

Research Article

Benefits of Testing for the G12D Mutation of the KRAS Gene in Hepatocellular Carcinoma (HCC) in Senegal

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Abstract

Background: The mutation of the KRAS proto-oncogene is frequently detected in various cancers, including hepatocellular carcinoma (HCC). This mutation often results in the failure of treatments based on tyrosine kinase receptor inhibitors. The study's objective was to investigate mutations of codon 12 and 13 of the KRAS gene and assess the risk of developing Hepatocellular Carcinoma. **Methods:** A prospective, cross-sectional, and analytical study was conducted from November 2018 to October 2021 at the biochemistry and molecular biology laboratory of Cheikh Anta Diop University's Faculty of Medicine. Mutations of codons 12 and 13 were identified using High Resolution Melting and confirmed by sequencing. Hepatic assessments (transaminases, GGT, PAL, bilirubin, and AFP) were performed on all patients, and histopathological analysis was conducted. **Results:** The study consisted of 34 patients with an average age of 44 ± 3.4 years and a sex ratio of 5.8. There was a predominance in the age group of 36 to 45 years (38.2%). The prevalence of the G12D mutation of the KRAS gene was 23.5% and was more common in subjects over 45 years ($n=5$; 11.7%). Hepatic impairments were less pronounced in carriers of the G12D mutation of the KRAS gene, albeit not significantly different. Additionally, tumors of grade 3 and type pT3 appeared more linked to the G12D mutation of the KRAS gene, with prevalences of 18.75% ($n=3$) and 30.8% ($n=4$), respectively. The relationship between chronic Hepatitis B Virus carriage and the KRAS gene mutation was not significant. **Conclusion:** Molecular biology significantly contributes to diagnosis and patient management by identifying molecular markers for pre-therapeutic evaluation.

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Keywords

Codon 12 and 13 KRAS, HBV, Hepatocellular Carcinoma, Senegal

1. Introduction

Primary liver cancer ranks as the sixth most commonly diagnosed cancer and the third leading cause of cancer-related deaths worldwide in 2020. Incidence and mortality rates are 2 to 3 times higher in men compared to women across most regions. In West Africa, the incidence stands at 11.4% for men and 5.6% for women [1]. The main risk factors of Hepatocellular Carcinoma are hepatitis B (HBV) and hepatitis C viruses (HCV), non-alcoholic steatohepatitis (NASH), alcohol, aflatoxin, hemochromatosis, diabetes, male sex and smoking. Its management depends on the stage at diagnosis, most often assessed by the classification of the Barcelona Clinic Liver Cancer (BCLC) [2]. Numerous molecular alterations have been described in the pathogenesis of this cancer. These range from disturbances in the cell cycle with p53 mutations to genes responsible for cell proliferation such as KRAS, BRAF, and others [3, 4]. The KRAS 4B isoform is predominantly implicated in KRAS gene mutations, with the majority of these mutations occurring at codons 12, 13, and 61. KRAS gene mutations are found in 90% of pancreatic cancers, 30 to 40% of colon cancers, and 15 to 20% of lung cancers. They are also present, albeit at a lower rate, in liver cancers and in various other cancer types [4]. The presence of these mutations implies a resistance to treatments involving tyrosine kinase receptor inhibitors [5]. Indeed, the presence of these mutations in tumors leads to the activation of RAS and MAPK proteins downstream of EGFR, causing the constitutive activation of signaling pathways dependent on the EGFR receptor [6]. The aim of our study was to investigate mutations in codons 12 and 13 of the KRAS gene and the risk of developing HCC. Additionally, we sought to elucidate the relationship between these mutations and the clinico-biological profile of the patients.

2. Materials and Methods

This is a prospective, cross-sectional, and analytical study conducted from November 2018 to October 2021 in the biochemistry and molecular biology laboratory at the Cheikh Anta Diop University's Faculty of Medicine in Dakar. The study was carried out in collaboration with the general surgery and pathology departments of the Idrissa Pouye Hospital in Grand Yoff and the International Center for Research, Training in Applied Genomics, and Health Surveillance (CIGASS) of UCAD.

