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# Molecular Clues for Prediction of Hepatocellular Carcinoma Recurrence after Liver Transplantation

Nourhan Badwei

*Tropical Medicine, Gastroenterology and Hepatology, Faculty of Medicine, Ain Shams University, Cairo, Egypt*

**Recurrence after liver transplantation (LT) for hepatocellular carcinoma (HCC) is one of the commonest causes of cancer-related mortality. Thus, advances in the HCC molecular features have paid researchers great attention to identifying the different risk factors that could aid in liver cancer initiation and progression for earlier prediction of post-operative HCC recurrence risk. Our review has focused on the possible molecular onco-drivers' for HCC recurrence post-LT that may represent diagnostic/prognostic tools and scoring models for the proper selection of LT candidates with HCC. (J Clin Exp Hepatol xxxx;xxx:xxx)**

Hepatocellular carcinoma (HCC) is the fifth most common and third lethal tumour globally. With a worldwide occurrence of 500,000 new instances annually. Its incidence has a characteristic geographical region mainly found in East Asia and sub-Saharan Africa; 82% of instances with mortality arise in developing regions.[1,2] Liver transplantation (LT) is a powerful alternative therapeutic option for both tumours and cirrhotic backgrounds. It is known that patients who fulfil both the Milan and University of California, San Francisco (UCSF) criteria standards can acquire favourable post-operative survival results, while there are others expanded criteria for selecting and treating patients who do not meet milan criteria (MC) that have been published but at the expense of survival. Saposochin *et al.* and Bruix *et al.* observed that the post-operative 5-year average overall survival (OS) rate should reach 75% for patients having HCC to assemble the standards.[3,38,40] Despite great advances in LT diagnostic and management techniques for HCC cases, organ shortage and recurrence constrained the LT choice options in HCC sufferers, and the recurrence rate continues to be excessive at twenty–forty% following LT, which represents a chief reason for poor outcomes after transplantation.[4]

Therefore, searching for HCC recurrence risk factors has been discussed thoroughly in various studies. However, an integrated prediction model for post-transplant survival of patients having HCC after LT has not yet been well defined. Current LT selection criteria are mainly based on bio-humoural markers, radiologic features, histology, response to therapy and tumour doubling times; at first, various medical risk factors have been identified as HCC recurrence predictors; tumour diameter, numbers, histological differentiation grades, micro- and macro-vascular invasions, alpha-fetoprotein (AFP)/AFP-mRNA/AFP-$L_3$/Des-gamma carboxy prothrombin levels, inflammatory markers levels, locoregional therapies locoregional therapies (LRTs) response (downstaging) and ischemia time represent important parameters. Moreover, sorafenib as adjuvant therapy and weight gain problems have been related to postoperative HCC metastasis. In addition, the use of immunosuppressants may be related to HCC recurrence after LT, whereas the mammalian target of rapamycin (mTOR) inhibitors (sirolimus) are known to have anticancer effects, while calcineurin inhibitors can promote cancer growth. Hence, a significant advance in the HCC molecular field pushes researchers to search for the possibility of detecting diagnostic/prognostic serological and molecular markers and scores including AFP, Des-gamma carboxy prothrombin, genes (allelic imbalance [AI] in microsatellites [MSs]), proteins and miRNAs that may act as red flags to aid in the early detection of HCC recurrence.[5–10,38,42,43]

## MOLECULAR KEYS DETECTED IN TUMOUR TISSUE

Molecular clues can be useful to properly select candidates who accurately meet LT standards by predicting high-risk HCC patients for recurrence as illustrated in Figure 1 and Table 1.[11]

### Proteins

Various studies have reported that several proteins were detected in metastatic or recurrent HCC after curative treatment that may aid in early detection. Ki-67/MIB-1

