Significance of the KRAS Gene Codon 12, 13 Point Mutation in Non-Small Cell Lung Carcinoma in Kashmiri Population

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Abstract
Lung cancer represents commonest cancer worldwide, with a high mortality rate as the disease becomes clinically apparent at advanced stages. The most common molecular alterations observed in NSCLC lie in the mutations of KRAS. These mutations occur in 15–30% of NSCLC and are more frequent in adenocarcinoma. The frequency and clinicopathologic significance of the K-ras gene point mutation in non-small cell lung carcinoma remain to be defined. We investigated the frequency of KRAS codon 12,13 point mutations in non-small cell lung carcinoma using a polymerase chain reaction (PCR)-based method in 70 DNA samples extracted from 70 different patients. The prevalence of KRAS mutation rate in NSCLC in the Kashmiri population was 30%. The significant association was seen between KRAS gene mutation and histological types of lung cancer. The higher frequency was seen in ADC (28.84%) than SCC (6%). The difference was statistically significant (OR=0.81, 95% CI=0.257-2.588, $p < 0.01$). Among the different stages, the higher frequency of KRAS (exon 2) mutation was reported in NSCLC patients in advanced stage (38.09%) than the early stages (17.85%). The difference was statistically significant (OR=0.353, 95% CI= 0.112- 1.116, $p<0.05$). A statistically significant difference was reported between smokers and non-smokers with respect to the KRAS (exon 2) mutation (OR= 4.899, 95%CI =1.273-18.77, $p < 0.01$). The significantly higher frequency of this mutation was reported in NSCLC patients (29.16%) with metastasis (OR= 0.941 95% CI= 0.319-2.775, $p < 0.03$). KRAS (exon 2) mutation is a common molecular alteration in NSCLC and occurs most predominantly on codon 12, 13, characterizing 30%. These mutations are significantly associated with clinicopathological characteristics
Keywords: Non-small cell lung carcinoma, KRAS, adenocarcinoma, squamous cell carcinoma, Kashmir, India

Introduction

Lung cancer represents the most common cancer worldwide, with a high mortality rate as the disease becomes clinically apparent at advanced stages (Aberle *et al*., 2011; Jemal *et al*., 2011). Lung cancer is one of the most critical types, accounting for 13% of cancer diagnoses in 2012 (Yang *et al*., 2016). More than 75% of lung cancers are diagnosed when the disease is locally advanced or metastatic, which results in a current 5 -year survival of less than 15% (Aberle *et al*., 2011). According to histological analyses, lung cancer is classified into non-small cell lung carcinoma (NSCLC), which consists of three main subtypes (adenocarcinoma, squamous cell carcinoma, and large cell carcinoma), and small cell lung carcinoma. Non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancer cases (Sun *et al*., 2007). It is now well known that ras oncogenes play an important role in the pathogenesis of various types of cancer. Point mutations at codons 12, 13, and 61 of ras genes result in equilibrium shift of Ras proteins towards the activated state, which constitutively activates the mitogenic signal transduction pathway. Frequency of mutated Ras genes varies among the different tumor types. Point mutations of the K-ras gene are found predominantly in adenocarcinomas (Johnson *et al*., 2012).

Materials and methods

Sample collection and DNA extraction

From April 2014 to November 2015, blood samples from newly diagnosed 70 NSCLC patients were selected from an ongoing molecular study of lung cancer in the University of Kashmir, Srinagar Jammu and Kashmir. Written informed consent was obtained from all participants and patient follow up was obtained through hospital records as well as by direct patient contact. Blood samples anti-coagulated with EDTA were stored at -80°C until use and genomic DNA was extracted from whole blood by DNA extraction kit (Quick- g DNATM MiniPrep supplied by Zymo Research).

Study population

A total of 47 males and 23 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 9 cases, and >45 years included 61 cases. A good proportion of the NSCLC cases 40 (57.14) that were recruited for this study had a monthly income of less than 6000 INR.

PCR amplification

PCR was performed in 25 µl reaction volume containing 3 µl of 100 ng template DNA, 0.3 µl 25 pmol each Primers, 0.6 µl 10 mM dNTPs, 2.2 µl of 20 mM $MgCl_2$, 0.3 µl of 5 U/µl Taq polymerase (Thermo scientific) with 2.5 µl of 10 × Taq Buffer (Thermo scientific) and 15.8 µl of nuclease free ddH2O. Specific primers designed for KRAS Exon 2 gene amplification are given below:

Forward primer KRAS 2F (Exon 2): 5´-TTCCTACAGGAAGCAAGT -3´

Reverse primer KRAS 2R (Exon 2): 5´-CACAAAGAAAGCCCTCCCCA-3´

PCR products were analyzed on 2.0% agarose gel, stained with ethidium bromide and photographed on a UV Gel doc system (**Figure 1**).