The study population consisted of patients diagnosed with HCC based on clinical, biological, radiological, and/or histological criteria according to international recommendations. All patients underwent surgical procedures, yielding both tumoral tissue and healthy liver tissue specimens. Patients diagnosed with other types of liver cancer, as well as those who were ineligible for surgery, were not included in the study.

The parameters studied were: age, gender, clinical data (hepatic pain, hepatomegaly, jaundice, general state of health, weight loss), biological markers (alpha-fetoprotein, Aspartate aminotransferase, Alanine aminotransferase, Alkaline phosphate, gamma glutamyl transferase, total bilirubin, direct bilirubin), and chronic HBV carrier status. Clinical data were unavailable for some patients. Patients with missing data were not included with analyses requiring the corresponding variable.

The samples consisted of tumoral tissues and healthy tissues from the surgical specimens. They were stored at -80°C. Genomic DNA (gDNA) extraction from the tissues was performed using the Promega Relia Prep gDNA Tissue kit.

The search for mutations at codon 12 of exon 2 of the KRAS gene was conducted by HRM (High Resolution Melting) using the Light Cycler 480 II (Roche) from the gDNA, following the protocol described in our previous work [7].

KRAS-F (92pb): 5' - TA TAA GGC CTG CTG AAA ATG ACT GAA - 3'

KRAS-R (92pb): 5' - GA ATT AGC TGT ATC GTC AAG GCA CT - 3'

Figure 1. Primer sequences used for HRM analysis of codon 12 of exon 2 of the KRAS gene.

Nucleotide sequencing was performed using the ABI 3500XL Genetic Analyzer (Applied Biosystems, Foster City, USA). The PCR products were obtained following the amplification protocol detailed in the table.

Table 1. PCR amplification protocol for exon 2 of the KRAS gene.

KRAS	Exon 2	E2F: GTGTGACATGTTCTAATATAGTCA E2R: GAATGGTCCTGCACCAGTAA	950C/10 mn; 40 cycles (940C/30s – 600C/30s – 720C/30s); 720C/10 mn
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Amplifications were conducted in a reaction volume of 20 µl comprising 10 µl of One Taq 2X MasterMix (New England Biolabs, UK), 1 µl of DNA, 1 µl of each primer (detailed in Table V), and the balance made up with PCR water.

PCR products were verified through electrophoretic migration on a 1% agarose gel, using 3 µl of the amplicons. The amplified marker size was estimated using a Lamda DNA size marker (Promega® Madison, USA). Samples with successful primer binding were subjected to sequencing. Sequence corrections were made using the FincTV analysis software.

Sequencing reactions were conducted in an MJ Research PTC-225 Peltier thermocycler using ABIPRISM BigDye TM Terminator Cycle kits. PCR products were purified utilizing the ExoSAP method according to the following protocol: firstly, an Exo/SAP master mix preparation step in a 0.6 ml microtube (50 µl of Exonuclease I (20 UI/µl) and 200 µl of Shrimp Alkaline Phosphatase 1UI/µl). We then prepared the reaction mixture with 10 µl of the PCR product and 2.5 µl of the Exo/SAP solution. This reaction mixture was incubated at 37°C for 15 minutes, followed by an inactivation step at 80°C for 15 minutes.

Each sample was sequenced using both forward and reverse primers with the Nimagen BrilliantDyeTM Terminator Cycle Sequencing kit V3.1. Fluorescent fragments were purified using the ZR-96 DNA Sequencing Clean-up Kit protocol. The samples were resuspended in distilled water and underwent electrophoresis in the ABI 3500XL Genetic Analyzer (Applied Biosystems, Foster City, USA).

Ethical Considerations

This study received approval from the research committee of UCAD, and all participants provided their consent.

Data Analysis

Data management and graph production were executed with Microsoft Excel software 2013, while data analysis was performed using SPSS v.25 software. The threshold for significance was set at a p-value < 0.05.

