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K-RAS GENE MUTATIONS AT CODON 12 AND 13 IN PANCREATIC CANCER AND CHRONIC PANCREATITIS PATIENTS FROM NORTH INDIAN POPULATION

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Abstract: To clarify the sensitivity and the validity of K-ras point mutational analysis at codon 12 and 13 in North Indian patients with pancreatic diseases, and the possible correlation between the presence of the mutation and the histopathological findings. Sixty-five pancreatic ductal adenocarcinoma and 50 chronic pancreatitis patients were enrolled in this study. Codon 12 and 13 K-ras mutations were examined using the two step polymerase chain reaction (PCR) followed by single strand confirmation polymorphism (SSCP) and further confirmed by automated DNA sequencing. The positive percentage of K-*ras* in pancreatic carcinoma was 72.30% (47/65) which was significantly higher than that in the chronic pancreatitis 6.0% (3/50) ($P<0.01$). The nucleotide sequences of K-*ras* demonstrate GGT to GGA (G>A) and GGC to GGG (C>G) transitions at codon12 and codon13 respectively. These results draw attention to the critical role of K-*ras* gene mutation for the detection of early stage pancreatic cancer from chronic pancreatitis. K-ras gene mutation can be used as an important biomarker for early pancreatic cancer diagnosis, but it needs to be confirmed in large number of chronic pancreatitis patients.

Key Words: K-ras, Pancraetic cancer, Chronic Pancreatitis, SSCP

INTRODUCTION

Pancreatic cancer is an uncommon tumor, but this form of cancer has now become a common cause of cancer mortality [1-2]. The causes of pancreatic cancer remain unknown, but there are several factors that increase the risk of this malignancy [1,3]. Tobacco smoking and diabetes mellitus are two important risk factors that have been firmly established. Smoking is the major known risk factor which accounts for ~25–30% of all the cases. Dietary factors are less significant in pancreatic cancer than in other digestive tract tumors[2]. It is a leading cause of cancer related deaths in developed countries and this is mainly attributable to the extremely poor survival rate[1-2]. Less than 20% of newly diagnosed patients survive first year, whereas less than 5% survival in all the stages of this cancer [4]. Despite this poor prognosis, considerable progress has been made in our understanding of the biology of pancreatic carcinoma. Different stages like diagnosis, staging, treatment and palliation of the disease has been well understood.

Point mutation of the K- ras gene is very common in many human cancers such as colorectal cancer, lung cancer [3]. Pancreatic adenocarcinoma shows the highest frequency of K-ras gene mutations among the common human cancers but the reasons are unknown [4]. Genetic mutations are associated with many types of human tumors, these changes mostly involved in proto-oncogenes and tumor suppressor genes (TSG), which control cellular growth and differentiation [5]. Ras genes (N-*ras*, H-*ras* and K-*ras*) are an important proto-oncogene which are able to code proteins commonly referred to as p21*ras* [6]. p21 ras act as molecular switches in the intracellular signal transduction process, binding GTP and GDP with intrinsic GTPase activity[4-9]. It is converted to an active oncogene by point mutations and plays an important role in tumorigenesis by maintaining the active GTP-bound form, thus favoring the constitutive transmission of a positive signal for cell growth.

In pancreatic cancer mutation usually occurs at codon 12, the hot spot of the gene, mutations also occur at codons 13 and 61with substitution of the correspondent amino acid in the *ras* protein [5,7,8]. The expression of these mutated genes leads to altered protein products which are capable of transforming cells into a malignant phenotype [8-9]. Mutational activation of the K-*ras* at codon 12 has been demonstrated in majority of the cases of pancreatic adenocarcinoma [10-11]. This very high prevalence of mutation has never been identified in other types of human tumors. The substitution of a nucleotide at the first or second base of codon 12 may precede the development of malignancy [12]. Moreover, K-*ras* gene mutation has been associated in the process of metastasis and aggressiveness of tumoral cells[13].