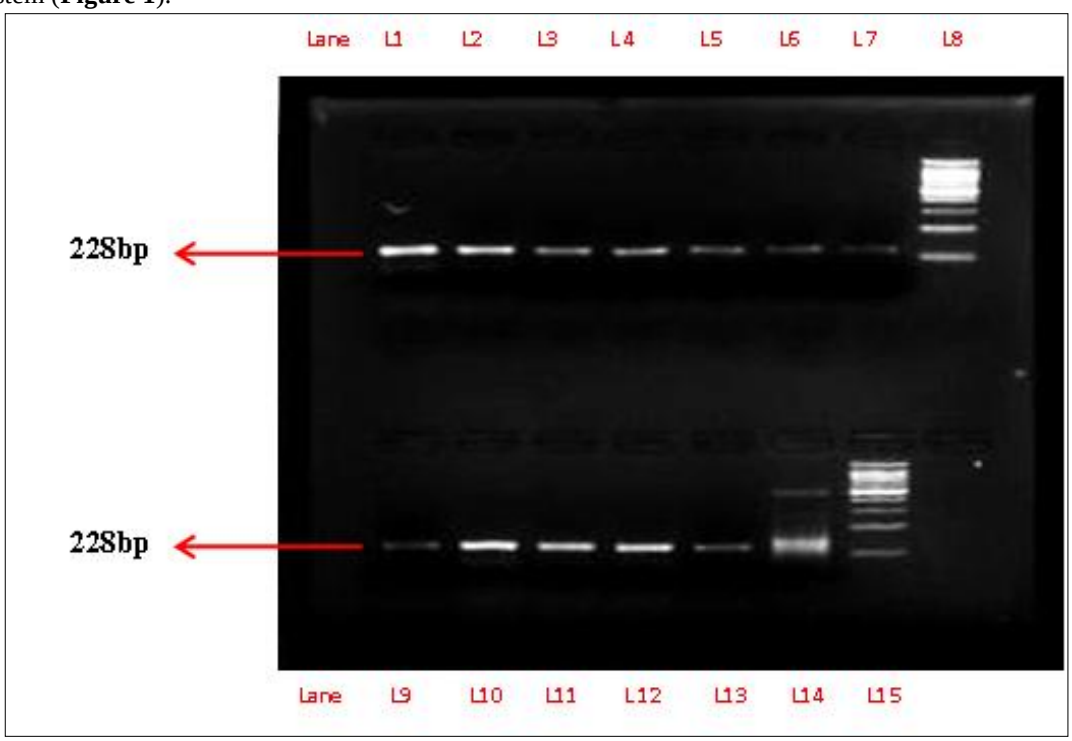

Figure 1: **Representative gel picture of the KRAS (exon 2) amplified by polymerase chain reaction. Lane no. L1 to L7 and Lane no. L9 to L14 represents 228 bp fragment of exon 2 of KRAS gene amplified by PCR, Lane no. L8 and L15 represent 200 bp ladders.**

Purification and DNA sequencing

The purification and sequencing was commercially done using the services of Eurofins Genomics, Bangalore employing sequence scanner software 2v2.0 (Applied Biosystems). For purification and sequencing, we sent 20 µl of unpurified PCR product samples along with 25 µl of 25 µM forward and reverse primers.

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 logistic regression method. The level of significance was set to $p<0.05$.

Result

KRAS mutation status

Out of seventy NSCLC patients, 21(30%) were positive for KRAS (exon 2) gene mutations and 49 (70%) were negative. The difference was statistically significant (p <0.001). The KRAS gene (exon 2) mutation status in relation to clinicopathological features is shown in the **Table 1.**

Table 1: Association of clinical and pathological characteristics with KRAS mutations in non- small cell lung cancer patients.