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*E-mail:* nourhanbadwei1990@gmail.com
*Abbreviations:* AFP: Alpha-fetoprotein; DCP: Des-gamma carboxy prothrombin; DFS: Disease-free survival; HCC: Hepatocellular carcinoma; lncRNAs: long non-coding RNAs; miRNAs: microRNAs; OS: Overall survival; OTL: Orthotopic liver transplantation; RFS: Recurrence-free survival; TERT: Telomerase reverse transcriptase
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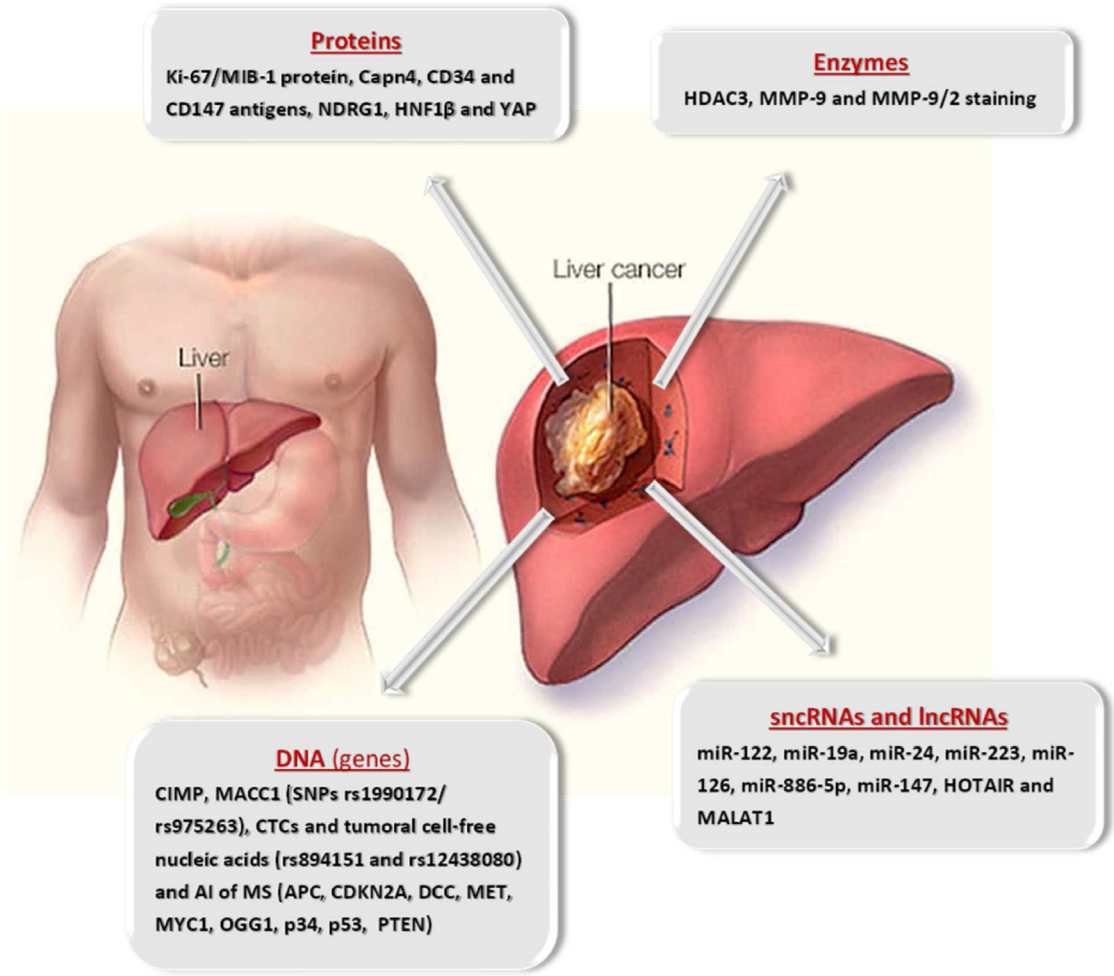


