Influence of E–cadherin Promoter Methylation and Mutation of β–catenin on Invasion and Metastasis of Nasopharyngeal Carcinoma Cells

Zhi Li, Suxia Lin, Huizhen Liang

Department of pathology, Sun Yat–sen medical college, Sun Yat–sen University, Guangzhou 510089, China

Abstract **Objective** To study the mechanisms of invasion and metastasis in early NPC to be associated with E–cadherin promoter methylation and mutation in exon 3 of β–catenin. **Methods** Methylation of E–cadherin promoter, mutation in exon 3 of β–catenin and differential expression of β–catenin in the primary lesion of 21 NPC and the metastatic lymph node of 21 NPC were investigated by DNA Methylation–Specific PCR, direct sequencing and immunohistochemical method. **Results** Methylation of E–cadherin promoter was showed in 23.8%(5/21) primary lesion and 61.9%(13/21) metastatic lymph node ($P<0.01$). Mutation in exon 3 of β–catenin was showed in 3 of 42 tissues: codon 37 (TCT→GCT), codon 41 (ACC→GCC) and codon 47 (AGT→ACT). However, there was no relationship between these mutations and invasion or metastasis ($p>0.05$). High β–catenin expression on the membrane without nuclear expression was observed in 42 tissues ($p>0.05$). **Conclusions** 1. In NPC, methylation of promoter is a main reason of down–regulation of E–cadherin which may lead to detachment and metastasis of neoplastic cells finally. 2. Mutation in exon 3 of β–catenin is a rare event in NPC. It may be an early event in the carcinogenesis of NPC, but have no significant role in invasion and metastasis. 3. High expression of β–catenin, as one of NPC characteristics, is not a key factor for invasion and metastasis.

Key words Nasopharyngeal neoplasm; Neoplasm metastasis; E–cadherin; β–catenin; Methylation; Mutation

Nasopharyngeal carcinoma (NPC) is a malignant tumor that is well known likely to invasive the surrounding tissue or metastasize to the cervical lymph nodes in the early stage of tumor development[1]. However, We don´t know the major relevant mechanism until now. Many factors are perhaps associated with local invasion and distant metastasis. E–cadherin is one of the important calcium–dependent cell adhesion molecules. Cooperating with cytoplasmic catenins (α–, β– and γ–catenin), E–cadherin plays a major role in the maintenance of intercellular junctions in normal epithelia cells by forming an E–cadherin–catenein complex. The expression of E–cadherin has been found to be downregulated in many human cancers [2,3], including NPC[4] . It suggests that E–cadherin downregulation may play a role in tumor progression and metastasis. In some cancer cells, aberrant gene promoter methylation of E–cadherin has been reported to be responsible for the decreased expression of E–cadherin[5,6]. However, little attention has been paid to whether E–cadherin promoter methylation is responsible for the early invasion and metastasis of NPC. The mechanism by which E–cadherin expression is altered remains obscure in NPC.

β–catenin is a major component of cadherin system and is also involved in the Wnt pathway as a transcriptional activator [7,8]. The change of β–catenin expression location and accumulation of the aberrant protein expression were usually found in human tumor[9]. However, the definite mechanism related was still unknown. Activating mutations in exon 3 of β–catenin gene have been described in many human malignant tumors[9,10] . If that occurs not received much attention yet.

To better confirm the major factors involved in the mechanism of tumor cells invasion and metastasis in early stage of the tumor, in the present study we investigated the gene promoter methylation of E–cadherin, mutation in exon 3 of β–catenin and the different expression pattern of β–catenin in pri-

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Correspondence to: Dr. Zhi LI (PhD), Department of pathology, Sun Yat–sen medical college, Sun Yat–sen University. 74#, Zhongshan Road II, Guangzhou, 510089, Guangdong province, China.
Tel: 8620–87331783, 8620–87331784.
Fax: 8620–87331679.
E–mail: pathol@gzsums.edu.cn

mary tumor and lymph node metastatic tumor of NPC.

