MUTATIONAL ANALYSIS OF *BRAF* GENE IN EGYPTIAN HEP ATOCELLULAR CARCINOMA PATIENTS USING *NGS*

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Hepatocellular carcinoma HCC is the fourth highest cause of cancer-related deaths globally. Cirrhosis caused by persistent infection with the hepatitis B or C virus accounts for 80-90% of HCC cases. Many individuals with HCC are not candidates for potentially curative therapy such as surgical resection and transplantation due to their advanced stage of the disease Russo *et al*. (2022).

BRAF is a protein kinase that targets serine and threonine residues. Mutations in this gene have also been linked to a variety of malignancies, including colorectal cancer, non-Hodgkin lymphoma, thyroid carcinoma, malignant melanoma, non-small cell lung carcinoma, and lung adenocarcinomas. *BRAF* is one of the essential cancer-associated genes in this pathway Gnoni *et al*. (2019). Cancer patients with abnormally activated RAS/RAF signaling pathways tend to have a bad prognosis. An innovative approach to treating HCC involves focusing on the RAS/RAF pathway. The RAF kinase inhibitor sorafenib helps treat HCC, so *BRAF* mutations are now the go-to target for HCC treatment. The therapy of advanced HCC has found a promising target in *BRAF* mutations Pope *et al*. (2021). The objective of the present investigation was to examine the relationship between *BRAF* and the progression of HCC in Egyptian HCC patients through the utilization of NGS technology.

MATERIALS AND METHODS

a. Study design and participants

This research comprised 21 HCC patients (eighteen males along with three females, having average ages of 62 years) recruited prospectively from the inpatient and outpatient clinics of the oncology unit at Egypt's Liver National Institute-Menoufia University.

All HCC patients had a family history taking, a clinical examination, tumor staging, and an exhaustive list of laboratory testing (liver enzymes, coagulation profile, renal function profile, and CBC) and chest X-ray.

The study was performed from January to November 2020, under the permission of Menoufia University's Ethics Committee (National Liver Institute). The study did not include any other cancer patients.

b. Sample collection and cell-free DNA extraction:

After collecting peripheral blood samples (1-3 mL) in EDTA-containing tubes, genomic DNA was isolated from a whole blood sample, and the plasma was frozen at -80ºC for cell-free DNA extraction. The QIAamp® DSP Virus spin kit Version 1 (QIAGEN, Hilden, Germany) was used to extract circulating cell-free DNA from plasma, as recommended by the manufacturer.

c Next-generation sequencing

Following the manufacturer's instructions, cell-free DNA was extracted from plasma samples using the QIAamp® DSP Virus spin kit Version 1 (QIAGEN, Hilden, Germany). Using the Gene JET Genomic DNA Purification Kit (Thermoscientific, Cat#K0), genomic DNA was extracted. Ion AmpliSeq HiFi Master Mix (Ion AmpliSeqTM Li-brary kit 2.0, Thermo Fisher Scientific, Inc.) and the Ion AmpliSeqTM Cancer Hotspot Panel (version 2) were used to amplify 10 ng of DNA for library preparation. Following the directions provided by the manufacturer, the library was quantified using qPCR with the ion library TaqMan® Quantitation Kit (Thermo Fisher Scientific, Inc.). Life Technologies' Ion OneTouchTM2 system was updated and installed on the templates. Ten percent to thirty percent of the ISPs produced were template positive; this was checked using the Ionosphere quality control kit made by Thermo Fisher Scientific, Inc. The template ISPs were loaded onto Ion 316TM chips after enrichment and sequenced using the IonPGMTM Sequencing Hi-Q view kit v2 and PGMTM (Life Technologies) according to the manufacturer's instructions.

d. Bioinformatics data analysis

To examine both normal and tumor samples, the ion ampliseq custom panel approach was used using the default plugin settings in Thermo Fisher's Ion

Reporter server 5.10. We used Torrent Suite (version 3.6.2; Thermo Fisher Scientific, Inc.) to compare the data to Human Genome Version 19 (hg19). Thermo Fisher Scientific, Inc.'s Coverage Analysis plug-in (version 3.6) was used. Allele frequency more than 10%, general uniformity greater than 80%, quality greater than 20, and average base coverage greater than 500x readings were the cutoffs. A plug-in called Variant Caller (version 3.6; Thermo Fisher Scientific, Inc.) was used to detect mutations. The Integrated Genome Viewer IGV at the Broad Institute was used to verify each mutation (www.broadinstitute.org).

