Promoter Methylation of *RASSF1A* in Non-small Cell Lung Carcinoma in Kashmiri, Indian Population

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Abstract

This study was aimed at defining the patterns of aberrant gene methylation in non-small cell lung cancer (NSCLC) in Kashmiri patients. To determine whether this gene is inactivated during lung cancer development, we studied 100 primary non-small cell lung cancers for the promoter methylation status of gene. The methylation-specific PCR (MSP) was used to study methylation of *RASSF1A* gene 100 NSCLCs. We found promoter hypermethylation at 41% in *RASSF1A*. Most of the patients have adenocarcinoma (72 patients) and squamous cell carcinoma is found in 28 patients who have NSCLCs (p=0.03). Age- based subgroup analysis demonstrated that higher *RASSF1A* promoter methylation levels were associated with NSCLC more in older patients (>45 years) than younger patients (≤45). In addition, the association of *RASSF1A* methylation with NSCLC was significantly associated with smokers, but not in non-smokers (p=0.01). It was also identified that *RASSF1A* promoter hypermethylation was significantly associated with advanced stage than early stage of NSCLC (p=0.05).

Keywords: *RASSF1A*, methylation, adenocarcinoma, squamous cell carcinoma, metastasis

Introduction

Lung cancer is the most frequent cause of cancer death worldwide and majority of which are non-small cell lung cancer (Greenlee at al., 2001: Malik *et al*., 2018). Lung cancer is the most common cancer amongst men in India with approximately 33,000 new cases ever year. Lung cancer constitutes 6.8% deaths in India (Ferlay *et al*.. 2010). Arsheed *et al*. 2012 recently reported lung cancer is the second most common cancer type in men and third among all (14.6%) in Kashmir valley. NSCLC arise by a stepwise acquisition of genetic and epigenetic alterations concomitantly with morphological changes as well that gives rise to the transformation of benign bronchial epithelium into neoplastic tissue. Molecular changes in proto-oncogenes and tumour suppressor genes have been detected in all stages of lung tumourigenesis. Recently, epigenetic silencing of gene expression by promoter CpG island hypermethylation has been shown to be important in cancer formation (Baylin *et al*., 2000). DNA methylation may be an alternative mechanism to mutations or deletions in disrupting tumor suppressor gene function. Aberrant gene methylation has also been frequently found in NSCLC. Activating point mutations in the *KRAS* gene have

been implicated in the pathogenesis of human cancers including those of lung (Boss, 1989). Histologically, about 80% are non-small cell and 20% are small cell lung cancers (Schiller *et al*., 2002). Among the NSCLCs, adenocarcinoma and SCC are the most common histological subtypes. In particular NSCLC has been shown to harbor KRAS mutations mostly in lung adenocarcinoms. However KRAS mutations were rarely detected in lung Squamous cell carcinomas (Rodenhius *et al*., 1988; Reynolds *et al*., 1991; Suzuki *et al*., 1990; Li *et al*., 1994; Mills *et al*., 1995; Shah *et al*., 2017). *RASSF1A*, a newly discovered Ras effector, is inactivated in a variety of human cancers including lung, colon, breast, prostate, thyroid, and renal cell carcinomas (Pfeifer *et al*., 2002; Dammann *et al*., 2001; Schagdarsurengin *et al*., 2002). The purpose of this study was to examine the association between KRAS mutation and *RASSF1A* hypermethylation in non-small cell lung carcinoma, and to determine correlation between genetic alterations of *RASSF1A* and KRAS with clinicopathological characteristics.

Material and Methods

Study population

A total of Hundred (n = 100) freshly diagnosed NSCLC patients between December 2016 to August 2018 at Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Soura, Srinagar were included in the study and equal number of control samples were included in the study. The NSCLC blood samples and their normal control blood samples were taken and properly stored for further processing. The study was approved by the Ethics Committee of SKIMS, Soura, India. Informed consent was obtained from all patients for blood sample collection. We have screened prospectively all newly diagnosed patients with NSCLC (n = 100) for the ability to be analysed for *RASSF1A* methylation. Staging of the NSCLC were performed according to the Union for International Cancer Control (UICC, Seventh Edition) (Riely *et al*., 2008) Demographic characteristics of the cases recruited for study are shown in **Table 1**. A total of 68 males and 32 females were included in the study. The patients were presented with average age 61.18 years; most of the patients were found within range of 50 to 84 years age group. Two age groups were made, patients with age ≤ 45 years included 13 cases, and > 45 years included 87 cases.