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The high prevalence of mutation in pancreatic tumor suggests that point mutations in the K-*ras* gene might be used for future screening protocols. Due to its critical role in pancreatic cancer, K-*ras* can be an important target for novel anti-cancer therapies. Therefore, the aim of the present study was to conduct an in-depth study to clarify the sensitivity and the validity of K-*ras* point mutations at codon 12 and 13 from pancreatic patients of North India.

MATERIALS AND METHODS

Tissue selection

A total of one hundred fifteen patients from Maulana Azad Medical College and Associated Lok Nayak & G. B. Pant Hospital, New Delhi, India with histological proven pancreatic cancer (65 patients), and chronic pancreatitis (50 patients) were enrolled in this study. The study was approved by the Institutes Ethical Committee and informed consent was taken from the patients. The tissues used in this study were derived from patients who underwent surgery for ductal adenocarcinoma of the pancreas and chronic pancreatitis. The control group constitute, normal pancreas from autopsy tissue who did not have pancreatic carcinoma or pancreatitis.

Analysis of K-ras Mutations

K-*ras* mutations were determined by a previously described method using a semi-nested PCR approach followed by mutation enrichment [14-15]. Two sets of primers were used for the first PCR and the second PCR amplification. The primer sequence for the first PCR were: 5′- GAA AAT GAC TGA ATA TAA ACTTGT GGT AGT TGG ACC T -3′ (sense) and 5′- TCA TGA AAA TGG TCA GAG AAA CC -3′ (antisense). For the second PCR, the sequence of sense primer was same as the first PCR and the sequence for the antisense primer was 5′- TCA AAG AAT GGT CCT GGA CC -3′. The first PCR was performed in a total volume of 25 µL containing: 2 µL of DNA, 0.1 µmol/L of each primer, 200 µmol/L of each dNTP, 1.25 units of DNA polymerase (PGC Scientifics, Frederick, Md), 1 × PCR buffer with 1.5 mmol/L of MgCl2. The PCR conditions consisted of 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The product was digested with 20 units of MvaI (New England Biolab, Beverly, Mass) under 65°C for 7 hours. MvaI specifically cuts the wild-type K-ras sequence but not any sequences Primer Amplicon size Sequence Primer A 150bp 5' GAA AAT GAC TGA ATA TAA ACTTGT GGT AGT TGG ACC T -F Primer B 151bp 5' TCA AAG AAT GGT CCT GGA CC – R Primer C 5'-TCA TGA AAA TGG TCA GAG AAA CC-3' that are mutant at codon 12 of K-ras. The product as resolved on 3% agarose gel. The second PCR reaction was performed in a total volume of 50 µL containing 1 µL of first PCR reaction product, 0.1 µmol/L of each primer, 200 Kmol/L of each dNTP, 1.25 units of DNA polymerase, and 1 × PCR ×buffer with 1.5 mmol/L of MgCl2. The PCR condition consists of 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The PCR product was digested with 20 units of BstNI under 65°C for 7 hours. The second PCR product (enriched PCR product) was subjected to direct sequencing.

Nonradioisotopic SSCP analysis

Nonradioisotopic SSCP Analyses were performed as described previously, with minor modification [16]. After denaturation at 100 for 5 min, a 10 L sample was applied and resolved by 120 g/L polyacrylamide gel electrophoresis at 35 V for 21 h at 4°C. The gels were silver-stained. Each sample was analyzed by SSCP repeatedly to confirm its accuracy.

Statistical analysis

A statistical analysis was performed using the chi-square test, the Fisher's exact test and Student *t* test with SPSS.

RESULTS

Out of 65 pancreatic cancer cases, 24 (36.92%) patients were female and 41 (63%) patients were male. The average age was 47.42 ± 11.32 years and range from 22-70. In 50 chronic pancreatitis only 7 (14%) were female and 43 (86%) were male having an average age of 36.02±10.55 years and range from 20-63. In the control group 35 were male and 15 were female with an average age of 36.75 ± 11.75years.