Statistical analysis

Frequencies and percentages were used to represent data from categorical variables, whereas mean Standard Deviation or median (IQR) was used to represent data from continuous variables. The significance between categorical variables was examined using the Chi-square test, while continuous variables were tested using Mann-Whitney U tests. $P<0.05$ was established as the criterion for statistical significance.

RESULTS AND DISCUSSION

The study population comprised of 18 (85.7%) males and 3 (14.3%) females. Of these, 13 (61.9%) were <60 years old, and 8 (38.1%) were ≥60 years old, with a mean age (of 62.19 ±8.85) and a median (of 63) years. A total of 19 patients had HCV and 1 patient had HBV, 13 (61.9%) had bilharzia antibodies. Over 47.6% of HCC patients had co-morbidities, diabetes (33.3%) and hypertension (14.3%) were among the common co-morbid conditions, (Table 1).

Mutant *BRAF* patients were significantly more likely to be older age >60 years (90%). BRAF gene mutations were also significantly more likely to be without family history (90%). All cases with muted BRAF had HCV (100%), 90% had no ascites, 10% had positive PVI, and 10% had lung metastasis. All pathological features were not significant, (Table 2).

A recent study found that 85.7% of HCC patients are men. The findings of the analysis of the influence of sex disparities on disease outcomes are inconclusive Braunwarth *et al*. (2020) and Rich *et al*. (2020). Increased exposure to risk factors, androgens (AR), and estrogens (ER), as well as male predominance in HCC Zhang *et al*. (2020). According to the current study, individuals 60 years old or older make up 61.9% of those with HCC. As a result, a variety of causes, including race, ethnicity, and genetic predisposition, could be implicated in the age disparity Mak and Kramvis (2021).

According to other studies, smoking has several harmful consequences on the liver, such as liver carcinogens Li *et al*. (2019). Hence, there was no statistically significant correlation between smoking and HCC. Increasing research indicates that HCC risk with an aggressive nature is significantly increased by a family history of liver

cancer Loomba *et al*. (2013) and Caruso *et al*. (2017).

In this study, 61.9% of the 21 HCC patients had bilharzia antibodies. Our findings are consistent with those of researchers, who found Schistosoma antibodies in 67.7% of Egyptian HCC patientsRamadan *et al*. (2021). In this study, bilharzia was identified as HCC risk factor (OR=1.625, 95% CI 0.558-4.73).

In this study, 90.5% of HCC patients had anti-HCV antibodies. HCV infection was linked to a 9.5-fold increased risk of HCC (OR= 9.50, 95% CI 1.96–46.01). Whereas only 4.7% of people tested positive for HBV (OR= 0.050, 95% CI 0.006-0.407). The study's findings are in line with those of earlier research, which found that HCV infection is the main cause of cirrhosis (93%) and a risk factor for HCC Mohamed *et al*. (2015) and Rashed *et al*. (2020).

In the current study, the mean serum AFP level was 2417.07± 9230.79, the median was 42 ng/dL as shown in (Table 3). According to Zhang *et al*. (2020), a blood AFP level of 400 ng/dL provides the highest sensitivity and specificity for detecting HCC.

The current study found a statistically significant relationship between ascites and HCC patients (P=0.023). This finding is consistent with Hsu *et al*. (2013) who found that 23% of patients had ascites at the time of diagnosis. Although the prevalence of macroscopic PVI varies across studies and is present in 30% to 62% of instances with advanced HCC, it is unquestionably underreported Shehta *et al*. (2021).

In a previous study, 14.3% of participants had a positive PVI that was significantly correlated with HCC (P = 0.01), Brain (2%), peritoneum/omentum (11%), adrenal glands (11%), bone (28%), local lymph nodes (53%), and lung (55%) are the most frequent extrahepatic HCC metastatic locations to their frequencies Becker *et al*. (2014).

Extrahepatic metastasis is a sign of advanced HCC, according to clinical standards. The lymph nodes and the lungs in this study were metastatic sites (14.3% for each). The classic Child-Pugh rating system has been the most popular way to evaluate liver function and determine the effectiveness of treatments for many years Zhao *et al*. (2020).

In 21 HCC patients, Child's A had a 76.2% preponderance, followed by Child's B with 14.3% and Child's C with 9.5%. This study's findings are consistent with those of Hassan-Kadle *et al*. (2022), who noted that 73.6% of patients were classified as Child's A, 17.2% as Child's B, and 9.0% as Child's C in the Child-Pugh classification. Patients with diabetes mellitus (DM), whose incidence is steadily rising globally, are two to three times more likely to develop HCC Li *et al*. (2017).

