATGTGGCAATGCGTTC-3); ß-catenin (F: 5-CAGATCTTGGACTGGACATTGG-3, R: 5-AACGGTAGCTGGGATCATCCT-3); MMP2 (F: 5-ATGGATCCTGGCTTTCCC-3, R: 5-GCTTCCAAACTTCACGCTC-3); MMP9 (F: 5-AATCTCTTCTAGAGACTGGGAAGGAG, R: 5-AGCTGATTGACTAAAGTAGCTGGA-3); p21(F: 5-GCAGACCAGCCTGACAGATTTC-3, R: 5-GGCACTTCAGGGTTTTCTCTTG-3); ß-actin (F: 5-TCCTTCCTGGGCATGGAGT-3, R: 5-GCCATGCCAATCTCATCTTG-3), ß-actin was used for normalization.

Western blot analysis. Western blotting was performed according to published methods (31). Briefly, cells were harvested and washed twice with PBS and scraped into a lysis buffer. Protein concentrations were determined with the Bradford method. Equal amounts of protein (20 μg/ lane) for each sample were electrophoresed through a 10% SDS-PAGE gel, and then electro-transferred to nitrocellulose membranes (Amersham, Uppsala, Sweden) by a semi-dry transferor. The membranes were blocked in 5% skimmed milk in PBS-T containing 0.05% Tween-20 at room temperature (RT) for 2 h, and subsequently incubated at RT for 2 h with corresponding primary antibodies including N-/E cadherins, ß-catenin, MMP2, MMP9, p21 and ß-actin (Santa Cruz Biotechnology) diluted in 1% skimmed milk in PBS-T buffer, respectively. For detection, horseradish peroxidase-conjugated secondary antibodies were used, followed by DAB solution development according to the manufacturer's instructions. Semiquantification of Band intensity was performed with Gene Tools software (UVP, Inc., Upland, CA, USA)

In vitro invasion assay. *In vitro* invasion assay was assessed using a Boyden chamber assay. Cells (10^5/ml) were suspended in 800 μl of serum-free medium supplemented with 0.2% bovine serum albumin (BSA) and placed in the upper compartment of the chamber for 6 h. After incubation, we collected cells that had migrated to the lower side of the filter to isolate highly invasive clones. To examine invasiveness, cells were fixed with methyl alcohol and stained with H&E at the end of the incubation. Quantification of the invasion assay was performed by counting the number of cells at the lower surface of the filters (30 fields at 200-fold magnification).

Cell proliferation assay. WST-8 is better than MTT for analyzing cell proliferation, because it can be reduced to soluble formazan by dehydrogenase in mitochondria and has little toxicity to cells. Cell proliferation was determined using WST-8 dye (Beyotime Inst. Biotech, China) according to manufacturer's instructions. Briefly, $5x10^3$ cells/well was seeded in a 96-well flat-bottomed plate, grown at 37˚C for 24 h,

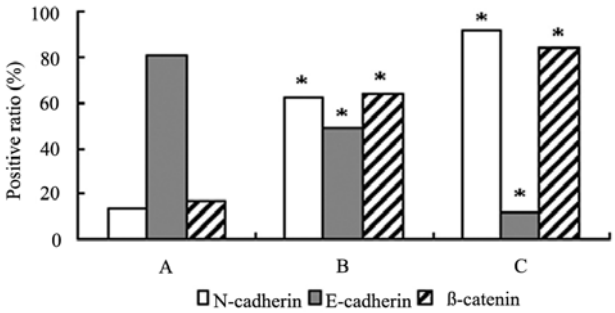

Figure 1. Positive ratio of expressions of N/E-cadherins, and ß-catenin proteins in the normal tongue epithelium, para-cancer tissues and SCC of the tongue. (A-C) showed normal tongue epithelium, para-cancer tissues and SCC of the tongue, respectively.

and then placed in serum-starved conditions for a further 6 h. Subsequently, cells were transfected with control siRNA, N-cadherin siRNA, ß-catenin siRNA and N-cadherin siRNA combined with ß-catenin siRNA, respectively. On the day of measuring the growth rate of untreated and treated cells (24, 48, 72, 96, and 120 h, respectively), 100 μl of spent medium was replaced with an equal volume of fresh medium containing 10% CCK-8, then cells were continued to incubated at 37˚C for 3 h, and the absorbance was finally determined at 450 nm using microplate reader.

Cell cycle analysis. Cell cycle analysis was carried out according to published methods (31). Briefly, $1x10^6$ of untreated and treated Tca8113 cells were harvested at 48 h after treatment and washed in PBS, then fixed in 70% cold ethanol for 30 min at 4˚C. After washing in cold PBS thrice, cells were resuspended in 1 ml of PBS solution with 40 μg of propidium iodide and 100 μg of RNase A for 30 min at 37˚C. Samples were then subjected to analysis of their DNA contents by Becton-Dickinson FACScan flow cytometer (FACScan, Becton-Dickinson, Mountain View, USA).

Cell apoptosis detection. Tca8113 cells untreated and transfected with control siRNA, N-cadherin siRNA, ß-catenin siRNA and N-cadherin siRNA combined with ß-catenin siRNA, respectively, were trypsinized at 48 h, and washed with cold PBS, then resuspended in PBS. Annexin V-FITC (BD Biosciences, USA) at final concentration of 1 μg/ml and 250 ng of propidium iodide were added to a mixture containing 100 μl of cell resuspension and binding buffer (BD Biosciences) each. After cells were vortexed and incubated for 15 min at RT in the dark, 400 μl of binding buffer was added to the mixture for flow cytometric analysis using Becton-Dickinson FACScan flow cytometer (FACScan, Becton-Dickinson).