In our study K-ras positivity was observed in 47/65 and mutations were observed in 31/65 and the remaining 34 were wild type while in chronic pancreatitis it was observed that 3/50 showed mutations and remaining 47 were wild type which shows statistical significance (p=0.0001) with OR=14.28 (3.88-77.25) at 95% confidence level. (Table 1)

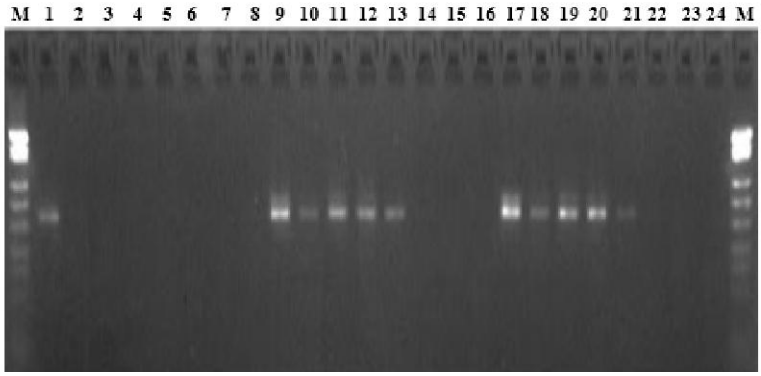

Figure 1: Analysis of pancreatic cancer samples by mutant enriched PCR for K-*ras*.
Lane M : ϕ X 174 Ha III digested Marker
Lane 1,9-13& 17-21:K-ras poitive

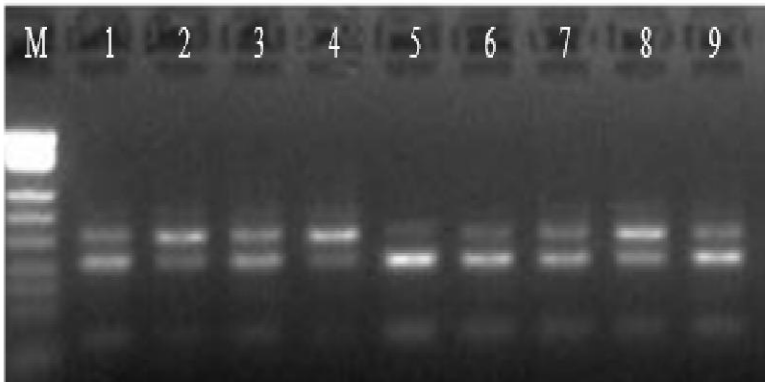

Figure 2: Amplified PCR product of Codon12 of K-*ras* digested with MvaI. The *top band*, the undigested mutant K-*ras* sequence (147 bp); the *lower band*, the digested wild-type K-*ras* band (111 bp).
Lane 1- 4 and 7-9: Mutant k-ras
Lane 5-6: Wild-type for K-*ras*
Lane M: Lanes loaded with *Msp*I-digested pUC18 as a size marker.

Table 1: Prevalence of K-ras gene mutations in pancreatic cancer cases, chronic pancreatitis and controls:

K-ras gene	Pancreatic Cancer (n=65)	Chronic Pancreatitis (n=50)	Control (n=50)	P value		
	1	2	3	1&2	1&3	2&3
Mutated	31(47.69%)	3(6%)	0(0%)	0.0001†	0.0001†	0.0001†
Wild	34(52.31%)	47(94%)	50(100%)			

K-ras gene 1&2 OR= 14.28(3.88-77.25)
† Significant

Detection of K-ras point mutation by non-radioisotopic SSCP analysis and direct sequencing of PCR product