In the present study, 33.3% of participants had DM that was not

significantly associated. HCC mortality has been related to primary hypertension, however, the reasons underlying this association are not fully understood Lopez-Lopez *et al*. (2020). In this study, 14.3% of 21 HCC patients had hypertension, and this association was significant (P = 0.01).

Various staging systems, including the BCLC staging system, have been proposed in recent years Hsu *et al*. (2013). The Child-Pugh score, tumor burden, and patient performance status are only a few of the factors the BCLC staging system considers. According to the study population's CT scan results, 81.0% of the 21 cases of HCC detected by CT scan had large tumors measuring more than 5 cm in diameter. Among these cases, 42.9% had a single lesion and 57.1% had multiple lesions. The BCLC staging revealed that stages A and C (33.35% for each) were more prevalent.

In this study, we investigated the prevalence of *BRAF* genetic alterations in a cohort of 21 human HCC patients. For the *BRAF* gene, somatic mutations were frequently found in 10 patients from 21 patients (47.6%).

Previous work reported the *BRAF* gene mutation in 65 HCC patients and reported that among 65 cases, the oncogenic mutations were detected in 15 (23%) patients for the *BRAF* gene Colombino *et al*. (2012).

In all 21 patients who underwent *BRAF* gene sequencing, mutations were identified in 10/21 (47.6%) of samples. There were 10 variants, out of them 8/10 (80%) were single nucleotide variants (SNVs), 1/10 (10%) were copy number variants (CNVs) and 1/10 (10%) were insertions/deletion variants (INDELs). Genomic variant annotation and filtering were further interpreted using the Ensembl Variant Effect Predictor (VEP). The program uses the Ensembl/GENCODE or RefSeq gene sets to forecast the molecular effects of variants. Among SNVs, 71.4% (5/7) were novel somatic mutations (missense variant), and 28.6% (2/7) were existing germline mutations (coding transcript intronic variants). The Sift and Polyphen Prediction by VEP showed that 100% were NA. Predicted ACMG Outcome by VEP showed that 100% Likely benign, (Table 4 and Figs. 1&2).

In a prior study on this subject, a tiny subset of human HCC patients did not have any BRAF mutations. Missense mutations made up 22.2% (2/9) of SNVs, while nonsense mutations made up 44.5% (4/9). A prior work investigated the MAPK/ERK pathway by means of an NGS panel and a copy-number array. The only recurring missense variation found in their group was a MAPK1 activating mutation, which happened twice. The classic BRAF-activating mutation was also detected in one tumor Haines *et al*. (2019).

Further study documented that among the 77 cases with RAF1 aberrations, 25 cases (32.5%) exhibited RAF1 copy number variants (CNVs) in their tumor samples. In addition, there are

differences in BRAF SNVs according to the type of cancer (HCC 2.3%, Bladder cancer 9.4%, and pancreatic cancer 4.5%) Lim *et al*. (2023).

Of the patients in the Chinese group, 35 cases had 39 different *BRAF* mutations. There were five different kinds of *BRAF* mutations in class 2, including four fusion mutations and one missense mutation. We identified six missenses in the individuals who had a class 3 *BRAF* mutation. In addition, eleven *BRAF* mutations were deemed undescribed. There was one frameshift mutation and one *BRAF* amplification mutation; six were missense kinds Huang *et al*. (2024)

Furthermore, it cannot be considered that distinct etiological variables may contribute to the development of HCC in various populations, which may lead to various methods of transformation. Clinicopathologic characteristics and *BRAF* mutations were significantly associated with (age, family history, HCV infection, ascites, portal vein invasion, and metastasis Tannapfel *et al*. (2003).

SUMMARY

Limitations: Found no evidence of portal vein invasion but did associate *BRAF* mutation to hypertension. Larger tumors tend to have more *BRAF* mutations. More research, including whole exome sequencing, is required to provide a complete explanation for the genetic changes seen in HCC.

The use of NGS led to the discovery of several unique gene mutations in HCC, including both confirmed and disproven mutations. The origin and progression of HCC are best understood because of these findings, which offer new views. Larger patient cohorts are required to fully comprehend *BRAF* genetic alteration and its impact on the development of HCC.

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Table (1): *BRAF* mutation in HCC cases according to demographic and clinical characteristics.