Statistical analysis. Correlations between the clinicopathological parameters and cadherins and catenin expression were analyzed using χ^2 tests and Fisher's exact tests. Results of RT-PCR and Western blotting were from at least three separate experiments. The data were performed by one-way analysis of variance using SPSS version 13.0 (SPSS, Chicago, IL, USA). Summary statistics were expressed at means ±

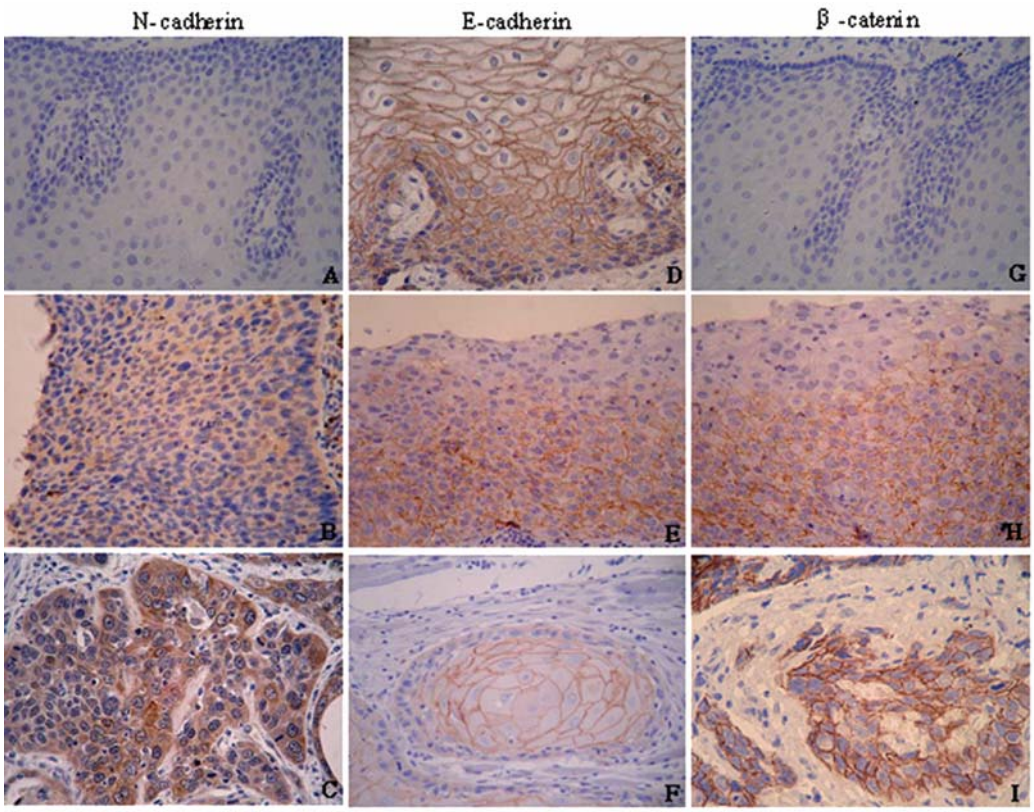

Figure 2. Representative patterns of N, E-cadherins and ß-catenin proteins in normal tongue epithelium, para-cancer tissues and SCC of the tongue tissues. N-cadherin negative in normal tongue epithelium (A); N-cadherin medium cytoplasmic staining in para-cancer tissues (B); N-cadherin strong cytoplasmic staining in SCC of the tongue tissues (C); E-cadherin strong membranous staining in normal tongue epithelium (D); E-cadherin medium membranous staining in para-cancer tissues (E); E-cadherin weak membranous staining in SCC of the tongue tissues (F); ß-catenin negative in normal tongue epithelium (G); ß-catenin medium membranous staining in para-cancer tissues (H); ß-catenin strong membranous staining in SCC of the tongue tissues (I). Original magnification x200 (10x20).

standard deviations, except when otherwise stated. $P<0.05$ was considered statistically significant, and all P-values were two-sided.

Results

Expression of N/E-cadherins, and ß-catenin proteins in normal tongue epithelium, para-cancer tissues and SCC of the tongue tissues. In 53 cases, only 7 showed cytoplasmic staining of N-cadherin in normal tongue epithelium tissues (Figs. 1A-C and 2A-C), and the positive ratio was 13.2% (7/53). However, positive ratios in para-cancer tissues and SCC of the tongue tissues were 62.3% (33/53) and 92.4% (49/53), respectively. Compared to that of normal tongue epithelium tissues, there was an evident increase in the expression of N-cadherin in para-cancer tissues and SCC of the tongue tissues ($P<0.001$) (Fig. 1A-C).

The expression of E-cadherin was generally located in the cell membrane (Fig. 2D-F). There was detectable strong membranous staining of E-cadherin in 43 of 53 cases of the normal tongue epithelium tissues. Expression of E-cadherin gradually decreased during the progression from normal to cancerous states, the positive ratios were: 81.1% (43/53), 49.1% (26/53), and 11.3% (6/53). The result of a comparison between groups indicated a significant difference ($P<0.01$). (Fig. 1D-F).

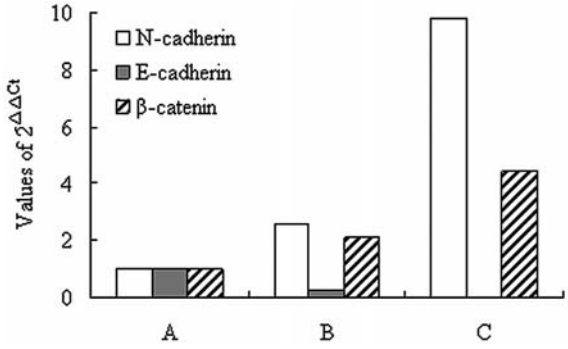

Figure 3. Real-time PCR analysis for the expressions of N/E-cadherins, and ß-catenin mRNAs in normal tongue epithelium (A), para-cancer tissues (B) and SCC of the tongue tissues (C).

