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ORIGINAL RESEARCH ARTICLE

Evaluating *NISCH* and *CDH1* Promoter Hypermethylation in Nonsmokers, Cancer Free Smokers and Lung Cancer Patients: A Case Control Study

Kritika Krishnamurthy[1] · T. K. Mishra[1] · Alpana Saxena[1] · M. K. Daga[2] · Nita Khurana[3] · Mirza Masroor[1] · Elvia Jamatia[1]

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Abstract Lung cancer has very high mortality due to late stage diagnosis not amenable to curative resection. Cancer specific methylation patterns of tumor suppressor genes may precede precursor lesions of lung cancer. Our aim was to evaluate the promoter hypermethylation of tumor suppressor gene NISCH and CDH1 in cfDNA from plasma of lung cancer patients and its possible correlation with smoking status and various clinicopathological parameters. Forty histopathologically confirmed lung cancer cases, thirty smoker and thirty nonsmoker controls were enrolled. Plasma cfDNA was extracted and subjected to bisulfite treatment followed by MS-PCR. Serum nischarin levels were estimated by ELISA. The frequency of promoter hypermethylation of NISCH and CDH1 was significantly higher in lung cancer patients as compared to lifelong non-smoker controls ($p < 0.05$). It did not vary with smoking status among cancer cases. No significant association was found with staging or histological grading. NISCH methylation was found to be significantly higher among smoker controls. Pack years and packs per day were significantly higher in the methylated group. Serum nischarin levels showed no significant association with NISCH methylation or clinicopathological variables. NISCH is highly methylated in both high risk smoker controls as well as cancerousnon-smokers and may mark the convergence of varied etiologies of lung cancer. Hence NISCH and CDH1 are highly methylated in plasma cfDNA of lung cancer patients.

Keywords NISCH · CDH1 · Hypermethylation · ccfDNA · Epigenetics

✉ Kritika Krishnamurthy
kritikakrishnamurthy@yahoo.com

T. K. Mishra
drtkmishra_mamc@rediffmail.com

Alpana Saxena
alpanasaxena@hotmail.com

M. K. Daga
drmraduldaga@gmail.com

Nita Khurana
nitakhurana@rediffmail.com

Mirza Masroor
mirzamasroor1986@gmail.com

Elvia Jamatia
ielvia.j@gmail.com

[1] Department of Biochemistry, Maulana Azad Medical College, 2 Bahadur Shah Zafar Marg, New Delhi, India

[2] Department of Medicine, Maulana Azad Medical College, 2 Bahadur Shah Zafar Marg, New Delhi, India

[3] Department of Pathology, Maulana Azad Medical College, 2 Bahadur Shah Zafar Marg, New Delhi, India

Introduction

Worldwide, lung cancer is the most common cancer, both in incidence and mortality. In 2012, there were 1.8 million new cases, and 1.59 million deaths due to lung cancer [1]. Smoking accounts for 80–90% of lung cancers [2]. Cigarette smoke contains over 60 known carcinogens [3]. Men smoking 15–24 cigarettes/day have 26-fold increase in lung cancer compared to never-smokers [4].

Lung cancer is divided into four major histological types by WHO: Adenocarcinoma (AD), Squamous Cell Carcinoma (SCC), Large Cell Lung Carcinoma (LC) and Small Cell Lung Carcinoma (SCLC). This classification is important for determining management and predicting outcomes. At presentation, 30–40% of non-SCLC

(NSCLC) are stage IV, and 60% of SCLC are stage IV [5]. The high mortality of lung cancer is attributable to the presence of metastatic disease in nearly two thirds of patients at diagnosis [6]. Detection of early stage lung cancer amenable to curative resection, could potentially increase survival rates by 10- to 50-fold [6].

Epigenetic alterations seemingly contribute to cancer initiation and progression. Cancer specific methylation patterns of tumour suppressor genes, which precede precursor lesions, could possibly herald earlier diagnosis of lung cancer and may even have important preventive or therapeutic implications.

