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KRAS and BRAF Mutations in Patients with Hepatocellular Carcinoma in Senegal

Thiam Souleymane[1, 2, *], Dia Fatou Kine Sy Thorpe[1], Sambiani Damigou Mawuli[8], Diop Papa Saloum[4], Ka Ibrahima[4], Deguenonvo Gabriel Nougnignon Comlan[5], Ndiaye Yaye Dié[6], Cisse Fatou[1, 2], Ndiaye Arame[1, 3], Samba Abdourahmane[1, 3], Coly Najah Fatou[7], Soumah Idrissa Yaya[1], Diedhiou Fatou[1], Agossou Hortence Honorine Médécé[1], Gaye Amy[6], Ndiaye Tolla[6], Dial Cherif Mouhame[5], Ndiaye Daouda[6], Diallo Fatou[1, 3]

[1]Laboratory of Biochemistry and Molecular Biology, Faculty of Medicine, Cheikh Anta Diop University, Dakar, Senegal

[2]Laboratory of Biochemistry, Dalal Jamm Hospital, Dakar, Senegal

[3]Laboratory of Biochemistry, Aristide Le Dantec Hospital, Dakar, Senegal

[4]Surgical Department, General Hospital of Grand Yoff, Dakar, Senegal

[5]Cytology and Pathology Department, General Hospital of Grand Yoff, Dakar, Senegal

[6]International Centre for Research, Training in Applied Genomics and Health Surveillance, Cheikh Anta Diop University of Dakar, Dakar, Senegal

[7]Pharmaceutical Biochemestry/Department of Medical Biology and Functional Exploration, Faculty of Health, Thies University, Thies, Senegal

[8]Surgical and Oncology Department, Sylvanus Olympio Hospital, Lome, Togo

Email address:

sthiam85@gmail.com (T. Souleymane)

[*]Corresponding author

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Abstract: Hepatocellular carcinoma (HCC) is a public health problem in developing countries where chronic HBV is endemic. The objective of our study was to determine the prevalence of KRAS and BRAF mutations in patients with HCC. Mutations in codons 12 and 13 of KRAS and the V600E mutation of the BRAF gene were searched by HRM on Light Cycler 480 and confirmed by direct sequencing. A total of 34 HCC patients underwent molecular testing for codon 12 and 13 mutations in the KRAS gene and the V600E mutation in the BRAF gene. Melting curve analysis showed a prevalence of 23.5% (n=8/34) for the KRAS gene and 41.2% (n=14/34) for the BRAF gene. The mean age of BRAF mutation carriers was lower compared with KRAS mutation carriers. Chronic HBV carriage appeared to play a role in the development of these mutations, increasing the risk by 2 (CI(95)=0.55-7.24; p=0.395) for BRAF and by 1.78 (CI(95)=0.23-13.5; p=1) for KRAS. KRAS and BRAF mutations do not appear to play a role in tumor metastasis. However, these results need to be confirmed by further studies with a larger sample size. Alterations in the RAS/RAF/MAP Kinase pathway appear to be more prominent in HBV-induced HCC. This may hinder management with receptor tyrosine kinase inhibitors, the basis for treatment of advanced HCC.

Keywords: KRAS, V600E BRAF, HBV, Hepatocellular Carcinoma, Senegal

1. Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer and the fourth leading cause of cancer-related death worldwide [1]. It develops mainly on the bed of chronic liver diseases [2]. The knowledge of somatic variants in HCC is continuously increasing with new diagnostic tools such as NGS [3].

Several signaling pathways are altered with a particularity according to populations and etiologies related to HCC. In countries with high exposure to aflatoxin and chronic HBV carriage, the most common alterations are mutations in the tumor suppressor gene, TP53 [4-6]. But with the heterogeneity noted in the development of HCC, other pathways may be involved as well. Studies in Asian, Italian, and American populations have shown alterations in other genes of the RAS/RAF/MAP kinase and PI3K/AKT/mTOR [7-10]. The MAPK and AKT/mTOR pathways are activated in more than 50% of HCC cases and are associated with aggressive tumors of the proliferative class [11].