**Figure 1** Molecular clues related to recurrent hepatocellular carcinoma HCC post-LT.

protein, a cellular marker for proliferation can be detected within the cell nucleus with monoclonal antibody MIB-1 and is known as a predictive factor for tumour development and its expression has been correlated with poor prognosis. A high expression of anti-MIB-1/Ki-67 clone index (ratio of MIB-1 positive nucleus concerning the whole wide variety of neoplasms) was proved in several studies to expect recurrence; in particular, if it was found with the tumour suppressor gene expression aggregate TP53 (Ki-67/TP53 proteins).[12] Also, the upregulation of calpain small subunit 1 (Capn4) acts as a protein with relevant interactions with many migrations–invasion-related proteins. Therefore, its identification in the tumour microenvironment was significantly related to the spread and invasion-related interactions that could predict recurrence.[13] Moreover, the identification of CD34 and CD147 antigens endothelial cell markers and positive immunohistochemically stained with anti-CD34/CD147 antibodies were reported to be strongly correlated with microscopic vascular invasion and recurrence.[14] On the other hand, N-myc downstream-regulated gene 1 acts as a multipurpose protein involved in several steps of carcinogenesis; positive protein expression with immunohistochemical staining had shown a significant association with shorter disease-free survival/OS recurrence.[15] In addition, the hepatocyte nuclear factor 1 (HNF1) proteins' family mainly HNF1$\beta$ modulates the AFP promoter activity strictly in hepatocarcinogenesis and regulates hepatic genes transcription. The hepatic expression of HNF1$\beta$ proved to be a useful aid for predicting HCC recurrence post-LT, it evidently affects post-transplantation survival and supports the known Milan criteria for the pre-LT selection and post-LT risk stratification of patients.[16] Lastly, the proteins involved in cellular survival and proliferation pathways have been reported in several studies such as Yes-associated proteins (YAPs), a major effector of the hippo signalling pathway that regulates cell proliferation and apoptosis during normal development; YAP overexpression has been considered as an independent prognostic marker for HCC recurrence post-LT as this protein




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**Table 1 Molecular Clues Related to Recurrent Hepatocellular Carcinoma HCC Post-LT.**

| Molecular clues | Role | Limitations |
|---|---|---|
| **Proteins** | | |
| Ki-67/MIB-1 protein upregulation (IHC) | _A cell proliferation marker, its high expression with p53 (IHC) in 1$^{ry}$ HCC lesions, as well as high serum AFP >100 ng/ml were associated with more rapid tumour recurrence in liver transplant recipients. | _Needs larger prospective cohort studies.<br>_PCR method to detect the mutant p53 is needed for better results |
| Positive Calpain small subunit 1 (Capn4) | _ Positive Capn4 tumours had a significantly increased risk of recurrence and reduced overall post-transplant survival.<br>_ It was significantly correlated with tumour number, larger sizes, encapsulation, venous invasion, and pTNM stage. | _ The exact Capn4 mechanism for promoting HCC cell migration and invasion remains to be elucidated for further studies. |
| Positive IHC of CD147 and MVD-CD34 | _The expression of MVD-CD34, as well as CD147 represent significant indicators for HCC recurrence and poor recurrence-free survival.<br>_These endothelial cell markers were found to be significantly associated with microscopic vascular invasion, also pTNM, tumour stage, venous invasion, and tumour size of HCC. | _They are promising biomarkers for a larger more rational selection of LT candidates with HCC. |
| Positive IHC of upregulated N-myc downstream-regulated gene 1 (NDRG1) | _NDRG1 promotes immune escape-related HCC through gene modulation involved in immune response, adhesion, and proliferation<br>_Positive NDRG1 high expression had been found in the recurrent/metastatic HCC tissues and associated with worse shorter disease-free survival/overall survival. | _The underlying pathogenesis of the immune escape hypothesis facilitating HCC metastasis/recurrence needs to be clarified for further studies on a larger scale. |
| Hepatocyte nuclear factor 1 (HNF1$\beta$) high IHC expression | _HNF1$\beta$ expression in HCC tissues is strongly associated with the time-dependent recurrence risk and HCC-specific death, independent of the Milan criteria.<br>_It's correlated with high AFP in the sera and tumour tissues, as it acts as a critical modulator of AFP promoter activity in hepatocarcinogenesis. | _The concept of pre-LT biopsy for further tumour characterization is a matter of debate, because the risk of seeding/disseminating malignancy may outweigh the contribution to selecting patients suitable for LT. |
| Positive Yes-associated proteins (YAP) IHC expression | _YAP expression in HCC tumours was significantly associated with tumour size, venous infiltration, and AJCC tumour stage, also HCC-specific DFS was significantly longer for patients with YAP negative expression<br>_It may serve as an independent prognostic marker for HCC recurrence post-LT | _Further investigations with a comparative analysis on a larger scale are warranted for better results. |
| **Enzymes** | | |
| Higher Class I histone deacetylases (HDAC3) expression | _Class I HDACs exert a tumour-promoter effect in HCC through the induction of cell proliferation, invasion, and metastasis; Higher HDAC3 expression had a significantly poor prognostic impact regarding recurrence-free survival in HBV-HCC patients.<br>_HDAC3 may serve as a promising biomarker for predicting HBV-related HCC recurrence after LT and even a potential therapeutic target through proliferation/invasion suppression. | _The studied population almost unavoidably consisted of patients with HBV-associated HCC, and so other etiological backgrounds might be very useful to ascertain the real predictive value of HDAC3 for HCC recurrence. |