DNA extraction

Total genomic (g) DNA was extracted from tissue specimens using quick gDNA MiniPrep kit (Zymo Research, USA) according to the manufacturer's protocol. DNA concentrations were calculated using a Nano Drop 2000c Spectrophotometer (Thermo Scientific, Asheville, NC, USA).

Bisulphite modification of genomic DNA: Genomic DNA (~1µg) was modified with sodium bisulphite using BisulFlash DNA Modification Kit (Epigentek, USA). Starting DNA ranged from 50-200 ng per reaction. Bisulphite conversion of DNA was carried out at 95°C for 20 minutes during which Unmethylated cytosine was converted into uracil completely. This was followed by the converted DNA clean-up and storage at -80°C

Methylation-specific PCR (MSP): Sodium bisulphite modification of gDNA was performed using Methylamp^{TM} DNA Modification Kit (Epigentek Group, New York). Methylation status in the CpG islands of *RASSF1A* promoter region was determined by methylation specific PCR. Bisulfite-modified DNA was amplified with primers specific for Methylated or Unmethylated sequences. The methylated DNA of *RASSF1A* was amplified using methylated (M) set of primers, 5′-GGGTTTTGCGAGAGCGCG3′ (sense), 5′-GCTAACAAACGCGAACCG-3′ (antisense), and the Unmethylated DNA of *RASSF1A* was amplified using Unmethylated (U) set of primers, 5′-GGTTTTGTGAGAGTGTGTTTAG-3′(sense), 5′-

CACTAACAAACACAAACCAAA-3′(anti-sense). The Universal Human methylated and nonmethylated DNA (Zymo Research, USA) was used as positive and negative control respectively. Two microliters of bisulfite-modified DNA was amplified in a total volume of 25 µL containing 10× PCR buffer 2.5 µL (Fermentas, USA), 10 mmol/L dNTP 0.5 µL, 10 mmol/L of each primer 0.5 µL, and 5 U/mL Taq DNA polymerase 0.1 µL (Fermentas, USA). Methylation-specific PCR reaction conditions of *RASSF1A* were as follows: initial denaturation at 95°C for 5 min, 40 cycles of amplification at 95°C for 30s, 60°C for both methylated and Unmethylated primers for 50s, and 72°C for 45s, followed by a final extension of 72°C for 10 min. 10 µl of each PCR reaction were directly loaded onto a 3% agarose gel, stained with ethidium bromide and visualized under UV illumination. A band size of 169 bp was observed in the *RASSF1A* methylation set **(Figure 1).**

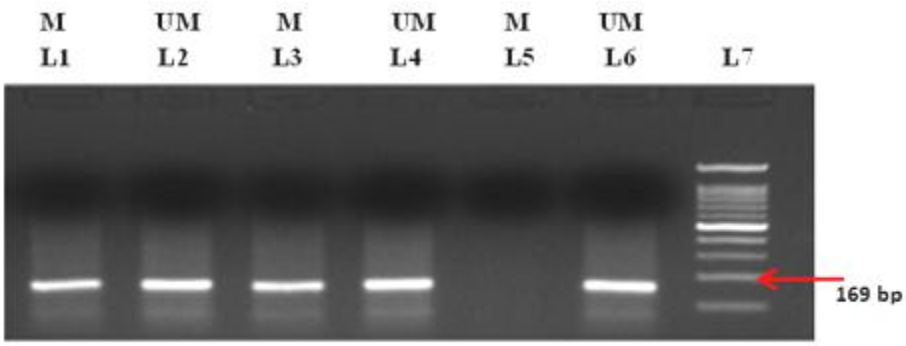

Figure 1: Gel Electrophoresis band pattern for *R4SSF1A* gene methylation visualized under 3% agarose gel under UV trans-illumination yielding 169bp PCR product.

Statistical analysis

All statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) version 20.0 (Chicago, IL, USA). The P value was computed using chi square test, odds ratio (OR) and confidence intervals (95% CI) was computed using unconditional Multivariate logistic regression method. The level of significance was set to $P < 0.05$.