The RAS/RAF/MAP kinase pathway controls, among other essential functions of the cell and the organism, cell cycle regulation, proliferation, and resistance to apoptosis [12, 13]. The constitutive activation of the RAS/RAF/MAP kinase pathway is dependent on several gene and epigenetic alterations.

In Senegal, no study on this signaling pathway has been done in patients with hepatocellular carcinoma. Such a study on the RAS/RAF/MAP kinase signaling pathway conducted in Senegal would be a first to establish the prevalence of KRAS and BRAF gene mutations in Senegalese patients with hepatocellular carcinoma and to determine their clinic-biological and histological profiles.

2. Materials and Methods

2.1. Setting and Type of Study

This was a descriptive and analytical study conducted between the Biochemistry and Molecular Biology Laboratory of the Faculty of Medicine in collaboration with the International Center for Applied Genomics Research and Health Surveillance at Cheikh Anta Diop University in Dakar and at the Departments the General Surgery and Anatomopathology of the Idrissa Pouye General Hospital (HOGIP).

2.2. Study Population

Patients were recruited at the Departments of Pathology and Surgery at Idrissa Pouye General Hospital (HOGIP). These patients were diagnosed with HCC based on clinical, biological, imaging and/or histological data. They had not received any chemotherapy or radiotherapy before hepatectomy.

Clinical and Paraclinical Data

Data were collected from the patients' files. They included age, sex, history, biological data (transaminases, GGT, PAL, total and direct bilirubin, AFP, HBV serology), imaging (Echo or CT). Chronic HBV carriage was considered when HBsAg was positive or when anti-HBc antibodies were present for more than 6 months.

2.3. Biological Specimens

We worked on kerosene blocks and fresh tissue stored at 8-0°C until DNA extraction.

2.4. DNA Extraction

The DNA extraction procedure has already been described in our previous work with the Promega gDNA [6] kit according to the recommendations of the manufacturer.

2.5. Analysis of KRAS and BRAF Mutations

The HRM technique has allowed the search for KRAS and BRAF mutations with the Light Cycler 480 (Roche Diagnostic®). It allows the detection of KRAS exon 2 mutations including codons 12 and 13 and codon 600 of BRAF. The oligonucleotide primers used were KRAS (92pdb) sense: TATAAGGCCTGCTGAAAATGACTGA and GAATTAGCTGTATCGTCAAGGCACT and for BRAF (147db). sense: GGTGATTTTGGTCTAGCTACAG and antisense: AGTAACTCAGCAGCATCTCAGG).

The protocol consisted of an initial denaturation at 95°C for 5 min followed by 50 PCR cycles of 15 sec at 95°C, 15 sec at 68°C and 20 sec at 72°C. For the melting curve, samples were denatured with an initial hold of 1 min at 95°C and 1 min at 40°C and a melting profile from 65°C to 95°C with a ramp rate of 0.02°C/sec. The analysis was performed in duplicate. Melt curve analysis was used to determine the mutated and non-mutated profile. Mutations were confirmed by Sanger sequencing with the ABI 3500XL Genetic Analyzers (Applied Biosystems, Foster City, USA).

2.6. Ethical Consideration

The study was approved by the ethics and research committee of Cheikh Anta Diop University in Dakar. All participants signed a consent form.

2.7. Statistical Analysis

Data were analyzed using SPSS v. 25 software (SPSS Inc, Chicago, IL.). File test and t-test were used to compare qualitative and quantitative data. A value of $p<0.05$ was retained as a statistically significant difference.

3. Result

Thirty-four patients were tested for KRAS and BRAF mutations. The mean age was 44±13.4 with a predominance of the 36-45 age group (38.2%). There was a male predominance (85%) with a M/F sex ratio of 5, 8 with 24 cases found with 4 histopathological reports that were not

available. The prevalence of chronic HBV carriage was 69.2% (n=18/26).

HRM melting curve analysis revealed a prevalence of 23.5% (n=8) for the KRAS gene and 41.2% (n=14) for the BRAF gene. Three patients (8.8%) had a concomitant KRAS and BRAF mutation.