MATERIALS AND METHODS

Tissue Samples and Histological Classification

The paraffin-embedded samples of 21 primary lesion of NPC and 21 metastatic lymph node of NPC were collected from the affiliated hospitals of Sun Yat-sen University of Medical Sciences, Guangzhou, China, during 1999-2001, all patients were not subjected to radiotherapy and chemotherapy. 28 patients were male, from 39 to 74 years old, and the average was 55.8 years old. 14 cases were female, from 40 to 70 years old, and the average age was 58.4 years old. All of them were classified according to the WHO criteria, 27 out of 42 cases were undifferentiated non keratinizing carcinoma (UNKC), and 15 cases were the subtype of differentiated non-keratinizing carcinoma (DNKC). There was no keratinzing carcinoma subtype found in all of sample tissues. Based on the growth pattern of the neoplastic cells, neoplastic cells of 22 cases showed so-called Schmincke growth pattern, the neoplastic cells mixed with the lymphoid cells, and no tumor nest had been seen. 12 cases were Regaud growth pattern, there was tumor cell nest with the clear region surrounding the lymphoid stroma. The other 8 cases was mixed pattern. A part of tissue samples was routinely stained with H&E, the rest was used to extract the genomic DNA.

Methods

Immunohistochemical staining Immunohistochemical staining was adopted EnVision plus system (Cat. No.K4011, DAKO, Glostrup, Denmark). Sections were incubated with anti-β-catenin monoclonal antibody (CAT-5H10, Code No. 13-8400, ZYMED Laboratories, San Francisco, CA), and the working dilution was 1:200. Antigen retrieval was preformed by microwaving in 10mM citrate buffer (pH 6.0) for 15 min. Slides were lightly counterstained with 1% methyl green. Two pathologist examined neoplastic cells membrane, nuclear and cytoplasmic staining independently, using double blank scoring method to evaluate the percentage of stained tumor cells.

Detection of gene promoter methylation of E-cadherin (1) DNA extraction and Sodium bisulfite modification: Genomic DNAs of 42 cases were isolated from 10μm-thick, formalin-fixed and paraffin-embedded serial sections. The sections were treated with xylene and alcohol to dewax and rehydrate the tissues. The genomic DNA of samples was extracted using QIAamp DNA Mini Kit (Cat. No. 51304, QIAGEN Inc. Valencia CA). First The aberrant methylation in rich CpG island of genomic DNA was modified by sodium bisulfite. After this chemical modification, all unmethylated cytosines (C) were deaminated and converted to uracils (U), but 5-methylcytosines (Cm) remain unaltered. The chemical modification was adopted CpGenome DNA modification kit (Cat. No. S7820, Invitrogen life technologies, Carlsbad CA). 1.0μg sample DNA was dissolved in 100μl H_2O, then added 3M NaOH and incubated at 37℃ for 10min. Fresh sodium bisulfite (pH5.0) was added into the mixture and vortex, followed by reaction in water bath at 50℃ for 16-20h. After finishing, reagent Ⅱ and reagent Ⅲ provided by kit were added into the reaction system in turn, mix and centrifuge at 5000×g for 10s to precipitate DNA. The DNA pellete was washed by fresh 70% alcohol for 3 times, then added 20mM NaOH/90% alcohol to rinse out the salt. Finally, the modified DNA was dissolved by 20-50μl TE solution (pH 7.5), and the DNA samples were stored at -20℃. (2) DNA methylation-specific PCR (MSP): Two pairs of primer were used to amplify each DNA sample. One for methylated E-cadherin promoter of: F 5′-TTAGGTTAGAGGGTTATCGCGT-3′, R 5′-TAACTAAAAATTCACCTACCGAC-3′, annealing at 57℃, 116bp. Another for unmethylated E-cadherin promoter, F 5′-TAATTTTAGGTTAGAGGGTTATTGT-3′, R 5′-CACAACCAATCAACAACACA-3′, annealing at 53℃, 97bp[11]. The samples were denatured at 95℃ for 10 min, followed by adding 1.25U Taq enzyme into the reaction system. The 40 thermal cycles were: 94℃, 30s, 94℃ 45s, 72℃ 45s. Final extension at 72℃ for 10min. The PCR products were analyzed by electrophoresis on 6% polyacrylamide gel. Samples were evaluated as methylation positive when obvious bands were observable on the gel.

