# SOMATIC MUTATIONS IDENTIFICATION OF *FGFR3* AMONG HCC EGYPTIAN PATIENTS USING NGS

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Hepatocellular carcinoma (HCC) is the most prominent liver cancer, accounting for 85% of primary liver malignancies. It is a very aggressive tumor, having a terrible prognosis and poor survival rate (Mir *et al*., 2021). HCC is ranked the sixth most common type of cancer and the third leading cause of cancer-related mortalities worldwide (Singh *et al*., 2020).

HCC incidences arise in complications associated with chronic liver disease like cirrhosis, endemic hepatitis B virus (HBV)/hepatitis C virus (HCV) infections, nonalcoholic fatty liver disease, and alcohol-related liver disease (Torre *et al*., 2015).

cfDNA was discovered in peripheral blood by normal and malignant cells. It was discovered that cancer patients' serum and plasma had considerably more cfDNA than did healthy controls (Song *et al*., 2021). Numerous investigations have shown that cancer patients' cfDNAs include genetic changes specific to their tumors and possess cancer-associated molecular characteristics and tumor cells can release DNA into peripheral blood (Thierry *et al*., 2014).

A DNA fragment known as ctDNA is a type of tumor-specific genetic mutation that is directly released into the blood from living or dead tumour cells in primary or metastatic tumour tissue. (Ocker 2018) and Alix-Panabieres and Pantel (2016). In cancer patients, ctDNA makes up a very minor portion of the total cfDNA. (Ye *et al*., 2019; Banini and Sanyal 2019). To gain genetic or epigenetic information, liquid biopsy can therefore analysis blood or other body fluids

to obtain ctDNA. This information can then be utilized for tumor screening, diagnosis, prognosis, therapy monitoring, or recurrence. (Banini and Sanyal 2019, Crowley *et al*., 2013).

Fibroblast growth factor receptor 3B is expressed by hepatocytes (Paur *et al*., 2015). Although FGFR3 plays an important role in HCC development and progression (Paur *et al*., 2015 and 2020) and FGFR3 expression is elevated in human cirrhotic livers (Kurniawan *et al*., 2020) the functional role of FGFR3 and its isoforms has not yet been investigated in the context of hepatic fibrosis.

FGF/FGFR signaling is impressionable to be hijacked by oncocytes. The underlying mechanism driving FGF/FGFR signaling is very cancer special and can be classified into FGFR amplification (Chang *et al*., 2014), FGFR mutation Greulich and Pollock (2011), abnormity of FGFR-involved ligands (Ahmad *et al*., 2012).

Our hypothesis was the possible role of cfDNA sequencing in carcinogenesis so the aim of our study was to identify the frequent deleterious somatic mutations of FGFR3 among HCC Egyptian patients by using NGS technology which has become a pioneer in the field of understanding and interpreting genetic alterations.

## SUBJECTS AND METHODS

### 1. Patients and samples

This study included 21 patients with HCC, 18 were males, and 3 were females. They were recruited from the oncology clinic at the National Liver Institute, Menofia University, Egypt. By analyzing these patients' cfDNA targets and comparing those with healthy genomes of 3 normal individuals free of any tumors, only somatic but not germline mutations could be filtered for study. They had only HCC and no other types of tumors.

The study protocol was approved by the local ethics committee of the National Liver Institute, Menoufia University (NLI IRB protocol Number 00232/2020, Dec. 2020- one year). Written consent was taken from the patients after explaining the aim and benefits of our study.

(6-8) ml of peripheral EDTA blood was collected from each patient. Genomic DNA was extracted from whole blood sample and the plasma was stored at -80ºC for cell-free DNA Extraction.

### 2. Methods

Patients were divided into 3 groups according to BCLC staging: group 1 (7 patients with stage A), group 2 (5 patients with stage B), and group 3 (9 patients with stages C&D). Medical history and clinical data were taken. In addition, clinical examination, chest X-ray, triphasic computed tomography (C.T.) and complete routine laboratory tests were obtained from medical records.

**Next-generation sequencing**

**cfDNA extraction**

Cell-free DNA was extracted from

patient plasma using (QIAamp DSP Virus Spin Kit, Cat.No.61704) according to the manufacturer's procedure. Genomic DNA was extracted using (PureLink™ Genomic DNA Mini Kit, Cat.No. K1820-00**)**.

