Molecular Evaluation of K-Ras Mutation in Egyptian Hepatocellular Carcinoma Patients

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Abstract

Background. The *RAS* gene family is among the most studied and best characterized of theknown cancer-related genes. Of the three human ras isoforms, *KRAS* is the most frequently altered gene, with mutations occurring in 17%–25% of all cancers.
Aim of the study. The purpose of the study was identification of point mutation in codon 12 of K-RAS in Egyptian hepatocellular carcinoma (HCC) patients.
***Patients and methods*.** Blood and urine samples were taken from 100 volunteers: 25 HCC patients, 25 HCC patients with HBV, HCV or schist soma infection and 50 healthy volunteers as control subject.
Mutant allele specific amplification (MASA), which is a highly sensitive method for detecting KRM, was performed, with the DNA_S extracted from these samples.
Results. K-Ras mutation detected in 13 (13%) HCC patients from total 100. There was 4 patients with positive KRM among 22 HCC patients with HBV, 5 patients with positive KRM among 22 HCC patients with HCV infection and 3 HCC patients with schist soma have KRM.
Conclusion. The KRM incidence was found to be relatively high for HCC patients especially who combine HBV infection. This result suggests that the detection of KRM by MASA is useful for screening of HCC.

KEY WORDS: K-Ras, codon 12, mutant allele specific amplification and hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common primary neoplasm and third largest cause of cancer-related death worldwide (**Jemal *et al*., 2005**).Hepatocellular carcinoma (HCC) is a particularly vascularized solid tumor where the Raf/MEK/ERK pathway is activated; suggesting that inhibition of this pathway may have therapeutic potential. Regulation of cell growth, differentiation and survival occurs through a complex interplay of extracellular signals and intracellular signaling cascades.

Ras genes consist of three families, namely, K-ras gene derived from Kirsten sarcoma virus, H-ras gene from Harvey sarcoma virus, and N-ras gene from neuroblastoma (**Hopkins *et al*., 1987**). These genes code for highly related proteins known as A/r 21,000 proteins, which are composed of 188 to 189 amino acids and are thought to control mechanisms of cell growth and differentiation (**Barbacid, 1987**). Dysregulation of Ras proteins by activating mutation, over expression or upstream activating is common in human tumor. Of the Ras proteins, K-Ras is the most frequently mutated and is therefore an attractive target for cancer therapy. Three ras genes are converted to active oncogenes by point mutations occurring in either codon 12, 13, or 61 (**Bos *et al*., 1987**).

It was reported that approximately 40% of colon cancer (**Barbacid, 1987**), 50% of adenocarcinoma of the lung (**Bos *et al*., 1987**), 30% of acute myeloid leukemia (**Rodcnhui *et al*., 1987**), and 90% of pancreatic adenocarcinoma (**Farr *et al*., 1988**) showed ras gene mutations. However, there was little information regarding ras gene mutations in tumors of the liver. So the aim of this study was to evaluate the K-Ras mutation in hepatocellular carcinoma and evaluate this mutation with the level of AFP as tumor marker and patient's clinical and pathological data.

Patients and methods.

Patients

Blood and urine samples were collected from 50 hepatocellular carcinoma (HCC) patients (13 females and 37 males) the average range between (35-80) years. 25 HCC patients previously infected with HCV & HBV or schistsoma as group I, and 25 HCC patients without viral infection or schistsoma infections as group II their age also range between (40-74) years. And 50 healthy volunteers as control group whose age range between (40-75) years. All patients were diagnosed by CT scan of the abdomen using intravenous amass shown on abdominal CT scan. The liver biopsies showed hepatocellular carcinoma. The history of the patients and their clinical data were considered for studying any correlation would appear with k-Ras expression in HCC as ALT, AST, Bilirubin, α-FP level, tumor size and cancer stages. Liver tumors stages were classified according to Barcelona clinic liver cancer staging (BCLC) that established by abdominal computed tomography, and endoscopic ultra sonography, magnetic resonance imaging and histopathological finding on the base of portal vein thrombosis, multifocal tumor, diffuse or massive disease, high alpha fetoprotein (AFP)

level and performance status. On the bases of Barcelona clinic liver cancer staging classification: 22patients were classified in tumor stage A, **15** patients in tumor stage B, **10** patients in tumor stage C, and **3** patients in tumor stage D. Transaminase test, serum total Bilirubin, Alpha-Fetoprotein (AFP), Detection of Hepatitis Viruses and schist soma all of that are detected for all patients

DNA extraction

Genomic DNA was extracted from blood samples by using Gene JET™ Purification Column according to manufacture (Fermentas Life Science, Thermo Fisher Scientific Inc., MA, and USA).