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**Table 1** (*Continued*)

| Molecular clues | Role | Limitations |
|---|---|---|
| Positive IHC of the matrix metalloproteinase-9/2 (MMP-9/2) | _MMPs, (MMP-2/-9) a group of zinc-dependent endopeptidases capable of degrading the extracellular matrix of the parenchymal and vascular basement membranes; Positive MMP-2/-9 immunoreactivity were detected in the marginal tissues of the tumours denoting the invasive potential and significant correlation with poor survival.<br>_ MMP-9 and MMP-2 expression in the stromal compartment, combined with pTNM tumour stages, may be helpful in predicting the poor prognosis in HCC patients. | _ The evaluation of MMP-2/-9 expression in the stromal compartment requires tissues from both tumour and stromal compartments through relatively larger pieces of biopsy samples may yield better results. |
| **sncRNAs and lncRNAs** | | |
| Low expression of Liver-specific microRNA-122 (miR-122) | _A tumour suppressor microRNA affecting intrahepatic metastasis of HCC by suppressing angiogenesis via regulation of ADAM17 (a potential modulator of HCC cell proliferation) disrupting EGFR signalling, however efficacy of EGFR-targeted chemotherapies in HCC is less than satisfactory. | _The role of the *miR-122*–ADAM17 link in hepatocarcinogenesis disrupting EGFR signalling needs to be clarified. |
| Lower miR-203 expression | _An antimetastatic tumour suppressor; miR-203 expression was lower in HCC tissues of patients with post-LT HCC recurrence compared to patients with no recurrence and so may be a prognostic marker in HCC patients' post-LT. | _Further studies are necessary to elucidate the molecular mechanisms that mediate miR-203 downregulation and its epigenetic alterations in HCC progression. |
| High miRNA expression signature panel | High-risk miRNA signatures including six miRNAs (miR-19a, miR-24, miR-223, miR-126, miR-886-5p and miR-147) have been related to larger sizes, angiogenesis, and poor differentiation<br>_They were associated with an increased risk of recurrence and decreased overall survival | _Further studies are needed to clarify the role of miRNAs in the molecular mechanisms of HCC recurrence. |
| Higher 1 metastasis-associated lncRNA (HOTAIR) expression | _The expression level of HOTAIR in cancer tissues was higher than in adjacent non-cancerous tissues predicting HCC recurrence in LT patients.<br>_ siRNA suppression of HOTAIR in a liver cancer cell line reduced cell viability and cell invasion, sensitized TNF-$\alpha$ induced apoptosis, and increased the chemotherapeutic sensitivity of cancer cells to cisplatin and doxorubicin, and so HOTAIR could be therapeutically targeted to reduce tumour recurrence and improve clinical therapies. | _The underlying molecular mechanisms of the HOTAIR regulation, gene expression microarray analysis involving tumour proliferation, apoptosis, and metastasis pathways, should be explained in further larger prospective studies with the detection of HOTAIR abundance in liver biopsy tissues' pre-LT candidates. |
| Higher Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) expression | _The lncRNA was found to be involved in cancer cell migration and invasion by influencing the expression of motility-related genes and disturbing the apoptosis pathways.<br>_The expression level of MALAT1 was higher in cancer tissues and associated with shortened DFS in HCC patients who underwent LT, especially those who exceeded Milan criteria.<br>_Silencing of MALAT1 by siRNA decreased cell proliferation, inhibited migration and invasion as well as sensitized cells to multi-stimuli-induced apoptosis (a therapeutic target). | _The molecular mechanism underlying the biological function of MALAT-1 is not well characterized. |