Interestingly, on average, the expression of ß-catenin showed membranous staining, however, the expression of ß-catenin gradually transferred into cell nuclei in cancerous states (Fig. 2G-I). Of 53 tongue carcinomas, 45 (84.9%) were strongly positive for nuclear and membranous staining of ß-catenin (Fig. 2G-I), in which nuclear and membranous stainings of ß-catenin were 77.8% (35/45) and 22.2% (10/45), respectively, suggesting nuclear staining of ß-catenin may be a molecular marker for the progression of tongue carcinomas. In addition, the positive ratios of membranous staining of

Association of clinicopathological characteristics with the expression of N-, E-cadherins and ß-catenin in SCC of the

Characteristic	Total (n=53)	N-cadherin expression (%)	P-value	E-cadherin expression (%)	P-value	ß-catenin expression (%)	P-value
Age							
≤50	18	17 (94.4)		13 (72.2)		10 (55.6)	
>50	35	20 (57.1)	0.013	18 (51.4)	0.246	31 (88.6)	0.018
Gender							
Male	32	25 (78.1)		22 (68.8)		21 (65.6)	
Female	21	12 (57.1)	0.186	16 (76.2)	0.782	14 (66.7)	1.000
Histology grade							
1	30	24 (80.0)		19 (63.3)		6 (20.0)	
≥2	23	13 (56.5)	0.123	15 (65.2)	1.000	18 (78.3)	0.000
Nodal status							
N0	24	20 (83.3)		15 (62.5)		12 (50.0)	
N1-3	29	19 (65.5)	0.250	17 (58.6)	0.996	21 (72.4)	0.164
Clinical stage							
II	20	15 (75.0)		11 (55.0)		8 (40.0)	
III and IV	33	22 (66.7)	0.740	23 (69.7)	0.432	20 (60.6)	0.241
Metastases							
Yes	12	4 (33.3)		6 (50.0)		7 (58.3)	
No	41	33 (80.5)	0.006	35 (85.4)	0.029	38 (92.7)	0.014

ß-catenin in normal tongue epithelium and para-cancer tissues were 17% (9/53) and 64.2% (34/53), representing a significant decrease compared with SCC of the tongue tissues (P<0.001). There was no detectable nuclear staining of ß-catenin in the normal tongue epithelium and para-cancer tissues.

Relationship between expression of N/E-cadherins, and ß-catenin proteins and clinicopathological characteristics in tongue SCC tumors. As indicated in Table I, the correlations between expression of N/E-cadherins, and ß-catenin and clinicopathological characteristics in SCC of the tongue were analyzed. The results showed that none of the clinicopathological parameters analyzed, including gender, nodal status, and clinical stage, was significantly correlated with N-/E-cadherins, and ß-catenin expressions. The comparison of N-/E-cadherins expressions between well-differentiated tumors and moderately or poorly-differentiated tumors did not show any statistical difference. In contrast, ß-catenin expression was closely associated with histology grade (P=0.000). In addition, N-cadherin and ß-catenin expression were related to age of patients (P=0.013 and 0.018, respectively), however, the expression of E-cadherin did not appear to differ significantly with the age of patients (P=0.246). Specifically, only the clinicopathological feature 'metastasis' was closely associated with the expressions of N-/E-cadherins and ß-catenin (P=0.006, 0.029 and 0.014, respectively). The data are summarized in Table I.

The results of real-time PCR in normal tongue epithelium, para-cancer tissues and SCC of the tongue tissues. A quantitative real-time PCR study revealed that N-cadherin and ß-catenin mRNAs were uniformly overexpressed in tongue cancer tissues studied (Fig. 3). The expression level of N-cadherin mRNA in tongue cancer tissues was ~10- and 4-fold higher than that expressed in the normal epithelial and para-cancer tissues, respectively. Whereas, the expression level of ß-catenin mRNA in tongue cancer tissues was ~4- and 2-fold higher than that expressed in the normal epithelial and para-cancer tissues, respectively. On the contrary, the expression level of E-cadherin mRNA in tongue cancer tissues was decreased compared to that in the normal epithelial and para-cancer tissues, the expression level of E-cadherin mRNA in normal epithelial and para-cancer tissues was ~125- and 4-fold higher than that in tongue cancer tissues, respectively.

The results of Western blotting in normal tongue epithelium, para-cancer tissues and SCC of the tongue tissues. According to the results of real-time PCR, to determine the expression patterns of N-/E-cadherins and ß-catenin in normal tongue epithelium, para-cancer tissues and SCC of the tongue tissues, we carried out Western blot analysis for their expression patterns in normal tongue epithelium, para-cancer tissues and SCC of the tongue tissues. As indicated in Fig. 4, the expressions of N-cadherin and ß-catenin in tongue carcinoma and para-cancer tissues was obviously higher than that in normal tongue epithelium (P<0.05), whereas expression level of E-cadherin protein in tongue carcinoma and para-cancer tissues was significantly decreased (P<0.01) compared to that in normal tongue epithelium, which was in accordance with the result of immunohistochemistry. In addition, the same expression patterns of N-cadherin and ß-catenin suggested that the combination of N-cadherin and ß-catenin played an important role in development and progression of the tongue carcinoma.