3. Results

Our study gathered data from 34 patients diagnosed with hepatocellular carcinoma. The average age of the participants was 44±3.4 years, with the most common age range being 36-45 years (38.2%). The study population was predominantly male, constituting 85%, with a sex-ratio of 5.8.

Mutation analysis of codons 12 and 13 of KRAS using the HRM technique revealed the mutation of codon 12 in 23.5% of the patients, predominantly among males (20.6%).

This mutation was further confirmed by sequencing to be the G12D mutation. This mutation was only identified in tumorous tissues.

Clinical data collected from 24 patients indicated a high prevalence of HBV in 75% of patients. Out of these, 38.8% carried the mutation, resulting in an almost twofold increased risk.(Table 2).

Table 2. Distribution of the G12D mutation of the KRAS gene according to age, gender, and chronic HBV carriage.

	KRAS Wild-type (%)	G12D Mutation (%)	P-value	RR (95% CI)
Sex				
Female	80	20	1	0.89 (0.1 - 5.4)
Male	75.9	24.1		
Age (years)				
< 35	62.5	37.5	0.310	—
36 – 45	92.3	7.7		
46 – 60	62.5	37.5		
≥ 60	80	20		
HBV Positive				
Yes	14 (77.8%)	4 (22.2%)	1	1.78 (0.2 -13.5)
No	7 (87.5%)	1 (12.5%)		

Clinical symptoms were primarily characterized by abdominal pain in 54.2% of patients, hepatomegaly in 45.8%, and weight loss in 20.8%. A general health deterioration was observed in 16.7% of patients, and jaundice in 4% of patients. (Table 3).

Table 3. Distribution of clinical data.

Clinical Signs	Presence	Number	Percentage
Abdominal Pain	Yes	13	54.2%
	No	11	45.8%
Jaundice	Yes	1	4.2%
	No	23	95.8%
General health deterioration	Yes	4	16.7%
	No	20	83.3%
Weight Loss	Yes	5	20.8%
	No	19	79.2%
Hepatomegaly	Yes	11	45.8%
	No	13	54.2%

The average values of biological parameters were all elevated, with AFP levels exceeding 10,000 times the norm. Moreover, age and other biological parameters, except for PAL (366.5 vs. 345), were slightly lower in mutation carriers. (Table 4).

Table 4. Average values of parameters based on the KRAS gene mutation.

	KRAS Wild-type	KRAS Mutated	P-value
Age (years)	44.15	42.5	0.79
AST (UI/l)	164.0272	138.5714	0.817
ALT (UI/l)	196.017	130.429	0.624
AFP (ng/ml)	203149.5173	8919.912	0.189
BT (mg/dl)	17.3089	7.48	0.061
BD (mg/dl)	9.0976	4.9571	0.232
PAL (UI/l)	345.144	366.5	0.892
GGT (UI/l)	185.261	150	0.591

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: Alpha fetoprotein; BT: Total bilirubin; BD: Direct bilirubin; PAL: Alkaline phosphatase; GGT: gamma glutamyl transferase.

The G12D mutation of the KRAS gene was predominant among patients with pT3 stage (n=4, 30.8%) and grade 3 (n=3, 18.75%). Furthermore, it was predominant among patients

without metastasis (n=4, 30.8%). However, no significant statistical link was found. (Table 5).

Table 5. Distribution of KRAS mutation based on tumor characteristics.

		KRAS Wild-type	KRAS Mutated	P-value
Location	Right	13 (50%)	3 (11.53%)	1
	Left	8 (30.8%)	2 (7.7%)	
Size (cm)	≥ 10	10 (47.6%)	2 (9.52%)	1
	< 10	7 (33.3%)	2 (9.52%)	
Grade	G1	0	1 (5.88%)	0,253
	G2	1 (5.88%)	1 (5.88%)	
	G3	8 (47.05%)	3 (18.75%)	
	G4	3 (17.6%)	0	
TNM	pT2	2 (15.38%)	1 (7.69%)	0,672
	pT3	5 (38.46%)	4 (30.8%)	
	Pt4	1 (7.69%)	0	
Metastasis	Yes	6 (46.15%)	1 (7.7%)	0,103
	No	2 (15.38%)	4 (30.8%)	