NISCH gene encodes a nonadrenergic imidazoline-1 receptor protein (nischarin) that localizes to cytosol and anchors to the inner layer of plasma membrane. The orthologous mouse protein has been shown to influence cytoskeleton organization and cell migration by binding to alpha-5 beta-1 integrin. In humans, this protein has been shown to bind to the adapter insulin receptor substrate 4 to mediate translocation of alpha-5 integrin from the cell membrane to endosomes. Studies have previously shown cancer-specific methylation of *NISCH* in the lung tumor tissue [7].

CDH1 gene encodes Cadherin-1 also known as CAM 120/80 or E-cadherin. It is localised on surfaces of epithelial cells in adherens junctions. Loss of E-cadherin function or expression has been implicated in cancer progression and metastasis. E-cadherin downregulation decreases strength of cellular adhesion within tissue, resulting in an increase in cellular motility. This may allow cancer cells to cross basement membrane, invading surrounding tissues. Multiple studies have shown methylation in the promoter region of the *CDH1* gene was correlated with tumor progression, tumor dedifferentiation, and prognosis [8].

The present study was designed to evaluate the promoter hypermethylation of putative tumours suppressor genes *NISCH* and *CDH1* in cfDNA from plasma of lung cancer patients and its possible correlation with smoking status and various clinicopathological parameters.

Methods

It was a hospital based case control study approved by the institutional ethics committee of Maulana Azad Medical College F.No./11/IRC/MAMC/2011/39.

Study Subjects

Forty histopathologically confirmed lung cancer cases were enrolled along with thirty age and sex matched smoker as well as thirty age and sex matched non-smoker controls from LNJP hospital, New Delhi. Patients with prior history of cancer or concomitant cancer at another site were excluded from the study. Written informed consent was taken from cases and controls.

5 ml blood sample was withdrawn under aseptic conditions, 2.5 ml in EDTA tube and 2.5 ml in plain tube. Plasma and serum were separated by centrifugation at 2600 rpm for 10 min and stored at − 80 °C till further analysis.

Staging and Grading of Lung Cancer

Staging was carried out as per the AJCC (7th Ed) recommendations. A histological assessment of the tumour biopsy was done for histopathological grade and type.

Methylation Analysis

Plasma cfDNA was extracted using serum FitAmpTM Plasma/Serum DNA isolation Kit Catalogue No. P-1004 obtained from Epigentek Group Inc. Eluted DNA was measured spectrophotometrically using nano drop (ND-1000 from Nanodrop Technologies Inc). Sodium bisulfite conversion of the extracted DNA was done using Bisul-FlashTM DNA modification kit Catalogue # P-1026 obtained from Epigentek. Bisulfite modification was followed by conventional methylation specific PCR for single CpG island. The primer sequences are shown in Table 1.

Each reaction was performed in a total volume of 25 µl containing 10 µl Master Mix, a working concentration of 25 pm for each primer and 3 µl of DNA.

Initial denaturation was performed at 95 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 45 s, Annealing at 52 °C in case of *CDH1* and 51.8 °C in case of *NISCH* for 45 s and extension at 72 °C for 45 s. This was followed by final extension at 72 °C for 10 min and cooling at 4 °C for 10 min.

In vitro methylated DNA was used as positive control and nuclease free PCR water was used negative control. The amplified product was resolved using electrophoresis in 2% agarose gel, stained ethidium bromide, visualized under UV illumination.

Serum Nischarin Levels

The serum nischarin levels were estimated by double-antibody sandwich enzyme linked immunosorbent one-step process assay using Human Nischarin (NISCH) ELISA Kit from QAYEE-BIO (Shanghai). Serum was diluted 1:5 as per manufacturers instructions.

Table 1 Primer sequences used in MSP analysis

Gene		Sequence		Amplicon size (bp)
		Forward	Reverse	
CDH1	Unmethylated	5′-TAATTTTAGGTTAGAGGGTTATTGT-3′	5′-CACAACCAATCAACAACACA-3′	97
	Methylated	5′-TTAGGTTAGAGGGTTATCGCGT-3′	5′-TAACTAAAAATTCACCTACCGAC-3′	116
NISCH	Unmethylated	5′-GAGTATTATTGTGTGTTGGGTT-3′	5′-TAAAACCTATACTTACCACCAAA-3′	144
	Methylated	5′-TTTTTTTCGTATAGAGTTCGT-3′	5′-CTAAACCTCTCTAAAATTCG-3′	155

Statistical Analysis

Statistical analysis was done using SPSS 22.0 software package. Parametric data was presented as mean and standard deviation and nonparametric data as median and range. Pearson's Chi square and Fisher's Exact Method were used to compare nominal variables. Mann–Whitney U test and Kruskal–Wallis test were used to assess differences between nonparametric data. These were followed by post hoc tests when indicated. A p value < 0.05 was considered to be significant.