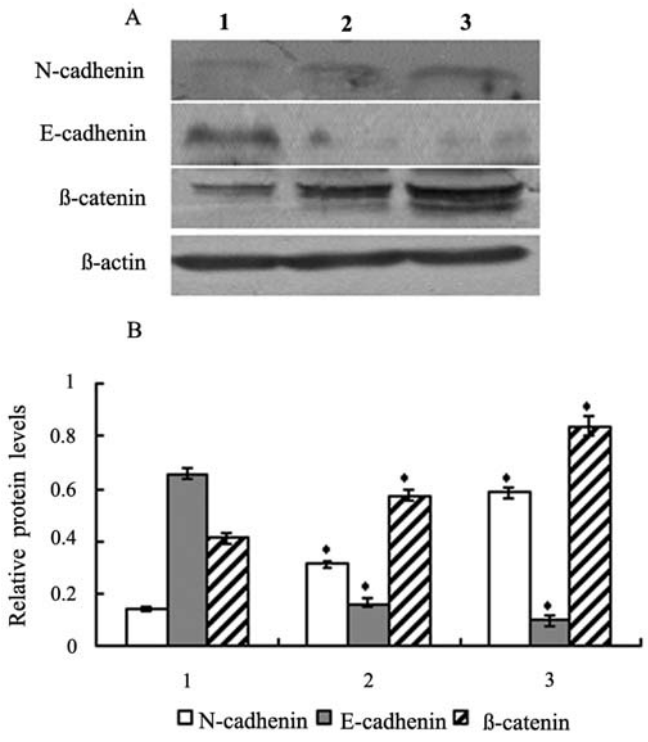

Figure 4. Detection of expression patterns of N-/E-cadherins and ß-catenin proteins in normal tongue epithelium, para-cancer tissues and SCC of the tongue tissues by Western blotting (A). Results from three independently repeated experiments, which were statistically analyzed by densitometry using Gene Tools software, are expressed as means ± SD (B). *P<0.05. Results of one representative experiment are reported. Levels of ß-actin are shown for equal loading conditions. 1, 2 and 3 represent normal tongue epithelium, para-cancer tissues and SCC of the tongue tissues, respectively.

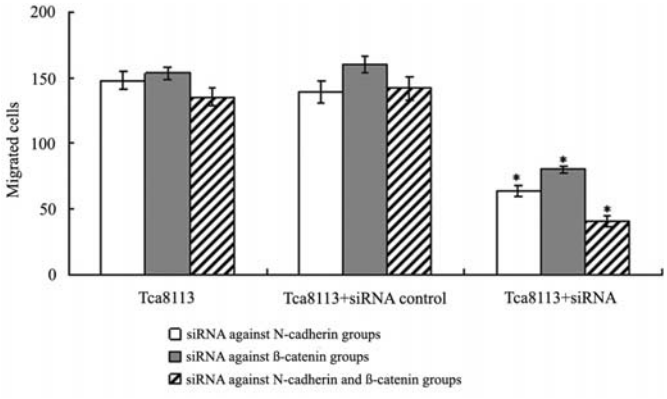

Figure 5. N-cadherin and ß-catenin promotes cell migration and invasion. Migrating cell numbers were counted in three individual experiments. The numbers represent mean ± SD for three experiments.

In vitro invasion assay after transfection with siRNA against N-cadherin and ß-catenin. Boyden chamber assay revealed that after treatment with the N-cadherin and ß-catenin, siRNA significantly (P<0.01) decreased the number of migrating cells (Fig. 5). However, when cells treated with N-cadherin siRNA combined with ß-catenin siRNA, the number of migrating cells were evidently less than those of cells treated with siRNA against N-cadherin or ß-catenin alone (P<0.05). These results indicated that N-cadherin and ß-catenin siRNAs could both obviously inhibit Tca8113 cell migration and combination of N-cadherin and ß-catenin had a synergistic effect on invasion and metastasis of Tca8113 cells, suggesting N-cadherin and ß-catenin play an important role in invasion and metastasis of SCC of the tongue.

Effects of siRNAs against N-cadherin and ß-catenin on the expression patterns of N-cadherin and its related proteins in Tca8113 cells. To investigate the invasive and metastasis mechanisms of N-cadherin and ß-catenin in tongue carcinoma Tca8113 cells. The total proteins and RNA were obtained from Tca8113 cells untreated and transfected with control siRNA and siRNA against combination with N-cadherin and ß-catenin at 48 h, respectively. The mRNAs and protein expression changes of N-cadherin and its related genes were analyzed using real-time PCR and Western blotting, respectively. As shown in Fig. 6, the results of real-time PCR demonstrated that the expression levels of E-cadherin and p21 mRNAs in Tca8113 cells transfected with N-cadherin and ß-catenin siRNAs were ~7- and 8.5-fold higher than that expressed in the control, respectively. Whereas, the expression levels of N-cadherin, ß-catenin, MMP2, and MMP9 in the control were ~6.5-, 8.0-, 6.0-, and 13.5-fold higher than that expressed in Tca8113 cells transfected with N-cadherin and ß-catenin siRNAs, respectively. However, there is no difference in Tca8113 cells untreated and transfected with control siRNA (Fig. 6 A).

In the Tca8113 cells transfected with siRNA against combined N-cadherin and ß-catenin, proteins levels of N-cadherin and ß-catenin were obviously reduced in all three separate experiments (P<0.01), whereas level of E-cadherin protein was significantly increased (P<0.01) compared to that of the cells untreated and transfected with control siRNA. In addition, compared to that of the cells untreated and transfected with control siRNA, protein expressions of MMP2 and MMP9 was down-regulated and p21 protein expression was up-regulated in the cells transfected with siRNA against combined N-cadherin and ß-catenin (Fig. 6B and C), suggesting that the expression inhibition of N-cadherin and ß-catenin may play an important role in invasion and metastasis of tongue carcinoma.

Cell proliferation assay. To determine whether siRNA against N-cadherin or ß-catenin in Tca8113 cells affects cellular proliferation, we utilized a CCK-8 kit, a kind of viable cell counts, as a substitute for MTT assays to analyze the effect of siRNA against N-cadherin or ß-catenin on cellular proliferation in Tca8113 cells. As shown in Fig. 7, there were no obvious differences in proliferation rates of Tca8113 cells untransfected and transfected with siRNA control (P>0.05). However, the growth of Tca8113 cells transfected with N-cadherin or ß-catenin was inhibited significantly (P<0.01) compared to that of Tca8113 cells untransfected and transfected with siRNA control. In addition, the growth of Tca8113 cells transfected with siRNA against N-cadherin was obviously inhibited compared to that of Tca8113 transfected with siRNA against ß-catenin (P<0.05). Interestingly,