4. Discussion

In sub-Saharan Africa, HCC increasingly affects the younger population, as evidenced by our study where the average age was 44 ± 3.4 years. These results mirror studies in Senegal where the average ages were 47.4 years for I. Diallo and 33 years for S. Diallo [2, 8]

Our study revealed a marked male dominance in HCC with a sex-ratio of 5.8. This male predominance was also reported in previous studies, which observed sex ratio of 6.6 and 2.2, respectively [2, 8]. A sex ratio of 2.03 indicating a higher prevalence among men is reported in West Africa [1]. Hormonal factors in women, such as estrogen, might provide protective effects against HCC [9]

We identified a mutation at codon 12 of exon 2 in 23.5% of the cases, a rather high rate compared to a study where mutations were present in 7.6% of patients [10].

In the series by Chaitanya et al., KRAS mutations are found in 23.6% of intrahepatic cholangiocarcinoma and 40% in extrahepatic cholangiocarcinoma [11]. In another study KRAS is mutated in 5% of the patients [12].

Experimental data suggest that uncontrolled activation of the RAS RAF MAP kinase pathway plays a role in resistance to sorafenib [5] implying that the G12D mutation of KRAS can induce resistance. The most effective means of treating HCC remains surgery, but less than 18% of patients undergo this procedure [13] mainly due to late diagnoses in our regions

[14]. For those with stage B or C HCC, as classified by the Barcelona Clinic Liver Cancer (BCLC) and ineligible for surgery [15], transarterial chemoembolization (TACE) or sorafenib is recommended [16].

In most high-risk HCC countries (China, Republic of Korea, and sub-Saharan Africa), the primary determinants are chronic HBV infection, exposure to aflatoxin, or both. In contrast, in countries like Japan, Italy, and Egypt, HCV infection is the likely primary cause [1].

Chronic HBV was found in 75% of our patients, a rate similar to that found in a previous study [2]. In our study, the link between chronic HBV carriage and KRAS mutation was not significant. However, the risk of having a KRAS mutation was 1.78 times higher in these patients.

Our study found that the majority of cases presented with abdominal pain (54.2%), weight loss (20.8%), and hepatomegaly (45.8%). Ascites, splenomegaly, and jaundice are sometimes observed. These clinical signs are consistent with other HCC studies [9, 17-19].

Increased transaminases, bilirubin, and alpha-fetoprotein were common in our study [9,17-19]. The majority had a tumor size greater than 10 cm (57.1%), with 3 stages pT2, pT3, and pT4, accounting for 23%, 69%, and 8%, respectively. In countries with high human development indexes, tumors can be discovered at very early stages, as evidenced by the French study which reported an average tumor size of 2.4 cm [20]. Similarly, a Japanese study found that 47.9 % of patients had tumors measuring between 2 and 5 cm ,and 9.9 % had tumors less than 2 cm [21].

5. Conclusion

The G12D mutation of the KRAS gene appears to be prevalent among HCC patients in Senegal. This mutation may contribute to therapeutic failure in adjuvant chemotherapy and should be considered in the pre-therapeutic assessment.

Abbreviations

HCC	Hepatocellular Carcinoma
CIGASS	International Center for Research, Training in Applied Genomics, and Health Surveillance
HBV	Hepatitis B virus
HCV	Hepatitis C virus
NASH	Non-Alcoholic Steatohepatitis
BCLC	Classification of the Barcelona Clinic Liver Cancer
AST	Aspartate Aminotransferase
ALT	Alanine Aminotransferase
AFP	Alpha Fetoprotein
BT	Total Bilirubin
BD	Direct Bilirubin
PAL	Alkaline Phosphate
GGT	Gamma Glutamyl Transferase
TNM	Tumor, Node, Metastasis

Author Contributions

Fatou Kine Sy Thorpe Dia: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing-original draft, Writing review & editing

Souleymane Thiam: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Project Administration, Resources, Supervision, Validation, Writing-original Draft, Writing -review & editing

Conflicts of Interest

The authors declare no conflicts of interest

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