Direct DNA sequence analysis for β-catenin mutation in exon 3 Gene mutations of β-catenin from 42 patients were performed by PCR to amplifying 201bp fragment of exon 3, followed by direct cycle sequencing. The primers′ sequence were: F 5′-ATGGAACCAGACAGAAAAGCGG-3′; R 5′-AGCTACTTGTTCTTGAGTGAAG-3′ [12]. The PCR conditions were as follows: 95℃, 5min, 35 cycles

of 94℃ 30s, 57℃ 30s and 72℃ 40s, extension at 72℃ for 10min. PCR product was purified by QIAquick PCR purification kit (Cat. No. 28106, QIAGEN Inc. Valencia, CA) and subjected to direct sequencing using a BigDye Terminator cycle sequencing Ready Reaction Kit (Cat. No. 403044, Perkin-Elmer, Foster City, CA). Sequencing reactions were run on an ABI Prism 3100 genetic analyzer, and sequencing was performed with the same PCR as above.The DNA and protein products obtained will be compared with sequence of exon 3 β-catenin in GenBank. All the mutation were verified by duplicate experiment.

RESULTS

β-catenin expression immunohistochemical Apart from 2 out of 42 cases were negative expression of β-catenin, 50%~100% neoplastic cell showed β-catenin protein expression on the membrane of cell in the other 40 cases. There was no detectable nuclear staining of β-catenin in any section of NPC tissues (Fig.1). There were no relationship between the expression of β-catenin with the patient´s gender, histological subtype, tumor cell growth pattern or lymph node metastasis. (Tab.1).

Detection of gene promoter methylation of E-cadherin In 42 cases, 39 cases (92.9%) displayed 97bp-unmethylated form of E-cadherin gene promoter. However, 116bp-methylated form of E-cadherin gene promoter was detected only in 18 out of 42 cases (42.9%) (Fig.2). Furthermore, in 21 cases of primary lesion, only 5 cases (23.8%) displayed gene promoter methylation of E-cadherin. In 21 cases of metastatic lymph nodes, 13 cases (61.9%) could be detected methylated form of E-cadherin. There was a significant different between primary lesion and metastatic tumor of NPC in gene promoter methylation of E-cadherin (χ^2=6.22, P<0.01; Tab.1). As showed in Table 1, the methylation degree in the tumor with Schmincke growth pattern (10/22, 45.5%) was more higher than that with Regaurd growth pattern (4/15, 26.7%). However, there was no a significant different found between two growth patterns (χ^2=4.75, P>0.05). The gene promoter methylation of E-cadherin also showed no correlate with histological subtype of tumor

β-catenin gene mutation in exon 3 Point mutations of the β-catenin in exon 3 gene were present in 3 of 42 (7.1%) NPC tissues. They were codon 37 Ser→Ala (TCT→TGT), codon 41 Thr→Ala (ACC→GCC) and codon 47 Ser→Thr (AGT→ACT) (Fig.3). The mutation in exon 3 of β-catenin gene had no relation to the tumor histological subtype, tumor cell growth pattern or lymph node metastasis. (Tab.1).

Table 1 The relationship between the alteration of E-cadherin, β-catenin and clinicopathological features in 42 cases of NPC (%)

Clinicopathological Features	Cases No.	β-catenin		Expression degree of β-catenin	Methylation of E-CD promoter	Mutation of β-catenin
		negative	positive			
Gander				80(50-100)	11(39.3)	2
male	28	2	26	75(50-100)	7(50)	1
female	14	0	14	>0.05	>0.05	>0.05
P value						
Histology type				85(50-100)	6(40)	1
DNKC	15	0	15	80(50-100)	12(44.4)	2
UNKC	27	2	25	>0.05	>0.05	>0.05
P value						
Growth pattern	22	0	22	85(50-100)	10(45.5)	3
schmincke	12	1	11	85(50-100)	4(26.7)	0
regaud	8	1	7	80 (60-90)	4(50)	0
mixed				>0.05	>0.05	>0.05
P value						
Tumor site				80(50-100)	5(23.8)	2
primary lesion	21	2	19	80(50-100)	13(61.9)	1
metastatic tumor	21	0	21	>0.05	<0.01	>0.05
P value						
Total	42	2	40		18(42.9)	3

