RESEARCH COMMUNICATION

Clinical Outcomes of Downregulation of E-cadherin Gene Expression in Non-small Cell Lung Cancer

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Abstract

Objective: To investigate the promoter methylation status of the E-cadherin gene in non-small cell lung cancer (NSCLC) and its association with clinical pathological parameters, and to explore the relationship between downregulation of E-cadherin gene expression and the methylation status of its promoter region. Methods: Nested methylation-specific PCR was performed to examine CpG methylation within the 5' CpG island of the E-cadherin gene in lung cancer and para-cancerous tissue from 37 patients with primary non-small cell lung cancer. Quantitative real-time PCR was performed to measure the level of E-cadherin mRNA. Results: Of thirty-seven cases, 12 (32.4%) samples showed aberrant CpG methylation in tumor tissues compared with the corresponding normal tissues. In addition, a reduction in E-cadherin mRNA levels was observed in 11 of the 12 (91.7%) tumor tissues carrying a methylated E-cadherin gene. However, only 10 (43.5%) cases displayed reduced mRNA levels in tumor tissues from the remaining 23 cases (excluding 2 samples from which mRNA was unavailable) without methylation events. Downregulation of E-cadherin gene expression significantly correlated with the promoter methylation status of this gene. Conclusion: These results provide strong evidence that the methylation status of E-cadherin gene contributes to a reduction in the expression of E-cadherin mRNA, and may play a role in the development and progression of NSCLC.

Keywords: E-cadherin gene - mRNA expression - paracarcinoma - methylation - non-small cell lung cancer

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Introduction

The E-cadherin gene (CDH1) is located on human chromosome at 16q22.1, and encodes a transmembrane glycoprotein with a molecular weight of 120 KD. It is often expressed on the surface of most epithelial cells and localizes in the center of cytoplasm. E-cadherin suppresses tumor metastasis, serving as an important metastasis suppressor gene (Oka et al., 1993). Recent studies have found downregulation of E-cadherin gene expression in non-small cell lung cancer (NSCLC) patients of different races (Kase et al., 2000; Fei et al., 2002). E-Cadherin-mediated cell-to-cell adhesion plays a critical role in the maintenance of cell polarity and environment (Hirohashi, 1998). E-Cadherin was reported to be downregulated and closely related to tumor invasion and metastasis in many cancers (Guarino, 2007; Mărgineanu et al., 2008; Alves et al., 2009).

Genetic and epigenetic alteration of E-cadherin was also reported (Hirohashi, 1998). Somatic mutation, loss of heterozygosity of the E-cadherin gene, and CpG methylation around the promoter region of the E-cadherin gene were noted in human gastric cancer, breast cancer, and hepatocarcinoma (Yoshiura et al., 1995; Kanai et al., 1997; Berx et al., 1998; Tamura et al., 2000; Cheng et al., 2001). However, E-cadherin promoter hypermethylation is not always associated with loss of expression (Tamura et al., 2000), and evidence has been presented that E-cadherin expression could be repressed by mechanisms other than promoter hypermethylation (Cheng et al., 2001).

These expression defects are significantly correlated with the extent of lung cancer differentiation and lymph node metastasis. We have analyzed the genotype of NSCLC patients and found no significant mutations. Tumor suppressor gene CpG island methylation is a hotspot of tumor molecular biology. We are interested in whether there is an association of E-cadherin gene promoter methylation with lung cancer differentiation and lymph node metastasis. It remains unclear if E-cadherin gene promoter methylation leads to the downregulation of E-cadherin gene expression.

This study sought to elucidate the CpG island methylation of exon 1 of E-cadherin gene in cancer and paracancerous tissue from NSCLC patients, and to analyze the correlation between E-cadherin gene DNA methylation, NSCLS differentiation and reduced E-cadherin gene expression. We hope this will provide important evidence for the possibility of applying molecular biomarkers to aid early diagnosis and tumor

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classification, for the prediction of tumor recurrence and metastasis, and for prognosis.

Materials and Methods

Subjects

Cancerous and adjacent normal tissues were harvested from 37 NSCLC patients (26 males and 11 females, aged 37-79 years). These patients had no history of chemotherapy before surgery. Samples were immediately stored in liquid nitrogen for DNA extraction. NSCLC pathology was classified according to the international lung cancer staging system (Mountain, 1997); clinical characteristics are shown in Table 1. All specimens were provided by Soochow University Affiliated First People's Hospital, China.