**Preparation of libraries**

10 ng of DNA was amplified using (Ion AmpliSeq™ Library Kits 2.0, Cat.No.4480441) and Ion AmpliSeq HiFi Master Mix (Ion AmpliSeq™ Library kit 2.0, Thermo Fisher Scientific, Inc.). The library was then quantified by qPCR with the (ion library TaqMan® Quantitation Kit) (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol (Morishita *et al.*, 2018).

**ISPs enrichment**

Template-positive Ion PGM™ Hi-Q™ View Ion Sphere™ Particles (ISPs) were Enriched using (Ion PGM™ Hi-Q™ View OT2 Kit, Cat.No. A 29900).

**FGFR3 sequencing**

Enriched template ISPs were then, loaded onto Ion 316™ chips and sequenced using (Ion PGM™ Hi-Q™ View Sequencing Kit, Cat. No. A30044) and Ion Personal Genome Machine System (Ion Torrent) (PGM™; Life Technologies) according to the manufacturer's protocol (Morishita *et al*., 2018).

**Data analysis**

In order to evaluate the matched standard and tumor sample for each utilizing the default plugin parameters, generated BAM files were uploaded to the cloud-based Ion reporter server version 5.10 on Thermo Fisher's website. Using the Torrent Suite program, the raw data were aligned to Human Genome Version 19 (hg19) (version 3.6.2; Thermo Fisher Scientific, Inc.). The Coverage Analysis plug-in was used for the coverage analysis (version 3.6; Thermo Fisher Scientific, Inc.). Cut-off values included a quality score of at least 20, an average base coverage of at least 500 reads, an allele frequency of at least 10%, and a total uniformity of at least 80%. Utilizing the Variant Caller plug-in, mutations were found (version 3.6; Thermo Fisher Scientific, Inc.). The Integrative Genome Viewer (IGV) from the Broad Institute was used to confirm each mutation. (www. broadinstitute.org) (Thorvaldsd'ottir *et al*., 2013).

**Statistical analysis**

Data were provided to the computer to investigate the correlation between *FGFR3* mutations and clinicopathological factors and analyzed using IBM SPSS software package version 20.0 (Armonk, NY: IBM Corp). The significance of the obtained results was judged at the 5% level.

## RESULTS AND DISCUSSION

**Study population**

Eighteen males and three females were included in this study. 13 were >60 years old, and 8 were <60 years old. Among patients: 19 were HCV-positive patients, one was HBV- positive, and 2 were non-viral hepatitis neither (B nor C) (Table 1). Group 1 (stage A) includes 7 patients with a percentage of 33.3%, while group 2 (stage B) includes 5 patients with

a percentage of 23.8%, and group 3 (stages C and D) includes 9 patients with a percentage of 42.9% patients using BCLC staging (Fig. 1).

Five patients (33.3%) in group1 (stage A) (according to BCLC1 staging) were *FGFR3* gene mutated, while three patients (20%) in group 2 (stage B) were *FGFR3* gene mutated, and seven patients (46.7%) in group 3 (stages C&D) were *FGFR3* gene mutated. Six patients had no mutations in *the FGFR3* gene: two patients (33.3%) were staged A, two patients (33.3%) were stage B, and two patients (33.3%) were stages C&D (Fig. 2).

### *FGFR3* mutations: germline & somatic

Fifteen patients (15/21) had a *FGFR3* mutated gene (Table 1). A total of 50 mutations were identified in the *FGFR3* gene in our HCC patient's liquid biopsy samples. Thirty-eight (76%) of these were somatic mutations as compared to their germline profile using gDNA, while twelve (24%) of the mutations were germline mutations. Among 38 SNV somatic mutations, 9/38 (23.7%) were synonymous mutations, 21/38 (55.3%) were nonsynonymous mutations and 8/38 (21%) were with unknown significance. Some of the nonsynonymous mutations may cause significant changes in protein structure and thus be more potentially deleterious or damaging to protein function as predicted by SIFT (Kumar *et al*., 2009) and poly phen (Adzhubei *et al*., 2013) (Table 3).

### Synonymous & nonsynonymous mutations

By using VEP to analyze the somatic mutations, 69% of somatic mutations were nonsynonymous, 30 % were synonymous, and 1% were stop-gained (Fig. 3). Furthermore, all of the nonsynonymous variants were missense as no-nonsense mutations were detected.