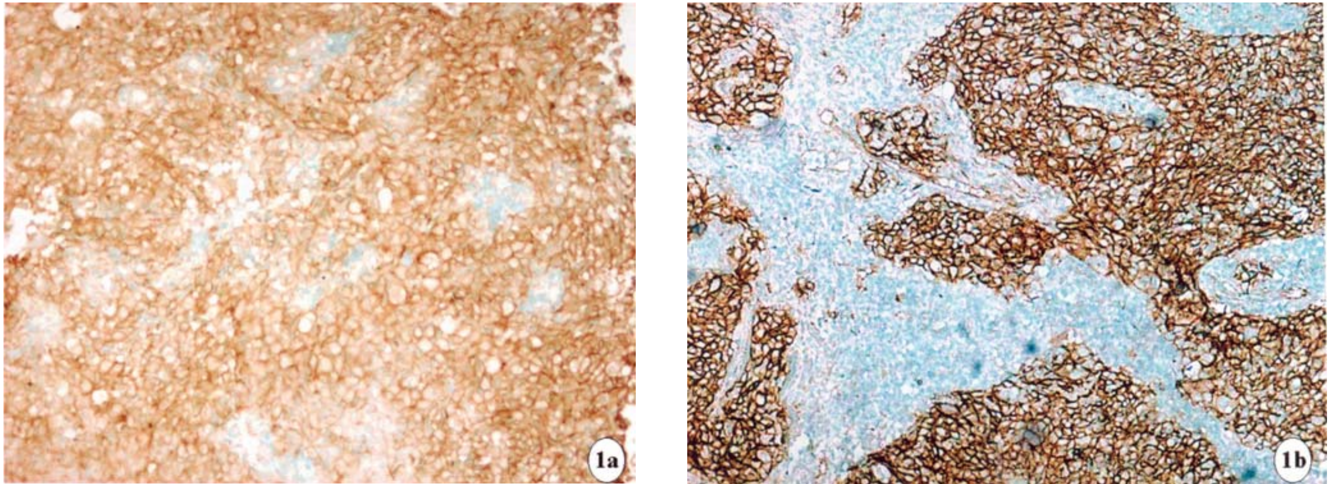


Fig. 1 Expression of β-catenin in primary lesion and lymph node metastatic tumor of NPC
1a: β-catenin showing high membrane expression in primary lesion of NPC. IHC 10×20.
1b: β-catenin showing high membrane expression in lymph node metastatic tumor of NPC. IHC 10×20.

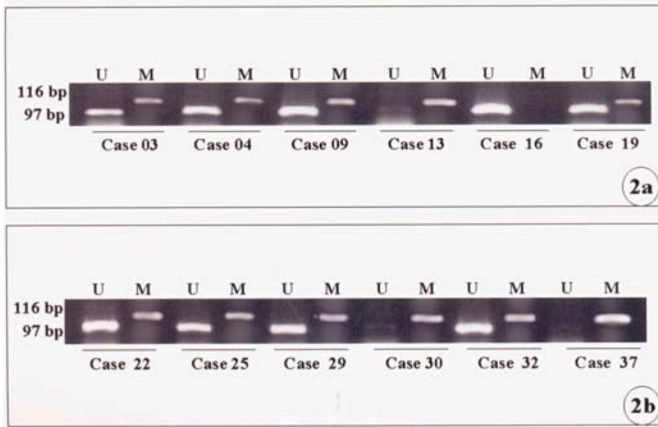


Fig.2 Methylation-specific PCR analysis for methylation of E-cadherin gene promoter in NPC.
U: unmethylated gene promoter; M: methylated gene promoter;
2a: primary lesion of NPC;
2b: lymph node metastatic tumor of NPC