Nested methylation-specific PCR (MSP)

Genomic DNA was extracted from NSCLC tissue using the phenol-chloroform-isoamyl alcohol method. Modification of genomic DNA was performed according to the MSP method described by Herman et al. (1996). Modified DNA was purified using a QIAGEN DNeasy Tissue kit to remove free sodium bisulfite, and then used as a PCR reaction template. Bisulfite treatment results in the conversion of cytosines to uracil in tissue DNA; this alteration does not occur if the gene of interest exhibits CpG island methylation. Methylation-specific PCR primers and non-methylation-specific PCR primers were utilized to detect if the gene is methylated. The sequence of E-cadherin gene methylation primers were described previously (Corn et al., 2001), and primers were synthesized by Shanghai Sangon Bioengineering Technology Service Co., Ltd., China (Table 2). Non-methylation-specific PCR and methylation-specific PCR primers used the same outer primer (E-cadherin-F/E-cadherin-R). The methylation and non-methylation-specific PCR primers were annotated E-cadherin-MF/E-cadherin-MR and E-cadherin-UF/E-cadherin-UR, respectively. SssI enzyme-treated genomic DNA from normal blood served as a positive control for methylation analysis, while unmodified DNA from normal blood served as a negative control for methylation analysis (Zhang et al., 2004).

Table 1. Clinical Pathological Features of 37 Cases of NSCLC

Clinical pathological features	Tumor[a] (%)	Gender[a]		Age
		Male	Female	(year)
Tumor size				
T1	6 (16.2%)	3	3	38-70
T2	20 (54.1%)	17	3	37-79
T3	8 (21.6%)	6	2	41-74
T4	3 (8.1%)	0	3	42-51
Lymph nodes				
N0	20 (54.1%)	13	7	38-79
N1	14 (37.8%)	12	2	37-72
N2	2 (5.4%)	1	1	48-52
N3	1 (2.7%)	0	1	42
Metastasis				
M0	35 (94.6%)	26	9	37-79
M1	2 (5.4%)	0	2	42-48
Pathological types				
Adenocarcinoma	17 (46%)	8	9	38-79
Squamous cell carcinoma	15 (40.5%)	13	2	45-74
Large cell carcinoma	5 (13.5%)	5	0	37-72

[a]represents the number of samples

TaqMan quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from NSCLC tissues using the chloroform-isoamyl alcohol method, and then reverse transcribed to cDNA. Target gene E-cadherin and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) qRT-PCR primers and probe primers were designed using Primer5.0 gene software, and synthesized by Shanghai Sangon Bioengineering Technology Service Co., Ltd., China (Table 3). Using cDNA as a template, PCR amplification was performed with a pair of primers and a specific fluorescent probe.

The relative expression of target genes was determined with the threshold method, and calculated according using the $2^{-\Delta\Delta Ct}$ formula. Ct is the fluorescence signal intensity detected by thermal cycler reaction device, $\Delta\Delta Ct$ = (Ct of target gene – Ct of housekeeping gene) experimental group - (Ct of target gene – Ct of housekeeping gene) control group (Livak and Schmittgen, 2001). $2^{-\Delta\Delta Ct}$ represents the multiple of target gene expression in the experimental group corresponding to that in the control group, and it directly quantizes the value of target gene to housekeeping gene.

Statistical analysis

Data were analyzed using SAS software and a value of $P < 0.05$ was considered significant.

Table 3. Primer Sequences Used for qRT-PCR Analysis

Primer name	Primer sequence (5 '- 3')
E-cadherin-F	ACCACGTACAAGGGTCAGGT
E-cadherin-R	GGCATCAGCATCAGTCACTT
E-cadherin-probe	fam+CGACGTTAGCCTCGTTCTCAGG C+tamra
GAPDH-F	CCACTCCTCCACCTTTGAC
GAPDH-R	ACCCTGTTGCTGTAGCCA
GAPDH-probe	fam+CCTCAACGACCACTTTGTC +tamara

Table 2. Primer Sequences Used for Nested Methylation-specific PCR

Primer name	Primer sequence (5'-3')	Size (bp)	Tm (°C)
E-cadherin-F	GTTTAGTTTTGGGGAGGGGTT	270	50
E-cadherin-R	ACTACTACTCCAAAAACCCATAACTAA		
E-cadherin-MF	TGTAGTTACGTATTTATTTTTAGTGGCGTC	112	62
E-cadherin-MR	CGAATACGATCGAATCGAACCG		
E-cadherin-UF	GGTTGTAGTTATGTATTTATTTTTAGTGGTGTT	120	64
E-cadherin-UR	ACACCAAATACAATCAAATCAAACCAAA		