### Clinical-pathological traits

The clinicopathological traits and the mutant *FGFR3* gene are correlated (Table 1), demonstrating no statistically significant link between them as the p-value is more than 0.05 for all of the features.

### Effect of FGFR3 genetic mutations

There are four main pathways acting as main downstream signaling and four main downstream pathways of the FGF/FGFR signal: MAPK, PI3-kinase, PLC, and STAT. The final effects of these activating downstream pathways are transcriptionally activating a series of target genes that are responsible for multiple hallmarks of HCC. *FGFR3* mutations detected in our study indicate the probability of involvement of one of these pathways in HCC development.

HCC incidence is a progressive pathological process regulated by multiple of genes. Thus, HCC could be prevented or even treated by understanding the molecular mechanisms of its pathogenesis according to the related factors in

high-risk groups. Hepatitis B virus, hepatitis C virus, and exposure to toxins (such as aflatoxin) are some of the familiar environmental risk factors that may lead to potential cirrhosis. Specific gene mutations have been isolated for each cause of HCC (Forner *et al*., 2018).

Liquid biopsy, the minimally invasive assay of circulating cancer-associated biomarkers such as circulating nucleic acids, circulating tumor cells, miRNAs, and exosomes, has several potential clinical applications (Felden *et al*., 2018 and Li *et al*., 2018). Of these, the analysis of cfDNA is currently the most promising in HCC. This is because circulating cfDNA refers to fragments of DNA detected in both healthy individuals and patients with cancer (Tran *et al*., 2021).

Most circulating cfDNA fragments are double-stranded, exist in plasma or serum, and are longer than 167 base pairs (Mouliere *et al*., 2018). In contrast, circulatory tumor DNA (ctDNA) fragments, which are released by necrotic or apoptotic tumor cells, are typically shorter than 150 base pairs; these size differences, as well as sequence variation or epigenetic modifications, may be exploited to identify tumor-specific sequences (Mouliere *et al*., 2018).

Potential clinical utilities of cfDNA/ ctDNA have been and are being investigated for detecting HCC, disease monitoring, and prognostication (Tran *et al*., 2021).

It was found that FGFR3-IIIb occurs predominantly in hepatocytes and FGFR3-IIIc in mesenchymal liver cells and that one or both splice variants are highly overexpressed in at least 50 percent of HCC cases investigated. Furthermore, aberrant expression of *FGFR3* variants was causally involved in the deregulated growth control and aggressive behavior in advanced stages of hepatocarcinogenesis (Paur *et al*., 2015).

Based on all pervious reasons, we specifically studied *FGFR3* and mutations by next-generation sequencing technique aiming this study helps molecular prediction and early diagnosis of HCC development among Egyptian patients.

We found that there was no significant statistical difference in the distribution of the *FGFR3* gene mutations among HCC stage A, HCC stage B and HCC stages C&D groups according to BCLC staging (p-value 0.836).

In the present study, nine patients: 3 with stage A (no. 2, 4, and 5), 2 with stage B (no. 11 and 12), and 4 with stage C (no.13, 16, 17 and 18), had normal unmutated *FGFR3* gene, with other altered genes and risk factors causing HCC.

These results were in agreement with a previous study of (Chang *et al*., 2012) who showed that overexpression of *FGFR3* in hepato-cellular carcinoma is not associated with genetic alterations of *FGFR3* gene and suggested that there could be another underlying

mechanism of aberrant FGFR3 expression in hepato-cellular carcinoma.

However our results were not in accordance with (Yan *et al*., 2017) who found a typical growth factor receptor tyrosine kinase *FGFR3* was predominantly mutated in HCC and higher FGFR3 protein levels occurred in 24% of HCC harboring *FGFR3* gene with single nucleotide mutations in exon 9, 11 or 12.

Also a study conducted by (He *et al*., 2019) who analyzed the correlation between the MAFs of specific genes in plasma cfDNA and the tumor load and found that the MAF values for *TP53*, *RET*, *APC*, and *FGFR3* were significantly higher in patients with multiple tumors or HCC with tumor metastasis than those with a single tumor HCC.

Patient 19, stage C, had given up tobacco smoking, with HCV, multiple liver lesions, lung metastasis and no lymph nodes. He had one existing nonsynonymous somatic mutation (chr4:1808939 _C/T) which was predicted to be detrimental and perhaps harmful.