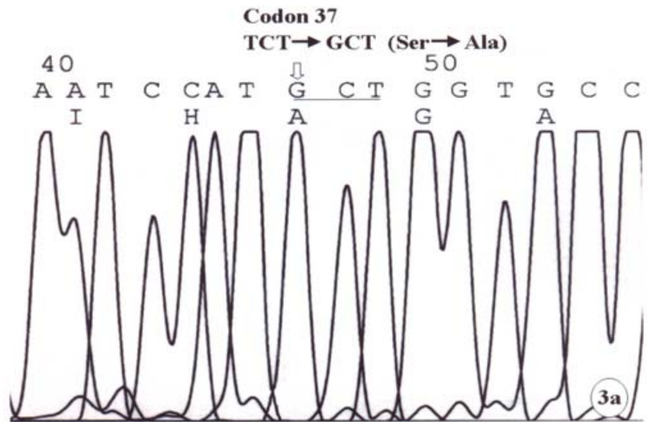
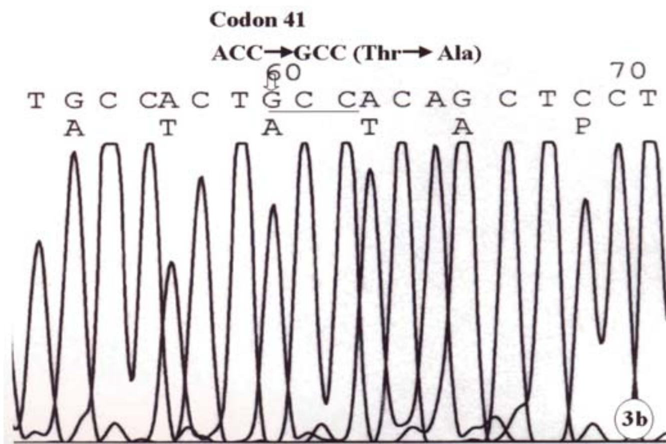


Fig. 3 DNA sequence analysis for β-catenin mutations in exon 3 in NPC.
3a: Codon 37 point mutation, Ser to Ala (TCT to TGT), Direct DNA sequence analysis.



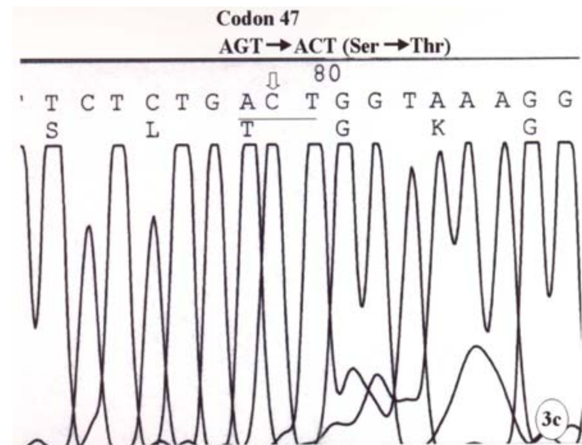


Fig. 3 DNA sequence analysis for β-catenin mutations in exon 3 in NPC.
3b: Codon 41 point mutation, Thr to Ala (ACC to GCC), Direct DNA sequence analysis.
3c: Codon 47 point mutation, Ser to Thr (AGT to ACT), Direct DNA sequence analysis.

DISCUSSION

The influence of methylation of E-cadherin gene promoter on invasion and metastasis of NPC