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**Table 1** (*Continued*)

| Molecular clues | Role | Limitations |
|---|---|---|
| **DNA (genes)** | | |
| CpG island methylator phenotype (CIMP) mutation | _Patients with CIMP + had higher AFP levels, multiple tumour numbers and worse recurrence-free survival, so it could serve as a prognostic biomarker of HCC recurrence after transplantation | _Future larger prospective studies are needed to focus on combining the CIMP pattern with other pre-LT criteria for proper candidate selection and prognostic prediction. |
| Higher Metastasis-associated in colon cancer-1 (MACC1) expression | _In terms of tumour recurrence; Heterozygous of SNP rs1990172 and SNP rs975263 showed a significantly high risk of relapse and may be potential biomarkers for predicting HCC recurrence in LT patients but not related to overall survival. | _Further large retrospective and prospective cohorts are necessary to demonstrate the link between the polymorphisms and the prognosis of HCC patients caused by various diseases, with trials of genomic DNA isolation from peripheral blood in pre-LT candidates. |
| Circulating tumoral cells-free nucleic acids | _Two SNPs (rs894151 and rs12438080) located in 8q22 and 15q26 were found in pre-transplant HBV-HCC patients' plasma circulating DNA had shown to be associated with HCC recurrence after LT. | _Further validated using a sufficiently large independent cohort from different transplantation centres including heterogeneous HCC samples of various aetiologies. |
| Allelic imbalance (AI) of microsatellites (MS) | _ LOH on chromosome 17p13.1 showed to contribute to the early metastatic recurrence of HCC.<br>_A panel of tumour suppressor gene markers of allelic loss (APC, CDKN2A, DCC, MET, MYC1, OGG1, p34, p53, PTEN) together with other clinicopathological features using artificial neural network ANN model could predict HCC recurrence after LT.<br>_LOH in the PTEN loci (involved in the PI3K/AKT/mTOR signalling pathway) was markedly correlated with a lower risk of HCC recurrence.<br>_ AI that was associated with HCC recurrence, was detected in 3 main *loci* (D3S2303, D9S251, and D9S254) | _Larger cohort studies with longer follow-ups and strict transplant standards selection are needed to validate their efficacy. |

Abbreviations: AFP, Alpha-fetoprotein; HCC, Hepatocellular carcinoma; IHC, Immunohistochemical; LOH, Loss of heterozygosity; LT, Liver transplantation; PCR, polymerase chain reaction; PTEN, phosphatase and tensin homolog; pTNM, pathological tumour-node-metastasis.