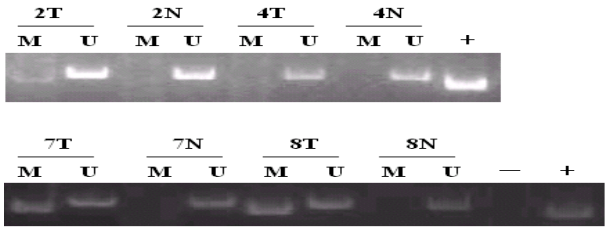

Figure 1. Electrophoresis of E-cadherin Gene Promoter Region Methylation Status in NSCLC (T) and Corresponding Adjacent Tissues (N) Using Nested MSP. M and U represent the methylation and non-methylation products of E-cadherin gene. + and - represent the positive and negative controls for methylation analysis. 2, 7, 8 represent samples exhibiting different degrees of methylation in non-small cell lung cancer tissue and non-methylation in adjacent tissue. 4 represents samples with no methylation in non-small cell lung cancer tissue and adjacent tissue

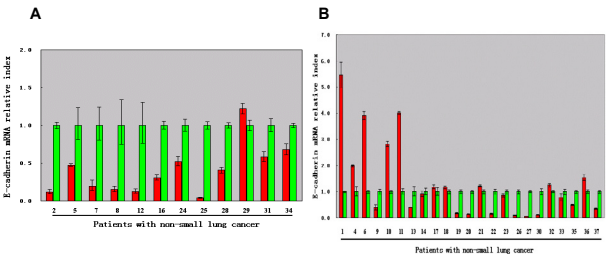

Figure 2. E-cadherin mRNA Expression in Non-small Cell Lung Cancer Tissue (red) and Corresponding Normal Adjacent Tissue (green)

Results

Nested MSP test of tissue DNA

Of 37 NSCLC patients, 12 (32.4%) patients exhibited E-cadherin gene promoter region methylation, while no methylation was detected in the adjacent normal tissues; 25 patients showed no abnormal methylation in the cancer and corresponding adjacent tissues (Figure 1).

E-cadherin mRNA expression in NSCLC tissues

12 NSCLC samples exhibited E-cadherin gene methylation; of these, E-cadherin gene expression in cancerous tissue was significantly lower than in the adjacent normal tissues in 11 cases. In the 23 cases that did not demonstrate E-cadherin gene methylation (including two cases where RNA samples were lost), E-cadherin gene expression was downregulated in only 10 cases (Figure 2).

E-cadherin gene promoter region methylation status is not associated with NSCLC patients' sex, age, differentiation and pathological types

Fisher's exact chi-square analysis showed no significant correlation between E-cadherin gene promoter region methylation status and gender, age, differentiation, metastasis and histological grade of NSCLC patients.

Correlation between E-cadherin gene promoter methylation status and E-cadherin expression

Fisher exact chi-square tests showed that E-cadherin gene promoter region methylation status was significantly associated with mRNA expression (P = 0.01; Table 5).

Table 4. Correlation Between E-cadherin Gene Methylation Status and Clinical Pathology in 37 NSCLC Patients

	Methylation[a]	Non-methylation[a]	Probability[b]
Sample total	12	25	
Gender			
Male	8	18	1
Female	4	7	
Age (year)			
≤50	5	5	0.24
> 50	7	20	
Tumor size			
≤T2	9	17	1
> T2	3	8	
Lymph nodes			
≤N2	11	25	0.324
> N2	1	0	
Metastasis			
M0	10	25	0.099
M1	2	0	
Pathological types			
Adenocarcinoma	4	13	0.308
Squamous cell carcinoma	5	10	
Large cell carcinoma	3	2	

[a]represents the number of samples; [b]represents Fisher chi-square test

Table 5. Correlation Between E-cadherin Gene Methylation Status and mRNA Expression in 35 NSCLC Patients

Methylation analysis	real-time PCR analysis	
	No expression decreased[a]	Expression decreased[a]
Methylation	1	11
Non-methylation	13	10
	P =0.010[b]	