MASA PCR

According to a modification of the MASA method (8) PCR through PCR mixtures consisted of DreamTaq Green PCR Master Mix (2X) (Fermentas, USA). All reactions were carried out in a Biometra thermal cycler (Biometra GmbH, Germany) and electrophoresis analysis were performed. The principle of the MASA method can be described briefly as follows: The oligonucleotide sequences of the primers for MASA are listed in Table 1. The 3'-terminal base of the sense primer was set to correspond to each of the 3 possible mutant bases of the first base of K-*ras* codon 12, and these 3 mutant sense primers were mixed. 1µg of template DNA was add to 25µL of PCR Master mix , 1µM of forward primers and 1µM of reverse primer deionized water was add, for a total volume 50µL. After the preheating phase (at 94 °C for1 minute), PCR was performed with 40 cycles (at 94 °C for 30 seconds, 63.5 °C for 90 seconds, and 72 °C for 90 seconds) followed by extension at 72 °C for 5 minutes.

Table (1): PCR different primers.

Oligonucleotide of K-*ras* codon 12 for MASA	**K-*ras* codon 12**
Sense (5'-3')	
Set1	
ACTTGTGGTAGTTGGAGCTA	**A**GT
ACTTGTGGTAGTTGGAGCTC	**C**GT
ACTTGTGGTAGTTGGAGCTT	**T**GT
Antisense (5'-3')	
CTCATGAAAATGGTCAGAGAAACC	
MASA: mutant allele specific amplification. Each 3'-terminal base of the sense primers for K-*ras* mutations at codon 12 corresponds to a possible mutant of the first base of K-*ras* codon 12. Set 1 primers include three mutant primers for the first base of K-*ras* codon 12, respectively	

Gel electrophoresis

Using 10 mL of each PCR product, electrophoresis (50 V for 1 hour) with a 2% agrose gel (FMC Bio- Products, Rockland, ME) and an electrophoretic device (Mupid 2; ADVANCE, Tokyo, Japan) was performed, followed by ethidium bromide (0.5 mg/mL) staining of the gel. When a specific single band of PCR product was visualized under ultraviolet illumination, as shown in Figure 1, the size of PCR products was determined relatively to the migration of a 50 bp step ladder (Promega Co. WI, USA). The presence of KRM was judged for Set 1. Each PCR product was 179 base pairs (bp) for Set 1.

Statistical Analysis

Statistical analysis was performed using a computer-assisted Student's *t*-test with SPSS ver 10 software. Statistical significance was found at $P < 0:05$.

Results.

K-Ras mutation has been detected by MASA PCR at 13% for all cases of total cases. This as illustrate at Table (2) and Figure (1) there were (84) 84% from total (100) without HBV infection and without K-Ras mutation. The HCC patients without HBV infection but have K-Ras mutation were 9% and there were 3% with HBV infection and without K-Ras mutation. The same Table show that 4% from total 100 were HCC patients with HBV and have mutation at K-Ras gene codon 12. .

Table (3) shows K-Ras mutation at 5% from total cases (100)100% in HCC patients with chronic HCV and 8cases (8%) have k-ras mutation while they did not have HCV infection . in the same table 70 cases (70%) without HCV infection and without K-Ras mutation .also, 17 patients(17%) who have HCV infection but did not have K-ras mutation.

Table(4) shows 10 patients without schistisoma infection but have K-Ras mutation at codon 12,and 3 patients with schistisoma infection and have point mutation at codon 12. Also, there were 79 cases without schistisoma infection and no mutation at K-Ras. And 8 patients with shistisoma and no mutation at K-Ras codon 12. This mutation was at codon 12 of K-Ras gene that converted guanine to cystein GGT→ CGT by substituting amino acid glycine by arginine that detect in HCC patients with viral infection or shist soma by perecent 32% and guanine to thymine transversion GGT → TGT by substituting amino acid glycine by cystein occour at HCC patients only by percent 20% and 12% in HCC patients with viral infection or schist soma, no detection of mutation at adenine first nucleotide of codon 12 at K-Ras gene (AGT)

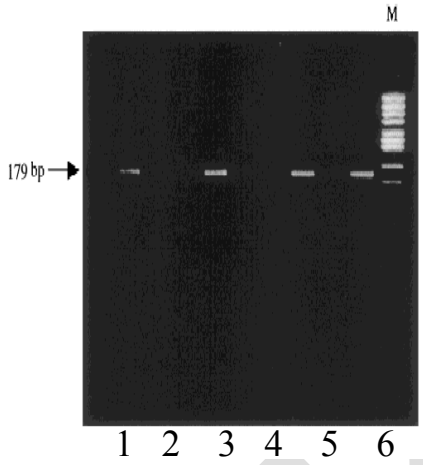

Figure (1): 3% (w/v) gel electrophoresis of PCR products for codon 12 K-Ras mutation by MASA-PCR

Lane 1, show products of HCC patients.
Lane 2, no band appear for HCC patient with schist soma
Lane3, show DNA from HCC with HCV patient with point mutation at base C
Lane 4, no band occur for HCC patient with chronic HCV
Lane 5 show DNA from HBV patient with point mutation at base T
Lane 6, is marker with molecular size 100 base pairs
Last well contain marker, each band equal 50 bp

Table (2): Detection of K-Ras mutation at codon 12 in HCC patients with HBV infection.