The positive percentage of K-*ras* in pancreatic carcinoma was 72.30 % (47/65) which was significantly higher than that in the chronic pancreatitis 6.0 % (3/50) (*P*<0.01). The nucleotide sequences of K-*ras* demonstrate GGT to GGA (G>A) and GGC to GGG (C>G) transitions at codon12 and codon13 respectively (Table 2). The control group showed wild type nucleotide sequence GGT and GGC at codon12 and codon13. The nucleotide sequences were submitted to the gene bank and were assigned accession numbers *EF471933 to EF471957.* Mutations of K-*ras* gene at codon 12 showed transition of GGT-GGA (G>A) in 28 cases and at codon 13 GGC-GGG (C>G) was observed in 3 cases while in chronic pancreatitis the mutations at codon12 were observed in 3 cases which was statistically significant (*p*<0.05) and not observed in codon13. (Table 2).

Table 2: Mutation Analysis of K-ras gene in cases of pancreatic cancer and chronic pancreatitis

	GGT-GGA	GGC-GGG	Wild
Pancreatic Cancer (n=65)	28 (90.32%)	3 (100%)	34 (41.98%)
ChronicPancreatitis (n=50)	3(9.68%)	0 (0%)	47 (58.02%)
P value	0.0001*	0.125*	0.0001*
OR	11.86 (3.21-64.35)	-	0.07 (0.01-0.26)

Expression profile of K-ras gene through immunohistochemistry

K-*ras* expression was localized to both the nucleus and cytoplasm of epithelial cells in all positive cases. In the lesions judged to be diffusely positive almost all neoplastic cells were strong and homogeneously positive for K-*ras* protein, whereas most lesions considered to be focal/scattered positive contained fewer than 30% neoplastic cells showing weak K-*ras*. Immuno-histochemical analysis for K-*ras* protein expression was performed in all specimens investigated with K-*ras* monoclonal antibody in this study. K-*ras* protein expression was 55.38% (36/65) in strong and medium expression in ductal adenocarcinomas stained positive for K-*ras* and 44.62% (29/65), with weak and nil expression Table 3 & Figure 3A-3D]). In chronic pancreatitis group it was noted in 100% (50/50) cases showing weak K-ras immunostaining showing statistical significance (*p*<0.05).

Table 3: Expression of K-ras in pancreatic cancer and chronic pancreatitis

	Weak-Nil Expression	Moderate -Strong expression
Pancreatic Cancer (n=65)	29 (44.62%)	36 (55.38%)
Chronic Pancreatitis (n=50)	50 (100%)	0 (0%)
P-value	0.0001*	0.0001*

*p value= Significant

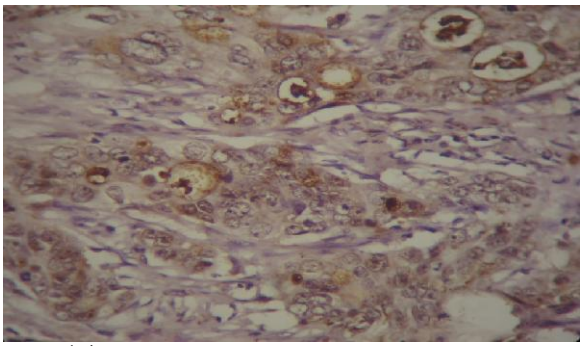

Figure 3(A): Photomicrograph of section from the tissue of pancreatic carcinoma. Immuno stained with K-ras antibody, showing strong degree of staining for K-ras protein in the tumor tissue.

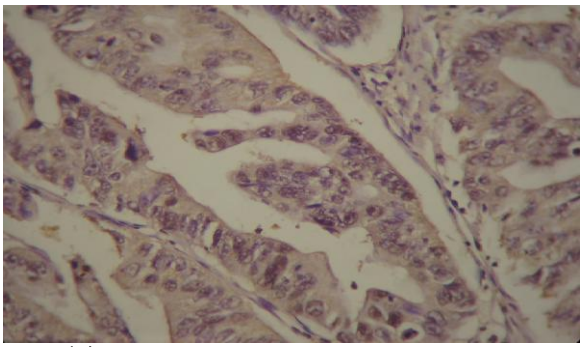

Figure 3(B): Photomicrograph of section from the tissue of pancreatic carcinoma. Immuno stained with K-ras antibody, showing medium degree of staining for K-ras protein in the tumor tissue.