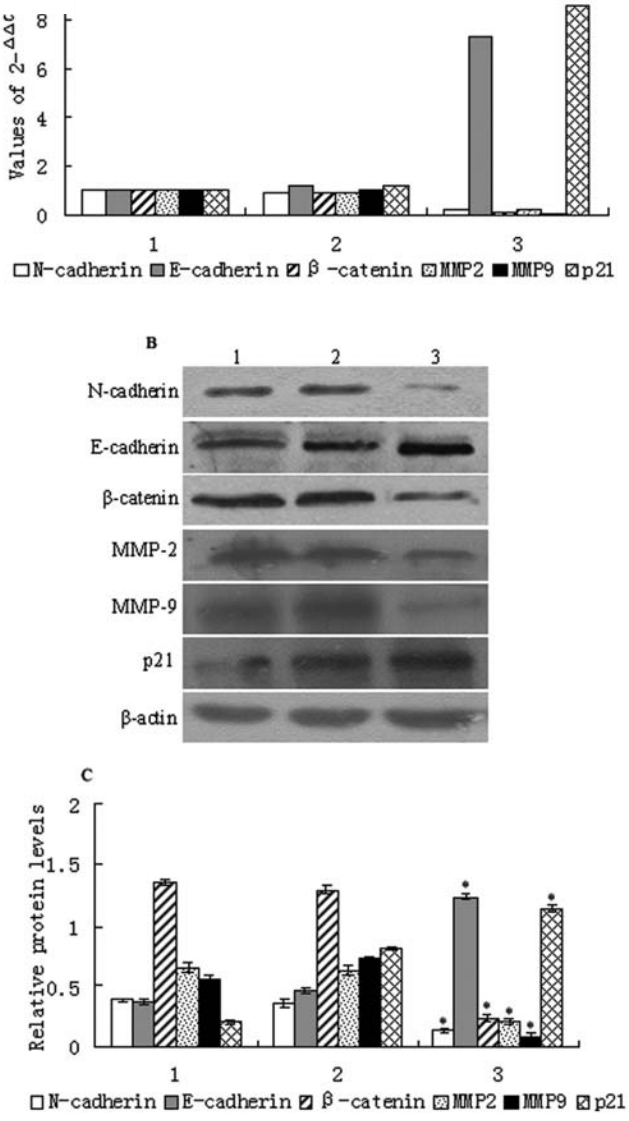

Figure 6. Expression of N-cadherin and its related genes. Numbers 1, 2, and 3 represent Tca8113 cells untreated and transfected with control siRNA and combination siRNA against N-cadherin and ß-catenin, respectively. To verify the specificity of the reactions, Tca8113 cells untreated and transfected with control siRNA was used as negative controls, respectively. The housekeeping gene ß-actin was used for normalization. Real-time PCR analysis of mRNA expression of N-/E-cadherins, ß-catenin, MMP-2/-9, p21 and ß-actin in Tca8113 cells (A). Analysis of proteins levels of N-/E-cadherins, ß-catenin, MMP-2/-9, p21 and ß-actin in Tca8113 cells (B). Semi-quantitated values of proteins levels of N-/E-cadherins, ß-catenin, MMP-2/-9, and p21 to ß-actin, respectively (C). Results from three independently repeated experiments, which were statistically analyzed by densitometry using Gene Tools software, are expressed as means ± SD. *P<0.05, compared to those of Tca8113 cells untreated and transfected with control. Herein, results of one representative experiment are reported. Levels of ß-actin are shown for equal loading conditions.

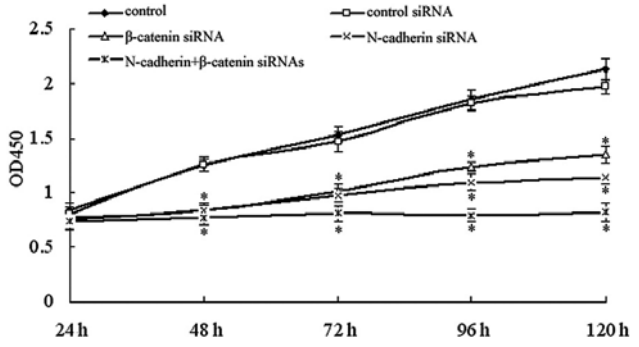

Figure 7. The effect of N-cadherin and ß-catenin siRNAs on the growth inhibition of tongue carcinoma cell line Tca8113 cells. The growth of Tca8113 cells was detected by CCK8 kit assay after transfection with siRNAs against N-cadherin and ß-catenin. Tca8113 cells had an obvious decreased growth rate compared to the control Tca8113 cells and Tca8113 cells transfected with control siRNA (P<0.01). *P<0.05, compared to those of Tca8113 cells untreated and transfected with control siRNA.

combination with siRNAs against N-cadherin and ß-catenin, the growth of Tca8113 cells was most obviously inhibited among the groups. These results demonstrated that introducing of siRNAs against N-cadherin and ß-catenin was able to inhibit obviously the growth of the Tca8113 cells.

Cell cycle assay. Since cell growth and death are closely linked to progression of the cell cycle, to characterize the mechanisms underlying the growth inhibition by siRNAs against N-cadherin and ß-catenin, we analyzed cell cycle kinetics in Tca8113 cells untreated, transfected with control siRNA, N-cadherin siRNA, and ß-catenin siRNA. As shown in Fig. 8, Tca8113 cells transfected with N-cadherin siRNA or ß-catenin siRNA showed a higher proportion of cells in G0/G1 phase (64.55% and 62.53%, respectively), compared to control (40.35%) and control siRNA (44.57%), suggesting that N-cadherin siRNA or ß-catenin siRNA effectively arrests Tca8113 cells in the G0/G1 phase of the cell cycle. On the other hand, the proportion of cells at S phase was decreased (P<0.01), compared to cells untreated and transfected with control siRNA.

In addition, to determine if combination with siRNAs against N-cadherin and ß-catenin has a collaborative effect, siRNAs against N-cadherin and ß-catenin were simultaneously transfected into Tca8113 cells for 48 h. The results of flow cytometry analysis revealed that cells treated with a combination of N-cadherin and ß-catenin siRNAs had higher proportion (73.16%) of cells at the G0/G1 phase than those only treated with N-cadherin siRNA or ß-catenin siRNA (Fig. 8A and B), and there was a statistically significant difference (P<0.05). Besides, cells treated with combination of N-cadherin and ß-catenin siRNAs showed the lowest proportion of cells at S phase (15.27%). Control siRNA had no effect on cell cycle of Tca8113 cells compared to Tca8113 cell untreated (P>0.05). These findings suggest that the combination of N-cadherin and ß-catenin siRNAs has a collaborative effect on the cell cycle.