[a]represents the number of samples; [b]represents Fisher chi-square test

Discussion

Cancer occurrence and development is a multi-step process involving the accumulation of mutations in a number of genes, and involves tumor suppressor gene inactivation, oncogene activation and apoptosis-related gene changes. The classical Knudson's two-hit hypothesis states that individuals susceptible to carcinogenesis have inherited variant cancer-causing genes from their parents, and then undergo loss of heterozygosity of the second allele of these mutant genes due to environmental factors, thus leading to tumorigenesis. However, studies regarding tumor molecular biology have advanced, and we found that the DNA of some malignant tumor cells is normal with no mutations, deletions or other genetic variations. However, the two-hit theory cannot explain the inactivation of tumor suppressor genes. Modern cancer theory believes that malignant tumors are a result of genetic defects and epigenetic changes. Genetic defects include mutations and deletions that induce damage to the coding region structure and function. Although epigenetic changes do not alter DNA sequences, they affect genetic expression through the chemical modification of DNA itself (DNA methylation and histone deacetylation/

acetylation) at the transcriptional level, thereby regulating DNA function. The inactivation of tumor suppressor genes are associated with promoter region CpG island methylation status (Leonhardt and Cardoso, 2000), indicating that CpG island methylation might play a key role in cancer development. Therefore, promoter CpG island methylation can be considered as second strike of tumorigenesis (Machado et al., 2001).

Reduced cell-cell adhesiveness allows cancer cells to disobey the social order, resulting in destruction of the histologic structure, the morphologic hallmark of malignant tumors (Hirohashi, 1998). Several reports have indicated that E-cadherin, an epithelial-specific cadherin, is a key molecule for the maintenance of epithelial integrity and of polarized states in association with alpha-, beta-, and gamma-catenin, and that the reduction of E-cadherin-mediated cell-cell adhesion favors the dispersion of cancer cells (Birchmeier and Behrens, 1994]. Consistent with this concept, immunohistochemical studies have revealed that decreased E-cadherin expression is associated with tumor dedifferentiation and progression in endometrial carcinoma5 and othertumors (Schipper et al., 1991; Shimoyama and Hirohashi, 1991; Shiozaki et al., 1991; Gamallo et al., 1993).

Numerous studies show that E-cadherin gene inactivation frequently occurs in a variety of epithelium-derived tumors, and plays an important role in early tumor invasion and metastasis of various cancers (Graff et al., 1995; Yoshiura et al., 1995; Li et al., 2001; Esteller, 2002; Chen et al., 2003), including NSCLC. There is increasing evidence to support the correlation between gene deletion expression of E-cadherin and gene promoter region CpG island methylation in a variety of tumors, but few investigations report this observation in NSCLC.

The current study is the first to explore E-cadherin gene promoter methylation status in tumors and adjacent normal tissues of patients with NSCLC. We examined the methylation status of 37 NSCLC patients using nested MSP. The experimental results revealed different degrees of methylation status of E-cadherin gene promoter region in 12 patients (32.4%). Our experimental results are similar to other human tumor studies, such as gastric cancer, esophageal cancer, breast cancer (Hiraguri, 1998; Tamura et al., 2000; Tsao, 2003). Statistical analysis showed no significant correlation between E-cadherin gene promoter methylation and clinicopathological factors of NSCLC patients. It is noteworthy that for NSCLC patients, the probability of E-cadherin gene methylation (32.4%) was significantly higher than that reported by Zochbauer-Muller et al. (2001) (19%). This may be due to two reasons. First, nested PCR is a sensitive technique and can detect one-thousandth of the methylated fragments, requiring minimal DNA template.. Second, Zochbauer-Muller et al. investigated a different E-cadherin gene promoter region to that investigated in the present study.

Although Zochbauer-Muller et al. (2001) found methylation abnormalities of the E-cadherin gene in NSCLC samples, they did not analyze the correlations between methylation abnormalities and reduced gene expression, which is also very rare in China. In this study, we determined E-cadherin mRNA expression in 12 NSCLC patients with abnormal methylation, through qRT-PCR. The experimental findings showed that E-cadherin gene mRNA expression was downregulated in 11 cases. Furthermore, mRNA expression levels in 23 NSCLC patients with no detectable abnormal methylation (2 RNA samples were lost) were measured, and 10 samples were found to have a relatively lower expression level. The statistical analysis showed that E-cadherin gene mRNA expression levels were significantly associated with the gene promoter region methylation status. This evidence suggests that E-cadherin gene promoter methylation status is closely related to its reduced expression, and E-cadherin gene 5' end CpG island hypermethylation is involved in NSCLC development.

Studies have shown that the complex pathogenesis of NSCLC is manifested not only through genetic changes caused by gene mutations or deletions, but also through epigenetic changes representative of DNA methylation. Thus, the methylation status of tumor suppressor genes such as the E-cadherin gene is of great significance in clinical treatment and diagnosis; understanding the gene methylation process will help us to better recognize the molecular mechanisms underlying gene regulation, in a broader attempt to perform early diagnosis and gene therapy for NSCLC.

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