Patient 15 has no metastasis, multiple liver lesions, and portal vein invasion. Its child Pugh was B and HCV infected, and had three new nonsynonymous missense mutations, one was not deleterious, but the other two were harmful with damaging effects. Also, patient 20, 67 years old, with old bilharzial infection, HCV infection, lung metastasis, no lymph nodes, no portal vein invasions and three liver lesions. He had two new nonsynonymous mutations in *FGFR3* gene (chr4:1803631_A/G and chr4:1808935_T/G) that were detrimental and probably harmful.

To the best of our knowledge, this study could be one of the first Egyptian studies focusing on whether the *FGFR3* gene profiling could be used as an approved biomarker for HCC risk among HCC patients.

The conflict between our results and other studies was attributed to a different type of cancer (HCC), different ethnic groups, genetic species, and the small sample size of our study compared to their large-scale multicenter studies. Furthermore, a limited number of Egyptian studies investigating the association of *FGFR3* gene mutations and HCC limited our chance for comparison, further explanations, and result validation.

## SUMMARY

One of the most prevalent malignancies in the world, hepatocellular carcinoma (HCC), has a high fatality rate. Noninvasive biomarkers are desperately needed to help in HCC screening and early diagnosis. Next-generation sequencing has advanced, and genetic indicators are now the mainstay of cancer detection. Early HCC diagnosis now focuses on genetic indicators such circulating tumour DNA in peripheral blood.

Overexpression of the fibroblast growth factor receptor 3 (FGFR3) splice variants FGFR3-IIIb and FGFR3-IIIc was

found in ~50% of hepatocellular carcinoma (HCC).

*FGFR3* gene mutations were not associated with an increased risk of HCC in the Egyptian population. However, it could have a probable role in the pathogenesis of liver cell failure, HCC development, and prognosis, as the present study identified several novel mutations involved in HCC using NGS. The results of the present study provide resources for understanding the molecular alterations underlying the development of HCC. However, further investigations with larger sample sizes are required to fully examine genetic alteration in HCC development.

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Table (1): Correlation of clinicopathological features and mutations of *FGFR3* in HCC patients.

| | FGFR3 | | | | p-value |
|---|---|---|---|---|---|
| | No mutation (n = 6) | | Mutated patients (n = 15) | | |
| | No. | % | No. | % | |
| Gender | | | | | |
| Male | 6 | 100.0 | 12 | 80.0 | 0.526 |
| Female | 0 | 0.0 | 3 | 20.0 | |
| Age (years) | | | | | |
| <60 | 1 | 16.7 | 7 | 46.7 | 0.336 |
| ≥60 | 5 | 83.3 | 8 | 53.3 | |
| BCLC1 | | | | | |
| A | 2 | 33.3 | 5 | 33.3 | 0.836 |
| B | 2 | 33.3 | 3 | 20.0 | |
| C & D | 2 | 33.3 | 7 | 46.7 | |
| AFP | | | | | |
| ≤20 | 3 | 50.0 | 3 | 20.0 | 0.291 |
| >20 | 3 | 50.0 | 12 | 80.0 | |
| Bilharziasis | | | | | |
| No | 2 | 33.3 | 6 | 40.0 | 1.000 |
| Yes | 4 | 66.7 | 9 | 60.0 | |
| Diabetes mellitus | | | | | |
| No | 3 | 50.0 | 11 | 73.3 | 0.354 |
| Yes | 3 | 50.0 | 4 | 26.7 | |
| HTN | | | | | |
| No | 5 | 83.3 | 13 | 86.7 | 1.000 |
| Yes | 1 | 16.7 | 2 | 13.3 | |
| Family history | | | | | |
| No | 5 | 83.3 | 12 | 80.0 | 1.000 |
| Yes | 1 | 16.7 | 3 | 20.0 | |
| Smoking | | | | | |
| No | 3 | 50.0 | 11 | 73.3 | 0.628 |
| Smoker | 1 | 16.7 | 2 | 13.3 | |
| Ex-smoker | 2 | 33.3 | 2 | 13.3 | |

BCLC= Bacelona clinic liver cancer, AFP=Alpha-feto protein, HTN = hypertension.