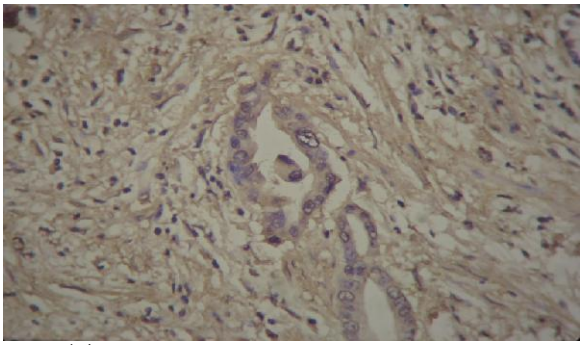

Figure 3(C): Photomicrograph of section from the tissue of pancreatic carcinoma. Immuno stained with K-ras antibody, showing weak degree of staining for K-ras protein in the tumor tissue.

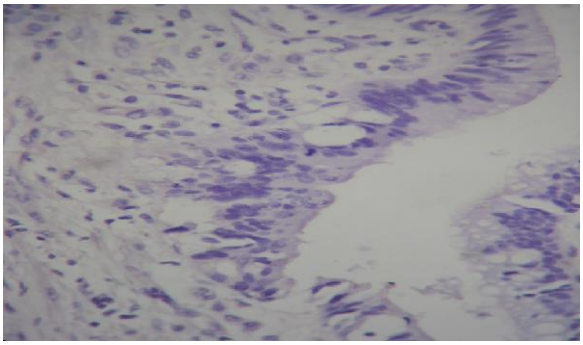

Figure 3(D): Photomicrograph of section from the tissue of pancreatic carcinoma. Immuno stained with K-ras antibody, showing absence of K-ras protein (No expression) in the tumor tissue.

Comparison of K-ras protein expression in relation to tumor differentiation, location, and metastasis of the tumor and K-ras gene.

K-*ras* is a proto-oncogene which is over expressed in precancerous lesions in pancreatic cancer. The expression of K-*ras* showed statistically significant correlation in well differentiated and non-differentiated compared to moderate and poorly differentiated (p=0.0006). The expression of K-*ras* protein and tumor location had statistically insignificant correlation (p>0.05). K-*ras* protein expression showed significant correlation in relation to the tumor progression in distant metastasis (p<0.05) than compared with Lymph node metastasis (Table 4).

DISCUSSION

The main problem in diagnosing pancreatic cancer, especially at the early stage, is characterizing and identifying which group of the population has a higher risk of tumor. Subjects with diseases such as chronic pancreatitis, mucinous ductal dilatation (intraductal tumor) and long-standing diabetes, have been considered as population groups having an increased risk of developing pancreatic cancer [17-18]. Using the advantages of the genomic amplification technique *in-vitro*, we have shown that mutations in the K-*ras* oncogene can be readily detected in fresh tumoral tissues. The sensitivity of K-*ras* in our study was approximately 72%, similar to the earlier findings [5,7-9,19]. This finding is very helpful in diagnostic purpose because of the specificity of the mutation. The unique nature of these mutations is usually limited to just one codon. Moreover, the incidence of K-*ras* point mutation in pancreatic tumor at codon 12 was higher in frequency (i.e.70–90%) compared to mutations at codon 13 or 61 [5,9]. Ninety percent of tumors in our study showed k-*ras* mutation at codon 12, majority of them had T>A transition, 5% with T>G while 5% had 2 nucleotide substitution mutations (i.e. G>A and T>A). Similarly, 2 cases showed mutations both at codon 12 and codon 13 with transition C>G (GGC→GGG), confirming earlier studies [20-22]. The mutation pattern of K-*ras* gene involved in chronic pancreatitis at codon 12 showed 60% T>A (GGT→ GGA) transition, identical to pancreatic carcinoma while rest were wild type. Most of the earlier study confirmed the prevalence of K-*ras* mutation in chronic pancreatitis patients with significant association with advanced age [23]. Although we have found K-*ras* mutations in five chronic pancreatitis cases but they were significantly younger than previous finding [23]. This confirms that patients who were evaluated for longer periods have more chances of harboring such mutations [24-26].