Cell apoptosis detection. It is well known that invasion and metastasis of tumor cells are closely associated with cell apoptosis. To examine whether N-cadherin siRNA or ß-catenin siRNA is able to affect apoptosis of Tca8113 cells, Tca8113 cells were, respectively, transfected with N-cadherin siRNA and ß-catenin siRNA, cell apoptosis detection was carried out by flow cytometry (Fig. 9). Cells untreated and transfected with control siRNA served as negative controls. The results showed that N-cadherin or ß-catenin siRNAs obviously induced Tca8113 cell apoptosis (P<0.05). After transfection

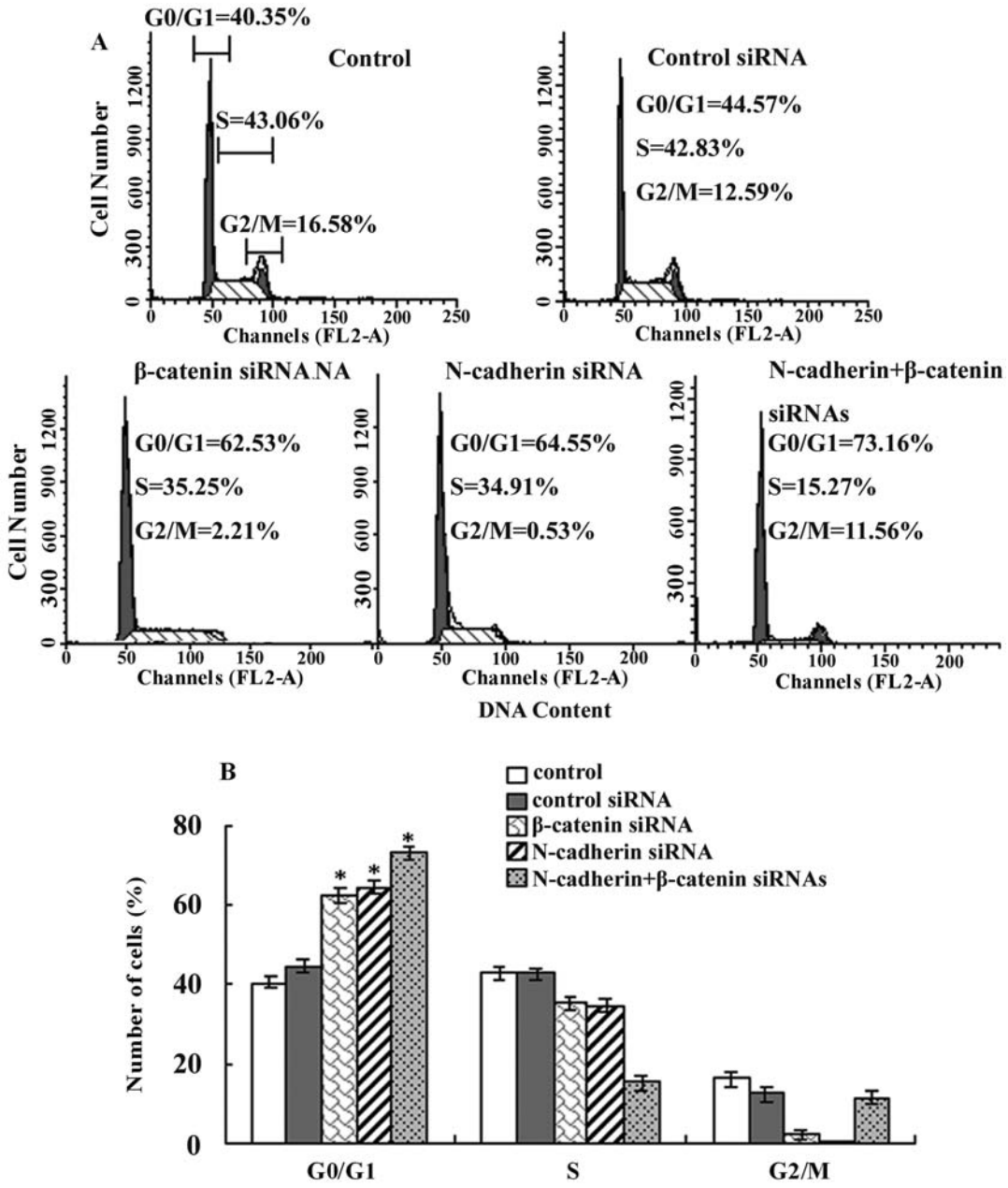

Figure 8. N-cadherin and ß-catenin siRNAs arrest Tca8113 cells in G0/G1 phase of cell cycle. Tca8113 cells were seeded into six-well plate containing 10% FBS RPMI-1640, Tca8113 cells were harvested at 48 h after transfection, and propidium iodide staining was used to analyze cell cycle distribution. (A) Tca8113 cells untreated and transfected with control siRNA, N-cadherin siRNA, ß-catenin siRNA, and combination of N-cadherin and ß-catenin siRNAs were harvested at 48 h. Cell cycle was analyzed using flow cytometry. (B) Results derived from three independent experiments are expressed as means ± SD. *P<0.05, compared to those of Tca8113 cells untreated and transfected with control siRNA.

with N-cadherin and ß-catenin siRNAs, respectively, the proportions of positive cells for Annexin V and propidium iodide (region II) (25.23% and 21.49%, respectively) were evidently increased (P<0.05), compared to cells untreated and transfected with control siRNA (2.37% and 2.85%, respectively). However, there was no difference between cells untreated and transfected with control siRNA (P>0.05). Besides, the apoptotic proportion of Tca8113 cells treated with a combination of N-cadherin and ß-catenin siRNAs (36.06%) was significantly higher (P<0.01) than those of Tca8113 cells treated with N-cadherin siRNA or ß-catenin siRNA alone (Fig. 9 A and B), suggesting the combination of N-cadherin and ß-catenin siRNAs has a collaborative effect on apoptosis of Tca8113 cells as well. Moreover, combination with the results of invasion experiment mentioned above, the inhibition of N-cadherin and ß-catenin siRNAs on invasion may be mediated by induction of cell apoptosis.