Table (2): Correlation of clinicopathological features and mutations of *FGFR3* in HCC patients.

| | FGFR3 | | | | p-value |
|---|---|---|---|---|---|
| | No (n = 6) | | Yes (n = 15) | | |
| | No. | % | No. | % | |
| **Metastasis** | | | | | |
| No | 5 | 83.3 | 13 | 86.7 | 1.000 |
| Yes | 1 | 16.7 | 2 | 13.3 | |
| **HCV** | | | | | |
| No | 1 | 16.7 | 1 | 6.7 | 0.500 |
| Yes | 5 | 83.3 | 14 | 93.3 | |
| **HBV** | | | | | |
| No | 6 | 100.0 | 14 | 93.3 | 1.000 |
| Yes | 0 | 0.0 | 1 | 6.7 | |
| **NBNC** | | | | | |
| No | 5 | 83.3 | 14 | 93.3 | 0.500 |
| Yes | 1 | 16.7 | 1 | 6.7 | |
| **P.S** | | | | | |
| No | 5 | 83.3 | 14 | 93.3 | 0.500 |
| Yes | 1 | 16.7 | 1 | 6.7 | |
| **P.V** | | | | | |
| No | 6 | 100.0 | 12 | 80.0 | 0.526 |
| Yes | 0 | 0.0 | 3 | 20.0 | |
| **Ascites** | | | | | |
| No | 5 | 83.3 | 12 | 80.0 | 1.000 |
| Mild | 1 | 16.7 | 2 | 13.3 | |
| Moderate | 0 | 0.0 | 1 | 6.7 | |
| **Child- pugh** | | | | | |
| Class A | 8 | 83.3 | 11 | 73.3 | 1.000 |
| Class B | 1 | 16.7 | 2 | 13.3 | |
| Class C | 0 | 0.0 | 2 | 13.3 | |
| **Number of lesions** | | | | | |
| 1 | 2 | 33.3 | 8 | 53.3 | 0.635 |
| >1 | 4 | 66.7 | 7 | 46.7 | |
| **Size of lesions** | | | | | |
| <3 | 1 | 16.7 | 4 | 26.7 | 1.000 |
| ≥3 | 5 | 83.3 | 11 | 73.3 | |

HCV= hepatitis c virus, HBV= hepatitis B virus, NBNC= no hepatitis B nor C, PS=Performance status, P.V = portal vein.

Table (3): Effects of nonsynonymous somatic mutations of *FGFR3* on HCC patients by using SIFT and PolyPhen.

| | Pt. I.D. | Age | Gender | BCLC | AFP | C - P | locus of mutation | mutation | E/N | mut. Type | SIFT | PolyPhen |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Group I (Stage A)** | HCC-1 | 80 | M | A | 4.9 | A | chr4:1803632 | C>C/T | E | synonymous | | |
| | | | | | | | chr4:1803633 | G>G/T | E | missense | deleterious | benign |
| | | | | | | | chr4:1803635 | G>G/T | N | synonymous | | |
| | | | | | | | chr4:1808935 | T>T/G | N | missense | deleterious | probably damaging |
| | HCC-2 | 63 | M | A | 586 | A | | no mutation | | | | |
| | HCC-3 | 61 | M | A | 143 | A | chr4:1803631 | A>A/G | N | missense | deleterious | possibly damaging |
| | | | | | | | chr4:1806090 | G>T/T | E | missense | tolerated | benign |
| | HCC-4 | 67 | M | A | 22.7 | A | | no mutation | | | | |
| | HCC-5 | 59 | M | A | 65.23 | A | | no mutation | | | | |
| | HCC-6 | 53 | F | A | 6.7 | A | chr4:1808937 | C>C/G | E | missense | deleterious | benign |
| | | | | | | | chr4:1808950 | G>G/A | N | synonymous | | |
| | HCC-7 | 63 | M | A | 325 | A | chr4:1808917 | A>A/G | N | synonymous | | |
| | | | | | | | chr4:1808920 | G>G/T | N | synonymous | | |
| | | | | | | | chr4:1808934 | T>T/C | N | missense | deleterious | probably damaging |
| ( | HCC-8 | 68 | F | B | 50.4 | A | chr4:1808935 | T>T/G | N | missense | deleterious | probably |

Table (3): Cont.'