Table 4: Comparison of K-ras IHC Expression in relation to tumor differentiation

	K-ras IHC Expression		P-value
	Nil/Weak (n=29)	Medium/Strong (n=36)	
Differentiation			
Well differentiated	2 (6.90%)	13 (36.11%)	0.005*
Moderate	15 (51.72%)	18 (50%)	0.89
Poor	2 (6.90%)	3 (8.33%)	0.83
Not-differentiated	10 (34.48%)	2 (5.56%)	0.003*
Location			
Periampullary	12 (41.38%)	15 (41.67%)	0.98
Head	12(41.38%)	14 (38.89%)	0.83
Body/Tail	4 (13.79%)	7 (19.44%)	0.55
Cyst	1 (3.45%)	0 (0%)	0.26
K-ras gene			
Mutated	15 (51.72%)	32 (88.89%)	0.0002*
Wild	14 (48.28%)	4 (11.11%)	
Lymph node metastasis			
Absent	29	32	
Present	0	4	0.06
Distant metastasis			
Absent	25	22	
Present	4	14	0.025*

*p value= Significant

Although the occurrence of k-*ras* mutations is linked to smoking in tumor types such as lung cancer [27], such relationship was also present in our study. Our study showed that k- *ras* mutation was present in

70% of cases with history of smoking in pancreatic cancer and chronic pancreatitis. In this study most of the patients with chronic pancreatitis were smokers and may increased the risk toward the progression to pancreatic cancer. Therefore, considering that chronic pancreatitis is possibly a risk factor for the pre-neoplasic process, further studies using patients with this etiology will be necessary to understand pancreatic tumoral behavior. Another important observation in this study was K-ras mutation in individual diagnosed with diabetes mellitus and progression towards pancreatic cancer. It was interesting to note that diabetes mellitus was a well-established risk factor for pancreatic cancer [12].

Immunohistochemical study confirms insignificant association of K-ras mutation with and tumor grade and staging. The K-*ras* point mutation occurs in the early stage of pancreatic carcinogenesis process, however it has not been clarified whether the frequency of this oncogene could be correlated with the grade of cellular atypism [28-31].

The mutation profiles of K-*ras* codon12 mutations in our study specimens were significantly different from those of European, Japanese and Chinese samples [32-35]. It seems that the T to A trans version in K-*ras* codon12 mutation may be important in the carcinogenesis of Indian pancreatic carcinoma. The heterogeneity of the K-*ras* mutation is not consistent with the interpretation that a single carcinogen is a causative factor. Rather, the distinct mutations are probably due to different exogenous or endogenous carcinogens. It is conceivable that international differences in the pattern of mutations may reflect ethnic peculiarities associated with distinct environmental or genetic factors [36].

CONCLUSIONS

In conclusion, the K-*ras* point mutation in our study is considerably prevalent in malignancies. These results draw attention to the critical role of K-*ras* gene mutation for the detection of early stage pancreatic cancer from chronic pancreatitis. K-ras gene mutation can be used as an important molecular biomarker for early pancreatic cancer diagnosis, but it needs to be confirmed in large number of chronic pancreatitis. These results encourage us to consider the possibility of treatment strategies of this oncogene in the future.

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