S.No	Variables	KRAS Mutation (Exon 2)		OR (95%CI)	*p* value
		Positive (%)	e g a t i v e (%)		
1.	**Age at Diagnosis**				
	≤45Y	2(22.22)	7(77.78)	0.632 (0.120-3.329)	0.58
	>45Y	19(31.14)	42(68.86)	1	
2.	**Gender**				
	Male	18(38.29)	29(61.17)	0.970 (0.327-2.873)	0.95
	Female	3(13.04)	20(86.95)	1	
3.	**Histological Type**				
	Adenocarcinoma.	15(28.84)	37(71.16)	0.811 (0.257- 2.588)	0.01*
	Squamous Cell Carcinoma	6(33.33)	12(66.67)	1	
4.	**Smoking Status**				
	Smokers	18(40)	27(60)	4.899 (1.273- 18.77)	0.01*
	Non smokers	03(12)	22(88)	1	
	Current smokers	08(26.66)	22(73.34)	0.182 (0.047- 0.697)	0.01*
	Ex-smokers	10(66.66)	05(33.34)	1	
5.	**TNM Staging**				
	Early stage (I and II)	5(17.85)	23(82.15)	0.353 (0.112- 1.116)	0.05*
	Advanced Stage (III and IV)	16(38.09)	26(61.91)	1	
6.	**Distant Metastasis**				
	Positive	07(29.16)	17(70.84)	0.941 (0.319-2.775)	0.03*
	Negative	14(30.43)	32(69.57)		
7.	**Smoking Level**				
	Mild (≤10)	02(66.66)	01(33.34)	1	0.64
	Moderate (≤40)	11(52.38)	10(47.62)	0.550 (0.043- 7.034)	
	Heavy (> 40)	08(38.09)	13(61.91)	0.308 (0.024- 3.968)	0.34

OR= Odd Ratio; *Statistically significant p-values (Chi-Square test); Odds ratio and 95% CI (logistic regression method).

Frequency of KRAS (exon 2) mutations with respect to various clinico-pathological characteristics.

Frequency of KRAS (exon 2) mutation with respect to histological type, gender and age

The significant association was seen between KRAS gene mutation and histological types of lung cancer. The higher frequency was seen in ADC (28.84%) than SCC (6%). The difference was statistically significant (OR=0.81, 95% CI=0.257- 2.588, $p < 0.01$). There was a much difference in frequency of KRAS gene mutation in NSCLC patients with respect to gender however also the higher frequency of those mutations was reported in higher age group >45(31.14%) than lower age group ≤ 45(22.22%) **(Table 1)**

Frequency of KRAS (exon 2) mutation with respect to stage

Among the different stages, the higher frequency of KRAS (exon 2) mutation was reported in NSCLC patients in advanced stage (38.09%) than the early stages (17.85%). The difference was statistically significant (OR=0.353, 95% CI= 0.112- 1.116, $p<0.05$). Also the progression was reported to be faster among the NSCLC patients in advanced stage with KRAS (exon 2) mutations **(Table 1).**

Frequency of KRAS (exon 2) mutation with respect to smoking type

It was found from the present study that the higher frequency of KRAS mutation was reported from the smokers (40%) than non-smokers (12%). The difference was statistically significant (OR= 4.899, 95%CI =1.273- 18.77, $p < 0.01$) between smokers and non-smokers with respect to the KRAS (exon 2) mutation. Also a significant association was seen between the frequency of KRAS gene (exon 2) mutations in ex-smokers (66.66%) and current smokers (26.66%) (OR= 0.182, 95% CI= 0.047- 0.697, $p < 0.01$) **(Table 1).**

Frequency of KRAS (exon 2) mutation with respect to metastasis

A significant association was seen between KRAS gene (exon 2) mutation and metastasis. The significantly higher frequency of this mutation was reported in NSCLC patients (29.16%) with metastasis (OR= 0.941 95% CI= 0.319-2.775, $p < 0.03$) **(Table 1).**

Frequency of KRAS (exon 2) mutation with respect to other clinico- pathological feature.

There was no significant association found between level of smoking (mild, moderate and heavy), family history and KRAS gene mutation **(Table 1).**

Mutational analysis.

As far as the mutation frequency is concerned, 70 patients were investigated through screening by direct sequencing, 30% showed mutation in exon 2 codons 12 and 13 of the KRAS proto-oncogene. Conversely 70% showed wild-type (WT) sequence (**Figure 2)**.

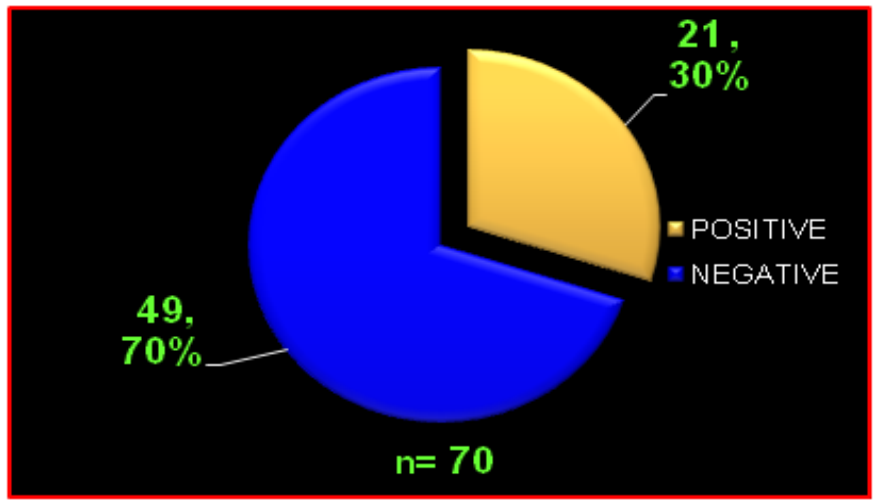

Figure. 2: Pie diagram showing the overall mutation frequency of KRAS gene in all analysed NSCLC patients.