The study of the characteristics of patients carrying the mutations showed a male predominance with 30% (KRAS) and 35% (BRAF). All KRAS/BRAF concomitant mutations were found only in men (n =3; 15%). KRAS mutations predominated in the 46-60 age group (n=3; 15%), whereas BRAF and KRAS/BRAF mutations predominated in the 36-45 age group (n=3; 15%) and in subjects younger than 35 years of age (n=2, 10%), respectively. The relative risk of carrying the mutations in chronic HBV carriers was 1.78 (CI(95)=0.23-13.5; p=1) and 2 (CI(95)=0.55-7.24; p=0.395) (Table 1).

Table 1. *Distribution of KRAS and BRAF mutations according to age, sex, and chronic HBV carriage.*

	KRAS		p	BRAF		p	KRAS/BRAF mutation		p
	Wild-type	Mutation		Wild-type	Mutation		No	Yes	
Gender									
Female	2 (10)	1 (5)	1	3 (15)	0	0,521	3 (15)	0	1
Male	11 (55)	6 (30)		10 (50)	7 (35)		14 (70)	3 (15)	
Age (years)									
< 35	1 (5)	2 (10)	0,31	1 (5)	2 (10)	0,462	1 (5)	2 (10)	0,041
36 - 45	6 (30)	1 (5)		4 (20)	3 (15)		7 (35)	0	
46 - 60	3 (15)	3 (15)		5 (25)	1 (5)		5 (25)	1 (5)	
≥ 60	3 (15)	1 (5)		3 (15)	1 (5)		4 (20)	0	
HBV positive									
Yes	14 (53,8)	4 (15,4)	1	9 (34,6)	9 (34,6)	0,395	17 (65,4)	1 (3,8)	0,529
No	7 (26,9)	1 (3,8)		6 (23,1)	2 (7,7)		7 (26,9)	1 (3,8)	

HBV: Hepatitis B Virus.

Clinical manifestations were predominantly hepatalgia (n=13; 54.2%) followed by hepatomegaly (n=11; 20.8%). Table 2 shows that patients with the mutations had a good general condition with occasional hepatalgia and hepatomegaly, especially for the BRAF mutation carriers.

Table 2. *Distribution of KRAS and BRAF mutations according to clinical signs.*

	KRAS		p	BRAF		p	KRAS/BRAF mutation		p
	Wild-type	Mutation		Wild-type	Mutation		No	Yes	
Hepatica									
Yes	9 (37,5)	4 (16,7)	0,327	6 (25)	7 (29,2)	0,24	11 (45,8)	2 (8,3)	0,482
No	10 (41,7)	1 (4,2)		8 (33,3)	3 (12,5)		11 (45,8)	0	
Icterus									
Yes	0	1 (4,2)	0,208	0	1 (4,2)	0,417	0	1 (4,2)	0,083
No	19 (79,2)	4 (16,7)		14 (58,3)	9 (37,5)		22 (91,7)	1 (4,2)	
DGC									
Yes	3 (12,5)	1 (4,2)	1	2 (8,3)	2 (8,3)	1	4 (16,7)	0	1
No	16 (66,7)	4 (16,7)		12 (50)	8 (33,3)		18 (75)	2 (8,3)	
Weight loss									
Yes	5 (20,8)	0	0,544	2 (8,3)	3 (12,5)	0,615	5 (20,8)	0	1
No	14 (58,3)	5 (20,8)		12 (50)	7 (29,2)		17 (70,8)	2 (8,3)	
Hepatomegaly									
Yes	9 (37,5)	2 (8,3)	1	4 (16,7)	7 (29,2)	0,095	9 (37,5)	2 (8,3)	0,199
No	10 (41,7)	3 (12,5)		10 (41,7)	3 (12,5)		13 (54,2)	0	

DGC: Detrioration of general condition.

Comparison of medians showed no statistically significant difference except for patients with a concomitant KRAS/BRAF mutation with bilirubin variations (p=0.01). Furthermore, patients with the BRAF mutation and both mutations have a relatively young age. For patients with the KRAS mutation or both mutations, the mean values of the biological parameters are somewhat lower compared to patients with the BRAF mutation except for LAPs (Table 3).