The factors responsible for invasion and metastasis in NPC have not been identified. This study has shown that the aberrant methylation of E-cadherin gene promoter may play a critical role in the process. The loss of cadherin-mediated cell-to-cell adhesion resulted from the down-regulated expression of E-cadherin was found to be an important step in promoting malignant metastatic potential for many human tumors, including gastric cancer, colorectal cancer and lobular breast cancer [13,14]. Our previous data had confirmed that the protein and mRNA expression of E-cadherin in NPC was indeed more less in the lymph node metastatic tumor than that in primary lesion[15]. However, the definite machanism of E-cadherin down-regulation remains obscure so far. In the present study, the methylation pattern of E-cadherin gene promoter in primary lesion and metastatic tumor of NPC was significantly different. Since the aberrant methylation in promoter of gene is an important factor to silence or inactivate the whole gene expression, which involved in the development and progression of tumor. This result indicated that the promoter methylation of E-cadherin plays one of the most important roles in acquiring the invasive and metastatic characterization of NPC cells through down-regulating the mRNA transcription and, down-regulating the protein expression of E-cadherin that lead to detachment and invasion of neoplastic cells of NPC finally. In some human malignant disease, hypermethylation of E-cadherin gene promoter has been reported to be responsible for the decreased mRNA expression of E-cadherin. Methylated E-cadherin promoter was found in 36.3%-64% of primary tumors, 67%-71% of recurrent and nodal metastases tumors of the head and neck malignant tumor [16,17]. Our data in present study were similar to these results, which indicated the methylation of E-cadherin gene promoter might be an important factor to induce tumor cell invasion and metastasis of human cancer. Although there was no correlation found between methylation of E-cadherin promoter and histological subtypes of tumor, the growth pattern of tumor cell showed closely relation to the alteration of E-cadherin gene promoter in present study. The scattered tumor cells were likely to be found more frequent methylation of E-cadherin promoter than that of adhesive cells, and there exist no statistic difference might merely resulted from less collected cases of NPC. These data indicated that methylation of E-cadherin might play a key role in growth pattern of the NPC cells, but it was not a critical factor to influence the differentiation of the NPC cells.

The influence of the alteration of β-catenin on NPC cells

β-catenin, as a component of cadherin-catenin complex, played an important role in cellular adhesion, tumor suppression, cell differentiation and cell migration. Some reports suggested that down-regulation expression of β-catenin might play a role in early and late tumor invasion and metastasis [18,19]. However, the present data showed that accumulated expression, but not down-regulated expression of β-catenin was one of NPC characteristics. There was no any alteration of β-catenin expression found between the primary lesion and metastatic tumor of NPC, and there was no any correlation to histological subtype and growth pattern of NPC cells. These results indicate that β-catenin might not be an important factor for the tumor cells invasion and metastasis of NPC. Since exon 3 of β-catenin gene encodes serine-threonine phosphorylation sites for the glycogen synthase kinase-3β (GSK-3) that regulate degradation of β-catenin by the ubiquitin-proteasome pathway. Mutation in exon 3 of β-catenin and alteration of these phosphorylation sites confer resistance to phosphorylation and lead to the accumulation of cytoplasmic and nuclear β-catenin, which acted as a transcription factor to activate the c-myc oncogene to induce cell proliferation[20,21]. The ratio of mutation of β-catenin in exon 3 was 57%-63% in human benign tumor and 4.8%-26% in malignant tumor[22,23]. In present study, only 3 cases (7.1%) could be found mutation in exon 3 of β-catenin, which suggested that it is a rare event in NPC. It might be an early event to promote cell carcinogenesis in NPC, but had no significant role in tumor cells´ progression and development, including invasion and metastasis.

In this study, all detectable mutation involved in the serine-threonine phosphorylation sites, but the tumor cells showed equally strong membranous expression of β-catenin at cell-cell boundaries. There was no detectable cytoplasmic or nuclear staining of β-catenin in any section of NPC tis-

sues. These results showed that once normal cell converted to malignance, the β-catenin protein was not able to enter the nucleus to regulate the cell proliferation and differentiation by activating related oncogene, so it seemed not to affect the tumor cell on invasion and metastasis in NPC.

In summary, our study showed that methylation of promoter was a major cause of down-regulation of E-cadherin, which might finally lead to detachment, and metastasis of neoplastic cells of NPC. β-catenin mutation was an infrequent event in NPC, β-catenin did not play an important role in influencing the progression of NPC, or just might acted as a assistant component of cadherin-catenin complex to induce invasion and metastasis of tumor cells in NPC.

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