The most common mutations that was found in NSCLC patients are glycine (G) to cysteine (C) on codon 12 (p.G12C, 33.33% of mutated samples; 07 of 21), glycine (G) to valine (V) on codon 12 (p.G12V, 23.80% of mutated samples; 05 of 21), glycine (G) to aspartate (D) on codon 12 (p.G12D, 19.04% of mutated samples; 04 of 21).The three mutations G12C, G12V, G12D account 76.19% of all the mutations.

Discussion

In the present study, 70 non-small cell lung cancer cases were analyzed. Out of 70 cases, 47 were males which corresponded to 67.14% and hence remaining 23 were females corresponded to 32.86% (Mandal *et al*., 2013). A significant proportion of the patients in the study were within range of 50 to 84 years, and the mean age was 61.18 ±10.98 years (Kashyap *et al*., 2003; Gupta *et al*., 2001).

This observation reconfirm the established fact of increasing incidence of lung cancer as the age advances and need of detailed evaluation of elderly patients who present features suggestive of lung cancer.

In the current study, 64.28% of male patients were smokers while none of the female patient had history of smoking (Wani *et al*., 2014). Mutations in codons 12 and 13 are the most common in lung cancers (Uchiyama *et al*., 2003).

In present study the frequency of KRAS gene (exon 2) mutations in NSCLC patients was 30%. The significant association was seen between KRAS gene mutation and histological types of lung cancer patients. The higher frequency was seen in ADC (28.84%) than SCC (6%). The difference was statistically significant (OR=0.81, 95% CI=0.257- 2.588, $p < 0.01$). KRAS mutations are detected mainly in lung adenocarcinomas and are less frequently observed in squamous cell carcinomas of the lung (Herbst *et al*., 2010; Ju *et al*., 2016; Brandao *et al*., 2012).

There was a much difference in frequency of KRAS gene mutation in NSCLC patients with respect to age however also the higher frequency of those mutations was reported in higher age group >45(31.14%) than lower age group ≤ 45(22.22%) (Riely *et al*., 2008).

Present study revealed that among the different stages, the higher frequency of KRAS (exon 2) mutation was reported in NSCLC patients in advanced stage (III &IV) (38.09%) than the early stages (I & II) (17.85%). The difference was statistically significant (OR=0.353, 95% CI= 0.112- 1.116, $p<0.05$), that means there is a significant association with increased risk of NSCLC if patients in advanced stage.

In addition it was found from the present study that higher frequency of KRAS mutation was reported from the smokers (40%) than non-smokers (12%); however, this difference was statistically significant (OR= 4.899, 95%CI =1.273- 18.77, $p < 0.01$). Also a significant association was seen between the frequency of KRAS gene (exon 2) mutations in ex-smokers (66.66%) and current smokers (26.66%) (OR= 0.182, 95%CI= 0.047- 0.697, $p < 0.01$)

A significant association was seen between KRAS gene (exon 2) mutation and metastasis. The significantly higher frequency of this mutation was reported in NSCLC patients (29.16%) with metastasis (OR= 0.941 95% CI= 0.319-2.775, $p < 0.03$) (Wagner *et al*., 2011).

There was no significant association found between level of smoking (mild, moderate and heavy), family history and KRAS gene mutation.

In a total of 70 cases diagnosed as NSCLCs, the KRAS mutations were found in 21 of 70 cases (30%). Nineteen of 21 (90.47%) were located in codon 12, and 2 (9.52%) was located in codon 13 (Macerellia *et al*., 2014).

Further, mutations in KRAS detected in this study were diverse comprising 21, out of which, 7 were KRAS c.34G>T transversion, and 5 were KRAS c.35G>T transversion, 4 were KRAS c.35G>A transition, 2 were KRAS c.34G>A transition, 1 was KRAS c.34G>C transversion on codon 12 and 2 were KRAS c.38G>A on codon 13(Li & Durbin, 2008).

Conclusions

The incidence of KRAS gene mutation in our NSCLC patients showed a tendency toward association with progressive disease status. The study revealed statistically significant association of KRAS gene mutation with stage of the disease, histological type, smoking, and metastasis.

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