Table 3. *Distribution of KRAS and BRAF mutations according to clinical signs.*

	KRAS		p	BRAF		p	KRAS-BRAF		p
	Wild-type	Mutation		Wild-type	Mutation		No	Yes	
Age (years)	44±13	43±15,5	0,79	46±14,1	41±12,2	0,298	45±13,4	34±11,1	0,240
AST (IU/l)	164±179,8	139±258	0,817	97±132,6	246±252,4	0,112	164±206,3	76±31,1	0,090
ALT (IU/l)	196±292,2	130±291,8	0,624	86±204,4	315±347,3	0,083	190±297	37±6,4	0,022
AFP (ng/ml)	203149±544033	8920±14385,4	0,189	234399±580833,2	6380±12459,7	0,182	16266±486549,3	1222	-
BT (mg/dl)	17±20,3	7±3,3	0,061	12,4±6,9	17,7±27,4	0,567	15,4±18,3	4,6±0,6	0,010

	KRAS		p	BRAF		p	KRAS-BRAF		p
	Wild-type	Mutation		Wild-type	Mutation		No	Yes	
BD (mg/dl)	9,1±12,8	4,96±3,4	0,232	6,7±4,3	9,5±16,6	0,617	8,5±11,3	1,6±0,6	0,010
PAL (IU/l)	345±449,1	367±209,4	0,892	494±549,4	231±200,8	0,205	358±427,4	271±196,6	0,657
GGT (IU/l)	185±204,8	150,00±104,7	0,591	202±226,9	139±94,7	0,361	178±187,3	135	-

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

Table 4 shows us that there is no statistically significant difference between mutations and tumor characteristics. But we can see that KRAS and BRAF mutations do not seem to have an impact on tumor size. In relation to histology, mutations seem to predominate in grade 3 and 4 tumors, pT3 class with lymph node localization. We also note that metastasis does not appear to be related to KRAS and BRAF mutations.

Table 4. *Distribution of KRAS and BRAF mutations according to tumor characteristics.*

	KRAS		p	BRAF		p	KRAS/BRAF mutation		p
	Wild-type	Mutation		Wild-type	Mutation		No	Yes	
Lacalisation									
Right	13 (50)	3 (11,5)	1	9 (34,6)	7 (26,9)	1	15 (57,7)	1 (3,8)	1
Left	8 (30,8)	2 (7,7)		6 (23,1)	4 (15,4)		9 (34,6)	1 (3,8)	
Size (cm)									
≥ 10	12 (52,2)	2 (8,7)	1	9 (39,1)	5 (21,7)	1	13 (56,5)	1 (4,3)	1
< 10	7 (30,4)	2 (8,7)		5 (21,7)	4 (17,4)		9 (39,1)	0	
Grade									
G1	0	1 (5)	0,562	1 (5)	0	0,426	1 (5)	0	0,79
G2	2 (10)	1 (5)		3 (15)	0		3 (15)	0	
G3	8 (40)	4 (20)		7 (35)	5 (25)		10 (50)	2 (10)	
G4	3 (15)	1 (5)		2 (10)	2 (10)		3 (15)	1 (5)	
pT									
pT1	0	1 (5)	0,466	1 (5)	0	0,516	1 (5)	0	0,929
pT2	3 (15)	2 (10)		4 (20)	1 (5)		4 (20)	1 (5)	
pT3	9 (45)	4 (20)		7 (35)	6 (30)		11 (55)	2 (10)	
pT4	1 (5)	0		1 (5)	0		1 (5)	0	
Nodes									
Zero	1 (5)	2 (10)	0,27	2 (10)	1 (5)	1	2 (10)	1 (5)	0,404
Multiple	12 (60)	5 (25)		11 (55)	6 (30)		15 (75)	2 (10)	
Metastasis									
Yes	6 (30)	1 (5)	0,329	5 (25)	2 (10)	1	7 (35)	0	0,521
No	7 (35)	6 (30)		8 (40)	5 (25)		10 (50)	3 (15)	

4. Discussion

Alterations in the RAS/RAF MAP kinase pathway are increasingly being studied in hepatocellular carcinoma with their likely implications in the personalized management of patients. In Senegal, hepatocellular carcinoma is one of the most frequent cancers with a very high morality [14]. In our context, it occurs most often in chronic HBV carriers with or without aflatoxin intoxication [4, 5]. The most described molecular alteration in this cancer is a mutation of the TP53 gene with especially the Ser249 mutation [4-6]. But in view of the molecular heterogeneity of HCC, other pathways are disrupted [15, 16]. It is in this perspective that we initiated this study to determine the prevalence of KRAS and BRAF gene mutations in HCC in Senegal.