Discussion

It is now well established that cell adhesion molecules of the cadherin superfamily are frequently altered during tumor progression. The loss of E-cadherin or its dysfunction leads to increased motility and invasiveness of carcinoma cells (32). Thus, a subset of tumors shows up-regulation of N-cadherin concomitant to their invasive stage, including in breast (15),

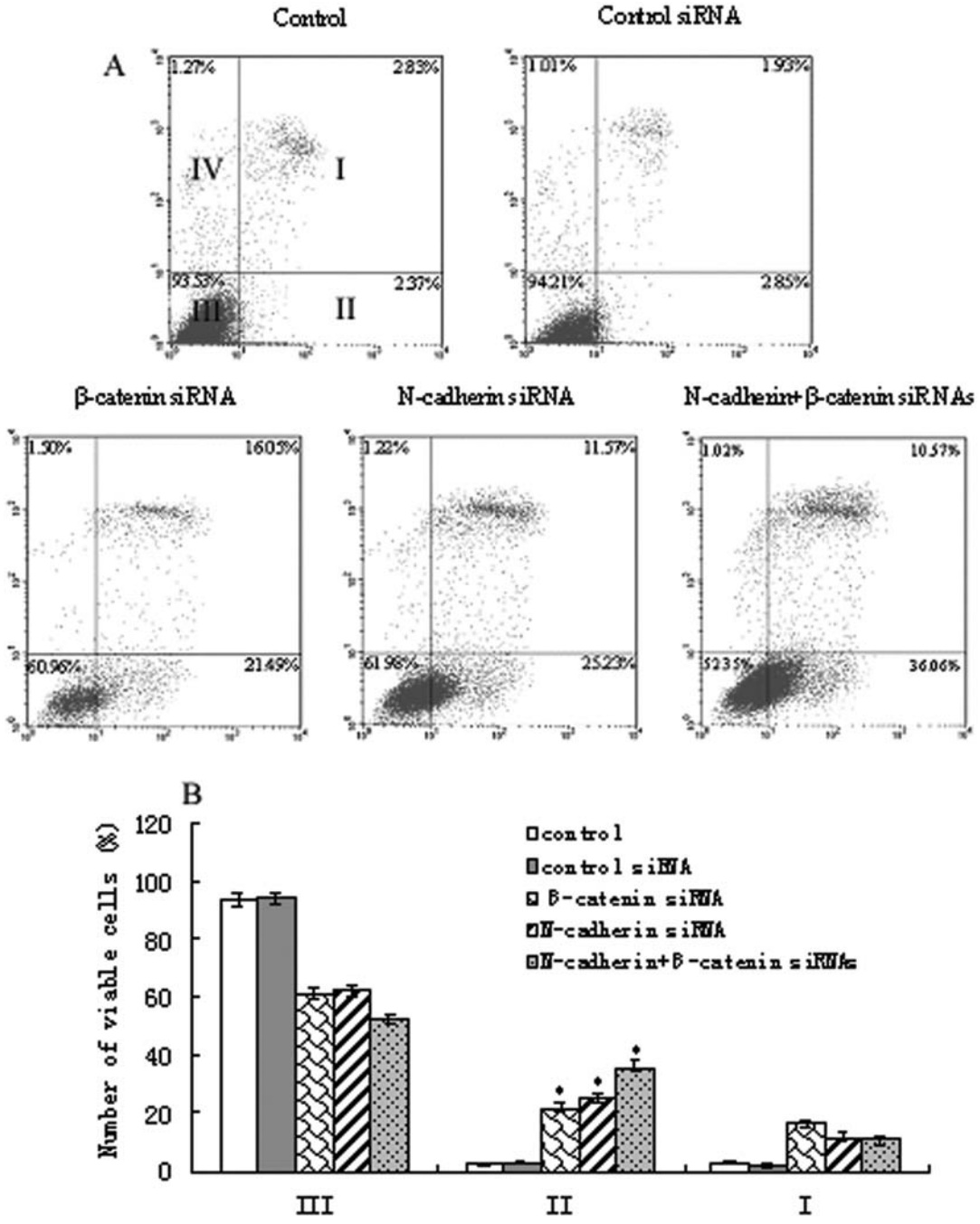

Figure 9. Induction of apoptosis in Tca8113 cells by N-cadherin and ß-catenin siRNAs. Tca8113 cells were seeded into six-well plate containing 10% FBS RPMI-1640, Tca8113 cells were harvested at 48 h after transfection, followed by apoptosis assay using the Annexin V-FITC apoptosis detection kit, as indicated in Materials and methods. (A) Tca8113 cells untreated and transfected with control siRNA, N-cadherin siRNA, ß-catenin siRNA, and combination of N-cadherin and ß-catenin siRNAs were harvested at 48 h. Cell apoptosis was analyzed using flow cytometry. (B) Results derived from three independent experiments are expressed as means ± SD. *P<0.05, compared to those of Tca8113 cells untreated and transfected with control siRNA. Note: I, late apoptosis cells; II, early apoptosis cells; III, live cells.

melanoma (33), prostate (18), bladder (34), pancreas (35), colon (36), but not in SCC of the tongue. Moreover, recent evidence demonstrates that N-cadherin is dominant over E-cadherin in metastatic progression and is overexpressed in a subset of cancer types in addition to the loss of E-cadherin (37,38). It was proposed that switching expression from E-cadherin to N-cadherin in tumor cells can enhance invasive and survival capabilities of tumor cells and also can promote cooperation between tumor cells and the surrounding micro-environment, a critical event in metastatic progression. Whereas, the role of ß-catenin in development of tumor remains controversial. Up to now, the role of N-cadherin and ß-catenin in SCC of the tongue has not been reported. In addition, N-cadherin and ß-catenin have respectively shown to mediate G1 cell-cycle arrest and/or apoptosis in different tumors. Therefore, N-cadherin and ß-catenin also seem to play an important role in the modulation of cell cycle progression and/or apoptosis.