Variables		Mutant type (n=10)		Wild type (n=11)		Total N=21		OR (95% CI)	*P*-value
		No.	%	No.	%	No.	%		
Sex	**Male**	7	%70	11	100%	18	85.7 %	0.70 (0.195-2.511)	0.584
	Female	3	%30	0	0.0%	3	14.3 %		
Smoking	**Yes**	1	10%	1	9.1%	2	9.5%		
	No	8	%80	6	54.5%	14	66.6%	1.467 (0.376-5.723)	0.581
	Ex. smoker	1	%10	4	36.4%	5	23.8%		
Bilharzia	**Yes**	6	%60	7	63.6%	13	61.9%	0.943 (0.236-3.772)	0.934
	No	4	%40	4	36.4%	8	38.1%		
Hepatic encephalopathy	**Yes**	0	0.0%	0	0.0%	0	0%	1.00 (0.298-3.357)	1.00
	No	10	100%	11	100%	21	100 %		
Family history	**Yes**	1	%10	3	27.3%	4	19.1 %	1.238 (0.344-4.454)	0.744
	No	9	%90	8	72.7%	17	80.9 %		

Viral infection	**HCV**	10	%100	9	81.8%	19	90.5 %	1.222 (0.353-4.235)	0.752
	HBV	0	.0%0	1	9.1%	1	4.7%	0.365 (0.013-9.979)	0.551
	NBNC	0	0.0%	1	9.1%	1	4.7 %	0.365 (0.013-9.979)	0.551
Comorbidities	**DM**	2	20%	5	45.5%	7	33.3 %	0.44 (0.069-2.798)	0.384
	HTN	2	20%	1	9.1%	3	14.3 %	2.20 (0.172-28.139)	0.544

Data are presented as frequency (%) or mean ± SD. HCC: Hepatocellular carcinoma, HCV: hepatitis C virus, HBV: hepatitis B virus, DM: diabetes mellitus. HTN: hypertension. *Significant. P value <0.05, CI: confidence interval, OR: odds ratio.

Table (2): Clinicopathological features of HCC patients according to *BRAF* gene mutation.

Variables	Mutant type (n = 10)		Wild type (n=11)		Total		*P*- value
	No.	%	No.	%	No.	%	
	Ascites						
No	9	90%	8	72.7%	17	90.0 %	0.744
Minimal	0	0.0%	1	9.1%	1	4.7 %	
Moderate	1	10%	2	18.2%	3	14.3 %	
	Portal Vein Invasion						
Negative	9	90%	9	81.8%	18	85.7 %	0.882
Positive	1	10%	2	18.2%	3	14.3 %	
	LN Metastasis						
Negative	10	100%	8	72.7%	18	85.7 %	0.622
Positive	0	0.0%	3	27.3%	3	14.3 %	
	Lung Metastasis						
Negative	9	90%	9	81.8%	18	85.7 %	0.882
Positive	1	10%	2	18.2%	3	14.3 %	
	Child PUGH class						

Table (2)Cont'							
A	8	80%	8	72.7%	16	76.2 %	0.195
B	0	0.0%	3	27.3%	3	14.3 %	
C	2	20%	0	0.0%	2	9.5 %	
	CT radiological findings						
	Tumor number						
Single	5	50%	4	36.4%	9	42.9 %	0.691
Multiple	5	50%	7	63.6%	12	57.1 %	
	Tumor Size						
Small (<3 cm)	3	30%	0	0.0%	3	14.3 %	0.691
Medium (3 - 5 cm)	0	0.0%	1	9.1%	1	4.7 %	
Large (>5 cm)	7	70%	10	90.9%	17	90.0 %	
	BCLC						
A	4	40%	3	27.3%	7	33.3%	0.085
B	4	40%	1	9.1%	5	23.8%	
C	0	0.0%	7	63.6%	7	33.3%	
D	2	20%	0	0.0%	2	9.5 %	

Data are presented as frequency (%) or mean ± SD. PVI: portal vein invasion. BCLC: Barcelona clinic liver cancer. *Significant. P value <0.05.

Table (3): AFP (ng/mL) level in the HCC population.

HCC	(n = 21)
AFP (ng/mL)	
Min. – Max.	4.9– 42443
Mean ± SD.	2417.07± 9230.79
Median	42.05 (18.4–107.5)

Table (4): Summary of *BRAF* gene variation in HCC detected by targeted sequencing.