The KRAS and BRAF genes are mutated in 8 patients and 14 patients respectively, which gives us a prevalence of 23.5% and 41.52%. Three patients out of 34 presented a concomitant mutation of KRAS and BRAF genes. Several authors have reported lower prevalence [10, 16, 17].

In their study on 65 Italian patients, Colombino et al [10] reported a single KRAS mutation of (2%) and 15 BRAF mutations (23%). In a Chinese study, Hou et al [18] reported a KRAS codon 61 mutation in 2 patients (5.6%). Mutation of codon 12 of the KRAS gene is the most common in HCC with a prevalence of 15% (n=3/20) reported by Weihrauch et al in Germany [17]. BRAF mutations in HCC seem to be less frequently reported. Zuo et al [16] reported a prevalence of 0% in 64 patients while Wheeler et al reported a prevalence of 0.3%. [1].

Concurrent KRAS and BRAF mutations are rarely reported in the literature [19, 20]. However, our team has already reported similar cases in patients with colorectal cancer (15%) [21]. These results further demonstrate that the molecular mechanisms involved in cancers vary between populations and that it is important that population-specific studies are further encouraged.

The implication of these mutations in our population is mainly the limitation of the prescription of receptor tyrosine kinase inhibitors such as sorafenib, which remains the treatment of choice in advanced stages of HCC [22, 23]. For a long time, authors have reported a response to sorafenib that is very variable. This variability could be explained by the

heterogeneity of HCC but especially by mutations of the pathways using tyrosine kinases such as the RAS/RAP/MAP Kinase pathway and its main effector the ERK protein [24-27].

Nevertheless, our results open prospects for personalized treatment with combination therapies as reported in several recent studies [28, 29]. But for this, we need a molecular characterization of our cancers by identifying the altered pathways to better adapt our treatments. Our study also reports a relatively young average age of HCC patients in Senegal (44±13.4 years), especially in patients with BRAF mutations (41±12.2 years) and concomitant KRAS/BRAF mutations (34±11.1 years). It seems likely that KRAS and BRAF mutations appear very early in liver carcinogenesis, especially in chronic HBV patients, especially since the risk of having the BRAF or KRAS mutation was respectively multiplied by 2 (CI(95)=0.55-7.24; p=0.395) and by 1.78 (CI(95)=0.23-13.5; p=1) in these patients, as reported by Xiong et al [30].

However, the advanced state in most patients (metastasis; n=7/20) and large tumors larger than 10 cm (n=14/23), contrasts with a general state that was almost well preserved (n=4/24), especially in patients with KRAS (n=1/4) and BRAF (n=2/10) mutations. These results may call into question Western guidelines on the management of HCC in our context [23]. They should be revisited to establish our own recommendations for a better management of our patients.

Although no statistically significant relationship was found, it appears that KRAS mutation carriers compared with BRAF mutation carriers had less disturbed mean values for liver biological parameters. The location, size and histological characteristics did not show any difference between mutation carriers and non-mutation carriers. On the other hand, metastases are mostly found in patients without mutations as reported by Silva et al in a Brazilian study [31]. These results still prove the tumor heterogeneity encountered during HCC. But further studies are needed to establish this. We plan to further analyze other signaling pathways involving RAS protein in HBV-induced HCC in other studies.

5. Conclusion

Alterations in the RAS/RAF/MAP kinase pathway appear to be involved in HBV-induced HCC. They may be responsible for therapeutic failures with receptor tyrosine kinase inhibitors. A high prevalence of KRAS and BRAF mutations are found with a probable link with HBV.

These mutations seem to appear at a very young age in our patients.

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