Results

General Characteristics of the Study Population

The study group comprised of forty histopathologically confirmed lung cancer patients with a mean age of 58.9 ± 11.2 years. Of the forty cases, sixteen were current or ex-smokers and twenty four were lifetime non-smokers.

The patients were recruited irrespective of clinical stage of the disease. There were 6 patients in Stage II, 2 patients in Stage III and 32 patients in Stage IV. There was no patient in Stage I of the disease in our study.

Histopathologically, eleven cases had squamous cell carcinoma, two had small cell carcinoma and twenty seven had adenocarcinoma. Twenty three patients had Grade I (well differentiated) tumor, nine had Grade II (moderately differentiated) and nine had Grade III (poorly differentiated) tumor.

The age, sex and smoking status among cases and healthy controls are summarised in Table 2.

Methylation Analysis

The frequency of promoter hypermethylation of *CDH1* was significantly higher in lung cancer patients as compared to non-cancerous smokers and lifelong non-smoker controls. Thirty-four cases (85%) showed methylation of *CDH1* ($p < 0.05$) with Cramer V value of 0.879 indicating a strong association (Table 3).

The frequency of promoter hypermethylation of *NISCH* was significantly higher in lung cancer patients and in non-cancerous smokers as compared to lifelong non-smoker controls ($p < 0.05$). Thirty cases (75%) and twenty-four (80%) smoker controls showed methylation of *NISCH*. The association was found to be strong with Cramer V values of > 0.71 (Table 3).

The cases were classified according to various clinicopathological characteristics and analysed by comparative statistics. There was no significant association between methylation status of tumour suppressor gene *NISCH* or *CDH-1* and clinicopathologic variables- staging, tumour size, lymph node status, metastasis and histopathological grading (Table 3).

We observed a higher frequency of *NISCH* methylation in small cell carcinoma and adenocarcinoma as compared to SCC. 85.2% of the twenty-seven adenocarcinoma cases and both the small cell carcinomas showed methylation of *NISCH* while only 45.5% of the eleven SCC cases were methylated ($p < 0.05$). The strength of the association was predicted to be moderate as the Cramer V value was 0.427 (Table 3).

Methylation status of tumour suppressor gene *NISCH* was found to be significantly higher among smokers as compared to non-smokers. 77.3% of the fifty-three smokers enrolled tested methylation positive ($p < 0.05$). A phi value of 0.498 predicted a moderate strength of association (Table 3).

But there was no significant difference in the frequency *NISCH* methylation between smoker and non-smoker lung cancer patients (Table 3).

The fifty-three smokers in both the groups were classified further based on type of smoking, duration of smoking and intensity of smoking in terms of packs per year and packs per day. There was no significant difference in methylation status of *NISCH* with type or duration of smoking. The pack years and packs per day were significantly higher in those in the methylated as compared to the unmethylated group. The methylated group smoked a mean of 1.7 ± 1.1 day as compared to 1.1 ± 0.6 packs per day smoked by the unmethylated group. There was a significant difference in the packs per year between the two groups

Table 2 Characteristics of lung cancer cases and controls

Characteristics	Cancer cases	Smoker controls	Non smoker controls
Number of subjects enrolled	40	30	30
Age (Mean ± SD)	58.96 ± 11.17	59.19 ± 9.52	59.57 ± 8.75
Sex			
Male	38	29	28
Female	2	1	2
Smoking status			
Smoker	16	30	0
Non smoker	24	0	30

Table 3 Comparison of *NISCH* and *CDH1* methylation across cases and controls and correlation with clinicopathological parameters