Results

Demographic characteristics of study population: The patient's demographic characteristics are summarized in **Table 1**. A total of 47 males and 23 females were included in the study. Two age groups were made, patients with age ≤ 45 years included 13 cases, and > 45 years included 87 cases. The patients were presented with average age 61.18 years; most of the patients were found within range of 50 to 84 years age group. The patients of non-small cell lung cancer belonged to different regions of Kashmir valley. Moreover, it was found that, of all the ten districts of the Kashmir division, highest number of NSCLC cases turned out from the central district of Srinagar, with total number of 25.72% of NSCLC patients followed by Budgam 11.43% recruited for the study. The demographic profile revealed that in our study 100 patients who were suspected for lung malignancy due their clinical signs and symptoms with positive radiological findings in favour of lung cancer. Among all suspected cases, 100 cases were diagnosed histopathologically as lung malignancy by the concerned Pathologists in the Department of Pathology SKIMS, Soura. In diagnosed malignant cases only non-small cell lung carcinoma were taken for the study. In NSCLC type of Lung cancer, adenocarcinoma was the most common and was found in 72 (72%) patients and

squamous cell carcinoma second most common type of non-small cell lungs cancer, found in 28 patients (28%). Majority of patients 57(57%) were diagnosed in the advanced stages (III and IV) of diseases. History of smoking was found in majority of patients.

Table 1: Demographic characteristics of the patients recruited for study

S. No	Characteristic	Sub group	No. of cases n (%)
1.	Sex	Male	68
		Female	32
2.	Age at diagnosis (yr)	≤45	13
		>45	87
3.	Age (Mean ± SD) yr	61.18±10	
4.	Smoking	Non-smokers	38
		Smokers	62
5.	Dwelling	Urban	43
		Rural	57
6.	Rural: Urban	1.32:1	
7.	Metastasis	Positive	18
		Negative	82
8.	Histological grade	Well	9
		Moderately	65
		Poorly	24
		Undifferentiated	2
9.	Histological type	Adenocarcinoma	72
		Squamous cell	28
10.	Clinical stage (TNM staging)	I & II (early)	43
		III & IV	57
11.	Smoking level	Mild (<10)	5
		Moderate (<40)	30
		Heavy (>40)	27
		No smoking	38

Methylation analysis of *RASSF1A*

Promoter hypermethylation in *RASSF1A* gene was observed in 41% (41/100) cases of non-small cell lung cancer (**Table 2**).

Association of *RASSF1A* gene promoter hypermethylation with age at diagnosis and gender:

Table 2 describes the association of *RASSF1A* gene promoter hypermethylation with age at diagnosis. In both groups ≤ 45 years and > 45 years, the association was found to be non-significant (OR= 0.824, 95% CI 0.261- 2.589, p<0.13.) although *RASSF1A* gene hypermethylation was found to be more evident (43.67%) in the age group >45 years as compared to the age group ≤ 45 years (23.08%). Aberrant methylation of the *RASSF1A* gene was more frequent in males (44.11%) than in females (34.37%), but the difference did not reach a statistical significance (p=0.60)

Table 2: Association of RASSF1A aberrant gene methylation with the clinicopathological features of NSCLC patients

S.no.	Variables	No. of patients	*RASSF1A* methylation status		OR (95%CI)	*p* value
			No. of methylated samples n (%)	No. of Unmethylated samples n (%)		
1.	**Age at Diagnosis**					
	≤45Y	13	3(23.08)	10(76.92)	0.824 (0.261- 2.589)	0.13
	>45Y	87	38(43.67)	49(56.33)	1	
2.	**Gender**					
	Male	68	30(44.11)	38(55.89)	0.540 (0.130-2.145)	0.60
	Female	32	11(34.37)	21(65.63)	1	
3.	**Histological Type**					
	Adenocarcinoma	72	34(47.22)	38(52.78)	0.621 (0.157- 2.183)	0.03*
	Squamous Cell Carcinoma	28	7(25)	21(75)	1	
4.	**Smoking Status**					
	Smokers	62	32(51.61)	30(48.39)	3.099 (1.167- 4.443)	0.01*
	Non smokers	38	9(23.68)	29(76.32)	1	
	Current smokers	20	12(60)	18(40)	0.282 (0.036- 0.856)	0.03*
	Ex-smokers	42	20(47.61)	22(52.39)	1	
5.	**TNM Staging**					
	Early stage (I and II)	53	11(20.75)	42(79.25)	0.359 (0.114- 1.129)	0.05*
	Advanced Stage (III and IV)	47	30(63.82)	17(36.18)	1	
6.	**Distant Metastasis**					
	Positive	18	10(55.55)	08(44.45)	0.821 (0.289-2.786)	0.02*
	Negative	82	31(37.80	51(62.20)	1	
7.	**Smoking Level**					
	Mild (≤10)	5	0(0.00)	05(100)	1	0.72
	Moderate (≤40)	30	16(53.33)	14(46.67)	0.460 (0.023- 7.045)	
	Heavy (> 40)	27	18(55.55)	9(33.34)	0.209 (0.014- 3.799)	0.22
	No-smoking	38	7(18.42)	31(81.58)	1	