| | Pt. I.D. | Age | Gender | BCLC | AFP | C - P | locus of mutation | mutation | E/N | mut. Type | SIFT | PolyPhen |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | damaging |
| | HCC-9 | 52 | M | B | 42443 | A | chr4:1808935 | T>T/G | N | missense | deleterious | probably damaging |
| | HCC-10 | 50 | F | B | 16.8 | A | chr4:1808917 | A>A/G | N | synonymous | | |
| | | | | | | | chr4:1808920 | G>G/T | N | synonymous | | |
| | | | | | | | chr4:1808933 | T>T/C | N | missense | deleterious | probably damaging |
| | HCC-11 | 79 | M | B | 10 | A | | no mutation | | | | |
| | HCC-12 | 57 | M | B | 20 | A | | no mutation | | | | |
| **Group III (Stages C&D)** | HCC-13 | 60 | M | C | 5.5 | B | | no mutation | | | | |
| | HCC-14 | 65 | M | C | 69 | B | chr4:1808388 | C>C/A | E | missense | deleterious | probably damaging |
| | | | | | | | chr4:1808390 | C>C/T | E | synonymous | | |
| | | | | | | | chr4:1808392 | C>C/G | E | missense | tolerated | benign |
| | | | | | | | chr4:1808920 | G>G/T | N | synonymous | | |
| | | | | | | | chr4:1808933 | T>T/C | N | missense | deleterious | probably damaging |
| | HCC-15 | 76 | M | C | 4370 | B | chr4:1803631 | A>A/G | N | missense | deleterious | possibly damaging |
| | | | | | | | chr4:1806105 | G>G/C | N | missense | tolerated | benign |
| | | | | | | | chr4:1808935 | T>T/G | N | missense | deleterious | probably |

Table (3): Cont.'

| | Pt. I.D. | Age | Gender | BCLC | AFP | C - P | locus of mutation | mutation | E/N | mut. Type | SIFT | PolyPhen |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | damaging |
| | HCC-16 | 48 | M | C | 25.1 | A | | no mutation | | | | |
| | HCC-17 | 68 | M | C | 72 | A | | no mutation | | | | |
| | HCC-18 | 63 | M | C | 46.1 | A | | no mutation | | | | |
| | HCC-19 | 54 | M | C | 38 | A | chr4:1808939 | C>C/T | E | missense | deleterious | probably damaging |
| | HCC-20 | 67 | M | D | 22 | C | chr4:1803631 | A>A/G | N | missense | deleterious | possibly damaging |
| | | | | | | | chr4:1808935 | T>T/G | N | missense | deleterious | probably damaging |
| | HCC-21 | 53 | M | D | 62 | C | chr4:1808912 | T>T/A | N | missense | deleterious | probably damaging |
| | | | | | | | chr4:1808927 | T>T/A | N | missense | deleterious | probably damaging |

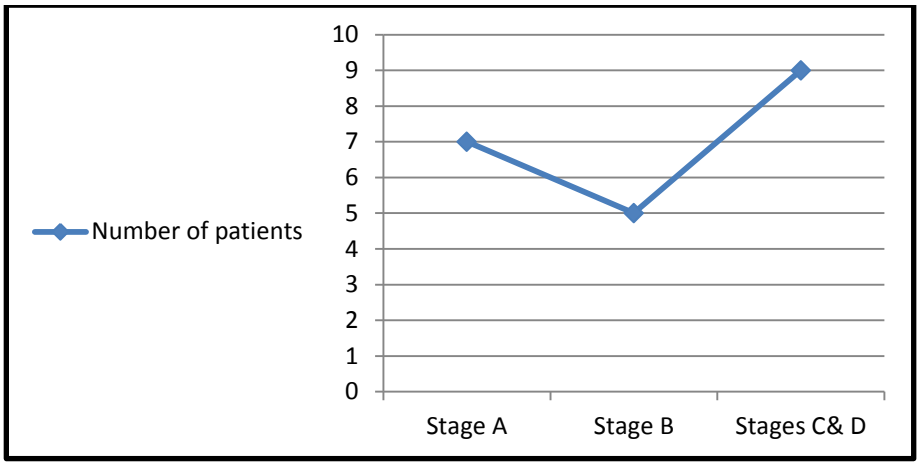


Fig. (1): Classification of HCC patients regarding to BCLC staging System.