	NISCH			*CDH1*		
	Methylated	Unmethylated	p value	Methylated	Unmethylated	p value
Subject groups						
Cases (40)	30	10	< 0.0001	34	6	< 0.0001
Smoker controls (30)	24	6		0	30	
Nonsmoker controls (30)	0	30		0	30	
Clinical staging						
II (6)	4	2	0.801	6	0	0.685
III (2)	2	0		2	2	
IV (32)	24	8		26	6	
Histopathology						
Squamous cell carcinoma (11)	5	6	0.025	8	3	0.517
Adenocarcinoma (27)	23	4		24	3	
Small cell carcinoma (2)	2	0		2	0	
General characteristics						
Age[b]	59.8 ± 10.75	55.4 ± 12.34	0.070	58.4 ± 10.98	60.0 ± 13.22	0.538
Sex (M:F)	24:6	8:2	1.000	26:8	6:0	0.318
Smoking status (all subjects)						
Smoker (53)	41	12	< 0.010	19	34	1.000
Nonsmoker (47)	13	34		15	32	
Smoking status (cancer cases)						
Smoker (23)	17	6	1.000	19	4	1.000
Nonsmoker (17)	13	4		15	2	
Smoking history						
Pack years[a]	30 (7.5–250)	20 (7.5–120)	0.043			
Packs per day[b]	1.74 ± 1.12	1.08 ± 0.63	0.011			
Duration of smoking[b]	28.9 ± 9.84	26.25 ± 10.03	0.383			

[a]As median (range)

[b]As mean ± standard deviation

with the methylated group smoking a median of 30 PPY (7.5–250) and the unmethylated group smoking a median of 20 PPY (7.5–150) (Table 3).

Smokers were further classified into current and former smokers with subjects who had quit smoking for greater than 6 months being allotted to the latter group. But no significant difference was found in the methylation status between the two.

CDH1 methylation did not show any significant association with smoking status or related variables. *CDH1* and *NISCH* methylation did not show any significant association with demographic variables—age, sex or religion.

When *NISCH* and *CDH1* promoter methylation were taken together as a panel, both genes were methylated in 64% lung cancer cases, 31% cancer cases had methylation of one of the genes while only 5% cancer cases had no methylation in either gene.

Serum Nischarin

The serum nischarin levels did not differ significantly between cancer cases and non-cancerous controls. Neither did it show any association with smoking status. Serum nischarin showed a median level of 1034.7 pg/ml (775.8–3124.3) among cancer subjects as compared to 957.3 pg/m1 (742.1–13,361.5) among smoker controls and 973.2 pg/ml (696.5–11,745.3) among non-smoker controls. The methylation status of *NISCH* did not show any significant association with the serum nischarin levels in our study (Table 4).

Discussion

NISCH gene is located on the short arm of chromosome 3 at the locus 3p21, a metastatic tumour suppressor locus. Functionally, the gene is thought to play an integral role in actin cytoskeleton organization, apoptosis, cell communication, negative regulation of cell migration and rac protein signal transduction.

In this study, the frequency of promoter hypermethylation of *NISCH* was significantly higher in lung cancer patients and in non-cancerous smokers as compared to lifelong non-smoker controls, similar to reported by Ostrow et al. [7]. These findings suggest that *NISCH* promoter methylation could be a precursor of disease irrespective of smoking status. Since lung cancer patients who were lifelong nonsmokers also showed *NISCH* promoter methylation, this gene may be the common pathway at which varied etiologies of lung cancer converge and should be investigated as potential therapeutic target for lung cancer irrespective of etiology.

NISCH promoter methylation should be scrutinised as a possible risk prognosticator for lung cancer among high risk smokers. Although there is evidence supporting the effectiveness of low-dose CT screening in individuals at high risk for lung cancer, the false positive rates of CT screening are high leading to more frequent follow-up CT scans and hence additional costs and radiation exposure. *NISCH* promoter methylation should be analysed as a possible adjuvant to low dose CT to improve the sensitivity and specificity of screening for early lung cancer.

Our study found that though there was no significant difference in methylation status of *NISCH* with type or duration of smoking, the pack years and packs per day were significantly higher in those with methylated *NISCH* as compared to the unmethylated group. In the absence of other studies, we concur that *NISCH* methylation is more strongly associated with the intensity of tobacco smoke exposure as compared to duration. In accordance with previous studies, we did not find any significant differences in *NISCH* methylation among former and current smokers [9].