Locus	Types	Variant frequency	genes	Amino Acid Change	Coding
chr7:140453102	CNV	0.44	*BRAF,EZH2*	0	0
chr7:140453106	SNV	0.04	*BRAF*	p.Phe610Ser	c.1829T>C
chr7:140453111	SNV	0.04	*BRAF*	p.His608Gln	c.1824T>G
chr7:140453147	SNV	0.04	*BRAF*	#N/A	#N/A
chr7:140453183	SNV	0.04	*BRAF*	#N/A	#N/A
chr7:140453207	SNV	0.04	*BRAF*	p.?	c.1742-14T>C
chr7:140453217	SNV	0.04	*BRAF*	p.?	c.1742-24T>C
chr7:140481398	INDEL	0.04	*BRAF*	p.Val471dup	c.1409_1410insGGT
chr7:140481479	SNV	0.04	*BRAF*	#N/A	#N/A
chr7:140481504	SNV,INDEL	0.12	*BRAF*	p.?	c.1315-12_1315-11insGCAGGC

Chr: Chromosome, SNV: single nucleotide variant, CNV: copy number variant, INDEL: insertions/deletion variants, N/A: not applicable

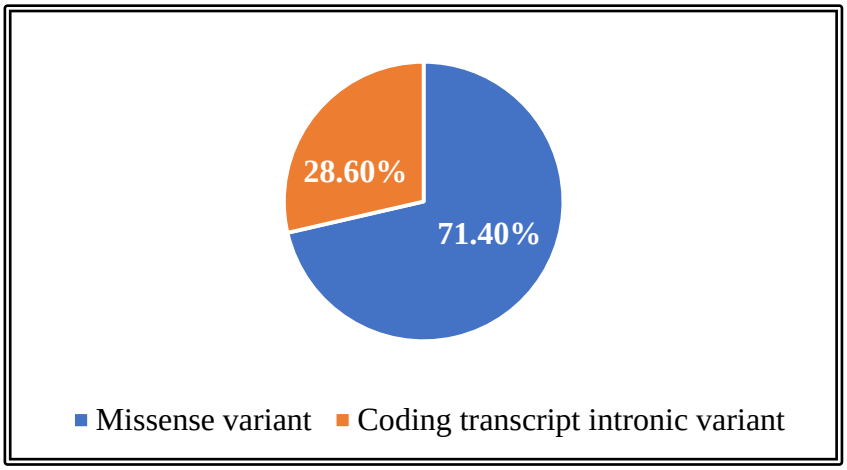

Fig. (1): Percentage of Variant effect of SNVs

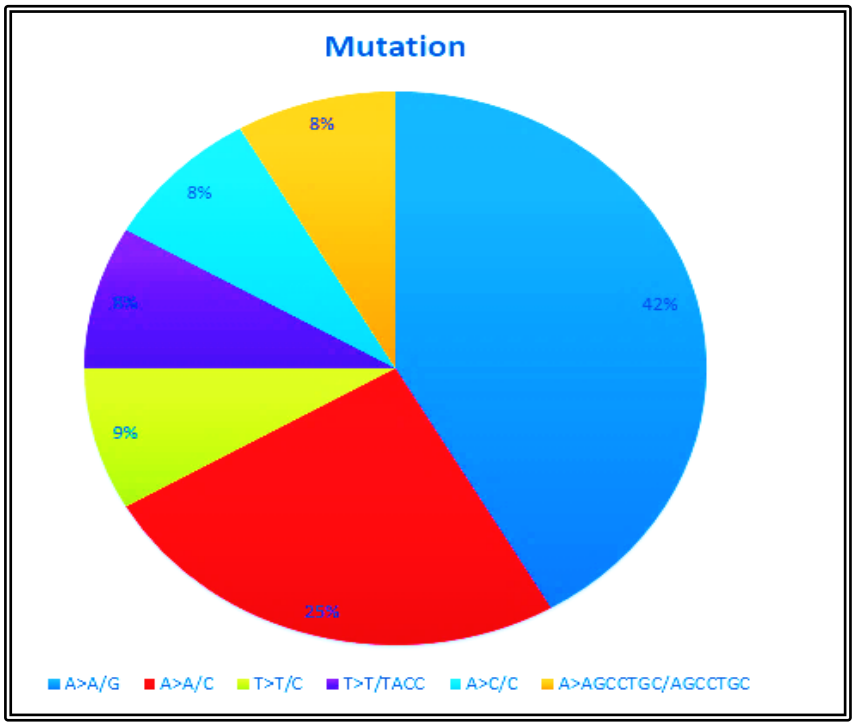

Fig. (2): Summary of *BRAF* mutations in HCC patients.