The purpose of the current study was to investigate whether N-cadherin or ß-catenin could be a molecular marker of

progression for SCC of the tongue, and they could affect invasion and metastasis, cell cycle arrest and apoptosis of SCC of the tongue. In the present study, we analyzed N-/E-cadherins and ß-catenin protein expression levels in 53 patients with SCC of the tongue and evaluated its correlation with the clinicopathological features. Using immunohistochemical analysis of SCC of the tongue from 53 patients, the results revealed that the expressions of N-cadherin and ß-catenin in SCC of the tongue were significantly higher than those in para-carcinoma and normal epithelium tissues. Conversely, the expression of E-cadherin in normal epithelium tissues is obviously higher than that in para-carcinoma and SCC of the tongue, and the results of real-time PCR and Western blotting showed the same characteristics with the result of immunohistochemistry. These findings suggested that the high expression of N-cadherin and ß-catenin and loss of E-cadherin expression result in progression of SCC of the tongue.

During the present study, ß-catenin accumulation in the nucleus was only shown in SCC of the tongue, not in the para-cancer and normal epithelium, suggesting that nuclear accumulation of ß-catenin in SCC of the tongue is closely related to tumor progression. Therefore, accumulation of ß-catenin increases the risk of tumor recurrence and indicates a poor prognosis. Furthermore, we analyzed the relationship between expressions of N/E-cadherins, and ß-catenin proteins and clinicopathological characteristics in SCC of the tongue. The results showed that none of the clinicopathological parameters analyzed, including gender, nodal status, and clinical stage, was significantly correlated with N/E-cadherins, and ß-catenin expressions. The comparison of N-/E-cadherins expressions between well-differentiated tumors and moderately or poorly-differentiated tumors did not show any statistical difference. In contrast, ß-catenin expression was closely associated with histology grade (P=0.000), which was contrary to some studies that ß-catenin were not associated with the histological tumor grade (39-41). In addition, N-cadherin and ß-catenin expression were related to age of patients ($P<0.05$), however, the expression of E-cadherin did not appear to differ significantly with the age of patients ($P>0.05$). Specifically, only the clinicopathological feature 'metastasis' was closely associated with the expressions of N-/E-cadherins and ß-catenin ($P<0.05$), indicating that N-/E-cadherins and ß-catenin play an important role in invasion and metastasis of SCC of the tongue.

The expression N-cadherin and ß-catenin is closely associated with metastasis of SCC of the tongue *in vivo*. We investigated whether interfering N-cadherin and ß-catenin could affect the invasion and metastasis of tongue carcinoma cell line Tca8113. Therefore, Matrigel invasion and metastasis experiments were performed. Our results revealed that siRNA against N-cadherin or ß-catenin both inhibited the invasion and metastasis of Tca8113 cells. Whereas, in Tca8113 cells treated with siRNA against N-cadherin combined with siRNA against ß-catenin, the cell numbers of invasion and metastasis were evidently less than those of cells treated with siRNA against N-cadherin or ß-catenin alone, indicating that the combination of siRNA against N-cadherin and ß-catenin has synergistic effect on invasion and metastasis of Tca8113 cells. These findings further suggest that N-cadherin and ß-catenin may play an essential role in invasion and metastasis of SCC of the tongue.

Cell invasion and metastasis are critical targets for therapeutic treatment of malignant solid tumors. One key mechanism involved in these pathological processes is the degradation of mechanical barriers represented by specialized extracellular matrices. Tools of election have been found to be certain tumor-cell-associated proteinases such as matrix metalloproteinases (MMPs) 2 (42) and 9 (43); their expression and activity against matrix macromolecules have been linked to the development of malignant phenotype (44). Therefore, we further determined whether the decreased invasion and metastasis of Tca8113 cells is due to decreased expression of MMPs. It is well documented that MMPs play an important role in cancer promotion and in pulmonary metastasis (45,46). Herewith, expressions of MMP-2 and MMP-9 were investigated by real-time PCR and Western blotting in Tca8113 cells transfected with N-cadherin and ß-catenin siRNAs. In the current study, we found that down-regulation of N-cadherin and ß-catenin in Tca8113 cells inhibited invasion and metastasis of Tca8113 cells *in vitro*, probably associated with down-regulation of MMP-2 and MMP-9 proteins and up-regulation of the cell cycle inhibitor p21cip1/Waf1 (p21).

In the present, p21 was perceived mainly as an anticancer protein because of its antiproliferative effects. Its role in human cancer has become more controversial because it may also possess anti-apoptotic (procancer) capabilities (48). Furthermore, proliferation experiments were conducted with CCK-8, the results demonstrated that siRNA against N-cadherin or ß-catenin both inhibited cell proliferation, but their combination inhibited Tca8113 cell proliferation significantly. Introduction of siRNA against N-cadherin or ß-catenin could both arrest Tca8113 cell at G0/G1 phase and induce cell apoptosis, possibly mediated by the p21-dependent signaling pathway.The mechanisms by which p21 may promote apoptosis are not currently understood but they could be related to its ability to interact with and possibly regulate components of the DNA repair machinery.

In conclusion, our findings in the present study suggest: i) N-cadherin and ß-catenin may play an essential role in the invasion and metastasis of tongue carcinoma; ii) down-regulated expression of N-cadherin and ß-catenin may give rise to cell cycle arrest and cell apoptosis; iii) N-cadherin and ß-catenin, especially N-cadherin may be useful for molecular target therapy of tongue carcinoma. We believe that the data presented in this study may prove helpful in the treatment and palliation of tongue carcinoma.

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