Association of *RASSF1A* gene promoter hypermethylation with stage of the disease:

There was a significant correlation established between *RASSF1A* gene promoter hypermethylation with stage of the disease although an increased percentage in the advanced stage disease can be observed (63.82%) and 20.75%) in early stage disease (OR=0.359, 95%CI=0.114- 1.129, p=0.05).

Association of *RASSF1A* gene promoter hypermethylation with different histological types:

A significant association (OR=0.621, 95% CI=0.157- 2.183, p<0.05) was found between the methylation status of *RASSF1A* gene with different histological types. Adenocarcinoma was found to be the highest (47.22%) as compared to squamous cell carcinoma (25%).

Association of *RASSF1A* gene promoter hypermethylation with smoking status:

Smoking was significantly associated with hypermethylation of the *RASSF1A* promoter. (OR =3.099, 95%CI =1.67-4.443; p<0.05; **Table 2**). However majority of the patients who have *RASSF1A* methylation belong to the smokers group with more than 10 years of smoking history.

Association of *RASSF1A* gene promoter hypermethylation with metastasis status:

A significant association was seen between *RASSF1A* gene promoter methylation and metastasis. The significantly higher frequency of this methylation was reported in NSCLC patients (55.55%) with metastasis (OR = 0.821, 95% CI: 0.289-2.786, p < 0.05) (**Table 2**).

Association of *RASSF1A* Promoter methylation and smoking level:

Patients with hypermethylation of the *RASSF1A* were mostly found in the group of patients who have smoked ≤40 years however no significant association was seen between *RASSF1A* promoter methylation and smoking level(OR=3.099, 95%CI= 1.167-4.443, p=0.01)

Discussion:

RASSF1A promoter hypermethylation has been observed in a variety of human cancers such as pancreatic endocrine tumor (Giorgio *et al*., 2011), colorectal cancer (Torbjorn *et al*., 2013), nasopharyngeal carcinoma (Joseph *et al*., 2002), prostate cancer (Limin *et al*., 2002), ovary and renal (Yoon *et al*., 2001) hepatocellular carcinoma (Pensri *et al*., 2010), breast cancer (Magdalini *et al*., 2009), NSCLC (Jie *et al*., 2004), gastric cancer (Do-Sun *et al*., 2001) as depicted in the **Figure 2.**