Our study did not find any correlation of *NISCH* methylation with advanced tumour stage, poor differentiation, lymph node metastasis as reported by Li et al. [10] in ovarian cancer tissues. However, due to the small sample size, these findings are not conclusive. The lower frequency of *NISCH* methylation in SCC as compared to adenocarcinoma and small cell carcinoma may be an overestimation due to the small sample size in this study.

Nischarin, the protein coded for by *NISCH*, associates with the cytoplasmic tail of the α5 subunit α5β1 integrin and affects cell migration as well as influences cytoskeletal organization [11]. The α5β1 integrin is a fibronectin receptor that plays a special role in regulating growth and survival in some cell types [12]. High expression of α5β1 has been linked with reductions in tumour cell growth rates both in vitro and in vivo [11]. There is no published work on Nischarin levels in serum prior to this study.

In the present study, the serum nischarin level was not significantly associated with methylation or disease status. Nischarin localizes to cell membrane and intracellularly. It is the low cell surface and intracellular expression of nischarin which predisposes to cancers. Thus, serum nischarin levels are unlikely to reflect the cellular expression. Chen et al. [13] reported that breast cancer tissues exhibited a significantly lower concentration of Nischarin compared with that of the adjacent non-cancerous tissues. But the current study did not evaluate the nischarin levels in

Table 4 Median and range of serum nischarin levels in lung cancer cases, smoker and nonsmoker controls

	Lung cancer patients (40)	Healthy controls		*p* value*
		Smokers (30)	Lifetime nonsmokers (30)	
Median (pg/ml)	1034.73	957.33	973.2	0.181
Range (pg/ml)	775.8–3124.3	742.1–13,261.5	696.5–11,745.3	

**p* value calculated by Kruskal–Wallis test

tumour tissue due to scantiness of tissue sample obtained by endobronchial biopsies.

CDH1 is a classical cadherin from the cadherin superfamily. Loss of cadherin activity is thought to contribute to progression of cancer by increasing proliferation, invasion, and/or metastasis. Loss of cadherin activity also leads to epithelial to mesenchymal transition which is an important mechanism for cancer development and cancer cell detachment and metastasis. Hence, *CDH1* is categorized as a tumour suppressor gene.

In our study, the frequency of *CDH1* methylation in cancer cases was found to be much higher than the 61.8% reported by Begum et al. [8] in western population. However, the study population enrolled in the American study was vastly different with majority of stage I subjects. The American study reported a methylation frequency of 7% in healthy controls contrary to our finding of lack of methylation in both smoker and non-smoker controls [8] supported by Tan et al. [14]. The variation in findings may also be due to differences in MSP assay design or sensitivity.

In this study, we did not find any significant association between *CDH1* methylation and clinicopathological or demographic parameters concurrent with Begum et al. [8]. Previous studies by Sebova et al. [15] in breast cancer have found significant association of *CDH1* methylation with metastatic disease and Melnikov et al. [16] reported increased frequency of *CDH1* methylation in SCC as compared to Adenocarcinoma. But no such association was found in our study.

Multiple studies have previously proven that abnormal *CDH1* methylation is associated with a decrease in E-cadherin expression, by multiple techniques including immunohistochemistry, quantitative PCR and western blotting [17]. In view of the outstanding evidence and due to paucity of tissue specimen, E-cadherin expression was not evaluated in our study.

Conclusion

Our findings suggest that *NISCH* and *CDH1* methylation occurs in high frequencies in cfDNA from plasma of lung cancer patients. Though it was not found to correlate with stage, tumour size, lymph node status, metastases or histological grade in our study. *NISCH* is found to be highly methylated in both high risk heavy smoker controls as well as lung cancer cases irrespective of smoking status, it can be hypothesized that *NISCH* methylation may be the common primogenitor at which varied etiologies for lung cancer converge. Since *NISCH* is found to be highly methylated in apparently healthy high risk heavy smokers, it may be investigated as a possible adjuvant to low dose CT for screening in high risk population.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by institutional ethics committee.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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