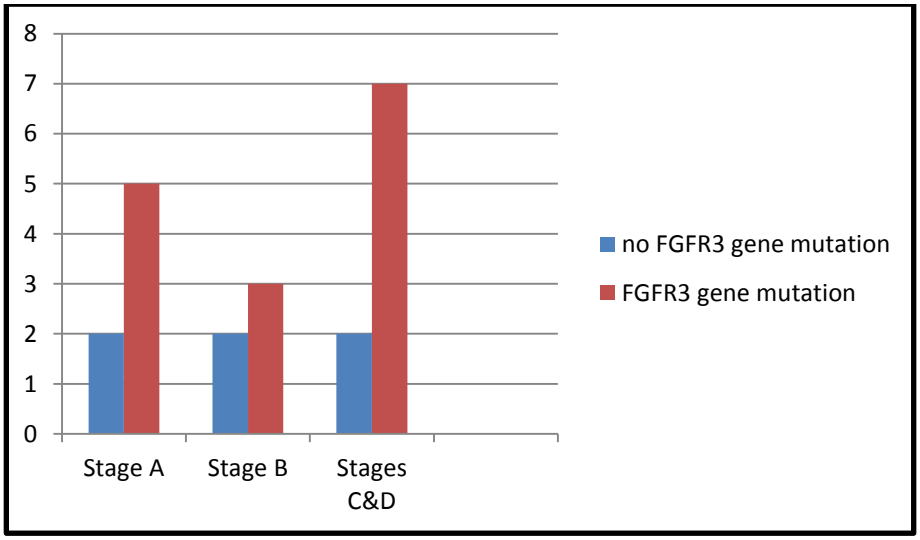


Fig. (2): *FGFR3* mutations in HCC patients stages A,B and C according to BCLC1 staging.

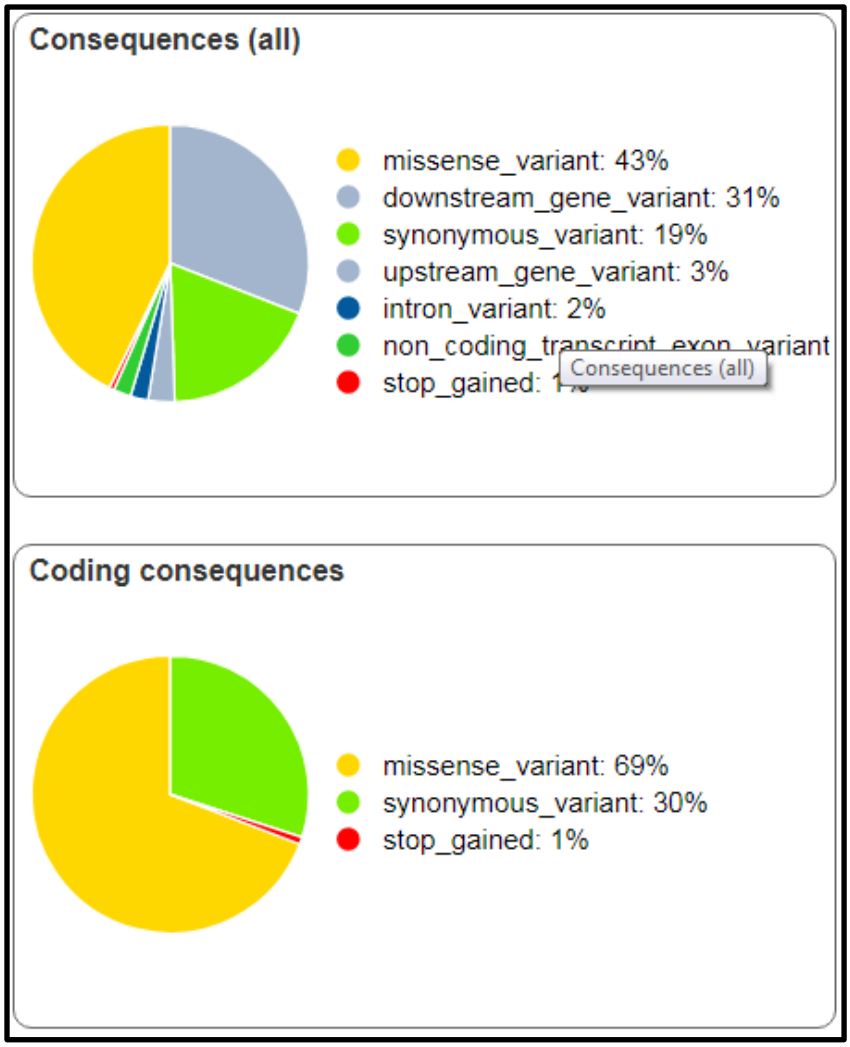


Fig. ( 3): *FGFR3* somatic mutations among studied HCC patients.