HBV	K-Ras Point Mutation at codon 12						Chi-Square	
	Negative		Positive		Total			
	N	%	N	%	N	%	X^2	P-value
Negative	84	84.00	9	9.00	93	93.00	8.580	0.003*
Positive	3	3.00	4	4.00	7	7.00		
Total	87	87.00	13	13.00	100	100.00		

Table (3): Detection of K-Ras mutation at codon 12 in HCC patients with HCV infection.

HCV	K Ras point mutation at codon12						Chi-Square	
	Negative		Positive		Total			
	N	%	N	%	N	%	X^2	P-value
Negative	70	70.00	8	8.00	78	78.00	2.109	0.146
Positive	17	17.00	5	5.00	22	22.00		
Total	87	87.00	13	13.00	100	100.00		

Table (4): Detection of K-Ras mutation at codon 12 in HCC patients with schistsoma infection.

Schist some	K-Ras Point mutation at codon 12						Chi-Square	
	Negative		Positive		Total			
	N	%	N	%	N	%	X^2	P-value
Negative	79	79.00	10	10.00	89	89.00	1.834	0.176
Positive	8	8.00	3	3.00	11	11.00		
Total	87	87.00	13	13.00	100	100.00		

Discussion.

In general, a single gene mutation rarely occurs in a specific tumor at a high incidence rate. The results from the present study indicate that 13% of HCC patients have KRM at codon 12, many investigators also have reported a high incidence rate of KRM in HCC ranging from 82% to 91% were in K-Ras codon 13(**Qingsu *et al.*, 1988**). (**Diego *et al.*, 2011) Diego F.** show activation of Ras in Hcc patients they demonstrated that No further up regulation of Ki-RAS and N-RAS in HCC, whereas an additional increase in Pan- and Ha-RAS levels occurred in HCC. In this study, Egyptian HCC patients show point mutation at K-Ras codon 12 at 4% from HCC patients combine HBV with a height significant value (p=0.003), also 5% HCC patients combine HCV and 3% HCC patients with schist soma .

The point mutation detected at first nucleotide of codon 12 at K-Ras gene that transverse guanine to cystein GGT→ CGT by substituting amino acid glycine by arginine that detect in HCC patients with viral infection or schist soma by percent 32% and guanine to thymine GGT → TGT by substituting amino acid glycine by cystein occour at HCC patients only by percent 20% and 12% in HCC patients with viral infection or shist soma ,no detection of mutation at adenine first nucleotide of codon 12 at K-Ras gene (AGT). The substitution of original amino acid into abnormal form play a big role in distribute cell growth and proliferation. It induce growth without control, Mutations activate proto-oncogenes through structural alterations in their encoded proteins. These alterations, which usually involve critical protein regulatory regions, often lead to the uncontrolled, continuous activity of the mutated protein. the abnormal protein make cell out of G_0 stage so liver cell easily convert into tumor cell. Also, hepatitis B virus and hepatitis C virus populations are at high risk for the development of hepatitis, cirrhosis, and finally HCC that result from its replication into normal cell. The virus itself is not cytopathic; hepatocyte destruction results from immune-mediated responses to viral antigens, leading to hepatic necrosis and inflammation. Or by integration of virus genome into host genome that result in activation of oncogene like Ras gene. Mutations have been described in sporadic hyper plastic colon polyps as well as those associated with chronic ulcerative colitis, a well known risk factor for colon cancer. K-ras mutations were described in 39% of patients with chronic pancreatitis, and these patients were more likely to subsequently develop pancreatic cancer. Activating mutations have also been identified in pre-cancerous actinic keratoses. Fifth, as described above, the high prevalence of K-ras mutations within a wide spectrum of cancer cells suggests a central role in the development of the malignant phenotype**. (Krane nburg *et al.*, 2004**).

The worldwide incidence of HCC is much higher in male compared with female individuals (**El serag and Rudolph, 2007**). In this study, male: female ratio was found to be 37:13. The observed gender difference may result from sex-specific differences in exposure to risk factors. Men are more likely to be infected with HBV and HCV, consume alcohol, smoke cigarettes, and have increased iron stores (**El serag and Rudolph, 2007).** Non-environmental endogenous factors that may affect male risk adversely

include higher body mass index and higher levels of androgenic hormones **(El serag and Rudolph, 2007).** In an earilier study carried out by (13) in rectal cancer The hot mutation areas of K-ras gene (in codon 5/12 and 13) were detected with polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) mutation was observed in codon 12 and 13 in 61 cases with a mutation rate of 62.9% (61/97).they proved that Malignant biological behaviors of rectal cancer are not enhanced by p53 and K-ras gene mutations. Co-mutation of p53 and K-ras gene has neither synergic carcinogenesis-promoting effect, nor prognostic effect on rectal cancer. In conclusion, MASA showed a relatively high incidence of KRM even in HCC patients. Therefore, the detection of KRM in by MASA is useful for the screening of HCC. Also, HBV is considered one of the etiological factor in Egyptian HCC according to a highly significant value occur between them.

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