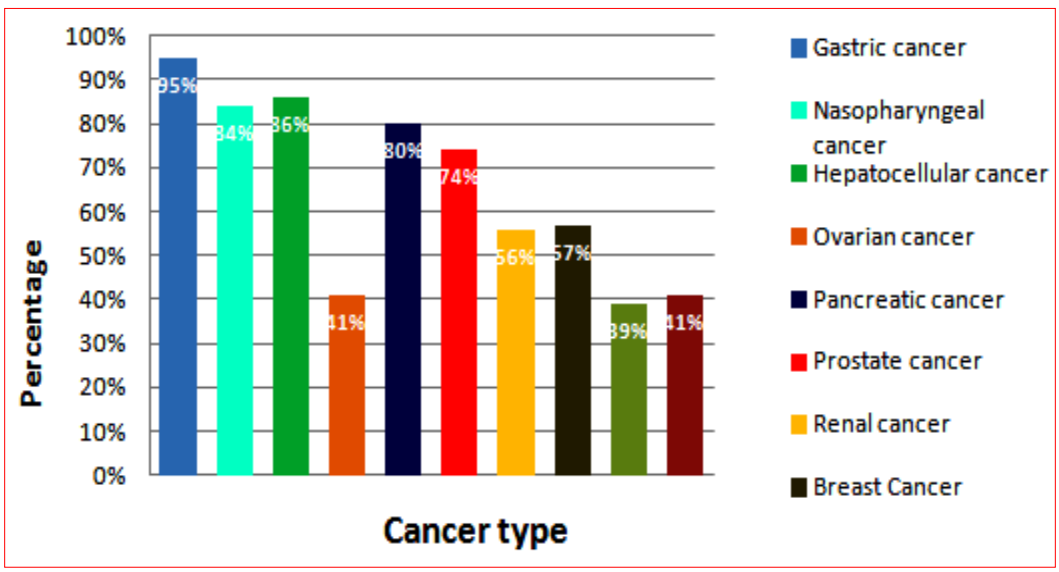

Figure 2: Distribution of *RASSF1A* methylation in various cancers all over the world.

The majority of recent studies have focused on the study of epigenetic changes resulting in many types of cancers. DNA methylation was the first epigenetic alteration to be observed in cancer cells. DNA methylation involves addition of a methyl group to the carbon 5 position of the cytosine ring. This reaction is catalyzed by DNA methyltransferase in the context of the sequence 5'-CG-3', which is also referred to as a CpG dinucleotide (Singal and Ginder, 1999; Jones and Laird, 1999). The methylation of gene, particularly the methylation of CpG-rich promoters, could block transcriptional activation. The potential contribution of DNA methylation to oncogenesis is mediated by one or more of mechanisms that include DNA hypomethylation, hyper-methylation of tumor suppressor gene and chromosomal instability in cancers (Jones and Laird, 1999; O'Neill *et al*., 1998; Chen *et al*., 1998).

In the present study, we used MS-PCR for analysis of the methylation status of *RASSF1A* gene. This method provided significant advantages over previous ones used for assaying methylation. MSP is much more sensitive than Southern analysis, facilitating the detection of low numbers of methylated alleles and the study of DNA from small samples. MS-PCR allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes.

The aberrant methylation of the CpG islands at promoter regions of, the *RASSF1A* gene that may play a role in the development of lung cancer. *RASSF1A* promoter methylation was detected in 41% in NSCLC patients. This prevalence of hypermethylation at the promoter region of this gene according to the present study is consistent with the findings of other groups (Kim *et al*., 2001; Esteller *et al*., 2000; Zochbauer-Muller *et al*., 2001; Dammann *et al*., 2000; Burbee *et al*., 2001; Virmani *et al*., 2000). *RASSF1A* aberrant promoter methylation was found to be significantly associated between adenocarcinoma and squamous cell carcinoma; histological types (p=0.03). The study conducted by Kim *et al*., 2003 and Li *et al*., 2003, shows the results that consistent with our study. We also found that aberrant methylation of the *RASSF1A* gene predominantly occurs in patients with advanced stage of NSCLC. Our study is consistent with the studies conduct by Sekido *et al*., 1998; Toyooka *et al*., 2001; Belinsky *et al*., 1998, showing the same results, consistent with our findings. In the present study, although we analyzed the prognostic significance of aberrant methylation of the *RASSF1A* gene and metastasis. These results suggest that aberrant methylation of the *RASSF1A* gene accompanies progression toward a more aggressive form of NSCLC. Oh *et al*., 2017 reported that *RASSF1A* is mostly methylated in non- small cell lung cancer patients with metastasis which is consistent with our study showing the same results. However there was no correlation found between *RASSF1A* aberrant promoter methylation and other clinicopathological factors like age, gender and smoking level.

This study demonstrates that smoking is significantly associated with hypermethylation of the *RASSF1A* promoter in NSCLC (OR=3.099, 95%CI=1.167-4.443, p=0.01) and that hypermethylation of the *RASSF1A* promoter may affect a patient's survival in NSCLC. The susceptibility to hypermethylation of a specific gene in smokers could be attributed to non-random changes in the environment around a gene. Several factors, including the activity of DNA methyltransferase and local-specific factors, such as SP1 transcription factor, chromatin structure, proximity to a methylation center, and the pre-existing methylation status of CpG islands (Vertino *et al*., 1996) may affect the methylation status of a gene.

In conclusion, the present study indicated that the association of higher methylation of the *RASSF1A* promoter with NSCLC may be specific to males, smokers, patients at advanced stage of the disease, patients who have adenocarcinoma histological type of NSCLC, metastatic stage of disease and patients aged >45years .

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