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contributes to the neoplastic formation, vascular invasion and poor cellular differentiation with larger sizes. On contrary, the other rest core components that are central to the Hippo tumour suppressive pathway to control tissue homeostasis, development and organ size. The MST-LATS kinase cascade including Mst1, Lats1/2, pLats1/2, pMst1/2 and pYAP do not seem to be related to recurrence.[17] So far, none of the previously mentioned proteins could be used as an independent risk factor for further evaluation.

### Enzymes

First, class I histone deacetylases have been correlated with malignant phenotype and poor prognosis in various human cancers; however, their expression patterns and prognostic role in HCC remain unclear. Histone deacetylase 3 plays a vital role in regulating tumour proliferation, indifferentiation and invasion as they may predispose to the nucleosome conformation of neoplasm exchange. Hence it may serve as a promising biomarker for predicting HBV-related HCC recurrence after LT and even a potential therapeutic target.[18] Second, the matrix metalloproteinases (MMPs), a family of secreted and membrane-anchored proteinasesand enzymes are essential for degrading the extracellular matrix and cell-associated proteins for local invasion. For example, MMP-9 can be released by neoplasms promoting the spread and so overexpression may be of prognostic value predicting metastatic manner, also positive MMP-9/2 staining in the surrounding adjacent tissues to the tumour had been associated with recurrence.[14,19] However, further studies are needed to prove the enzymes' efficacy as prognostic biomarkers.

### Small Non-Coding RNAs and Long Non-Coding RNAs

There is a growing trend that HCC tumour biology evaluation via molecular characterization holds promising results in achieving accurate clinical risk stratification of patients. It is known that microRNAs (miRNAs) have been involved in the regulation of post-transcriptional hundreds of genes expression and so affecting various biological mechanisms such as cellular proliferation and differentiation increasing the risk of carcinogenesis. Liver-specific microRNA-122 (miR-122), a tumour suppressor miRNAs affecting intrahepatic metastasis of HCC by suppressing angiogenesis via regulation of a disintegrin and metalloprotease 17 (ADAM17), hence the loss of *miR-122* expression has been associated with a worse prognosis for HCC. Moreover, miR-203, an antimetastatic tumour suppressor and higher miR-203 expression had been significantly related to better recurrence-free survival (RFS) and OS hence it could be a prognostic marker in HCC patients' post-LT and might also be a potential therapeutic target. In addition, the use of the differential miRNA expression signature panel detected the high-risk miRNA signature that was significantly correlated with tumour recurrence and HCC patients' survival following orthotopic liver transplantation (OLT), including six miRNAs (miR-19a, miR-24, miR-223, miR-126, miR-886-5p and miR-147) that have been related to larger sizes, angiogenesis and poor differentiation.[20–24] Regarding long non-coding RNAs (lncRNAs), they have been involved in various cellular processes including cell-cycle regulation, immune surveillance and stem cell pluripotency. The expression level of 1 metastasis-associated lncRNA (HOTAIR) in cancerous tissues was higher than in adjacent noncancerous tissues, also related to a significant short RFS. Moreover, specific small interfering RNA suppression of HOTAIR in a liver cancer cell line showed reduced cell viability, invasion, sensitized TNF-$\alpha$ induced apoptosis and increased cellular chemotherapeutic sensitivity to cisplatin and doxorubicin. and so over HOTAIR expression was proved to be an independent prognostic factor for predicting HCC recurrence in LT patients. Additionally, metastasis-associated lung adenocarcinoma transcript 1, an lncRNA is up-regulated in many solid tumours and associated with metastasis and recurrence. Higher levels of metastasis-associated lung adenocarcinoma transcript 1 expression had a significant risk of HCC recurrence after LT especially those that exceeded the Milan criteria.[25,26]

### DNA (genes)

Tumour suppressor genes inactivation via methylation of DNA has been associated with various malignancies' prognoses. The CpG island methylator phenotype has been shown to be associated with progression, high recurrence and low overall survival, so it could serve as a prognostic biomarker of HCC recurrence after transplantation.[27] On the other hand, the hepatocyte growth factor-MET signalling pathway is mainly involved in regulating cellular proliferation, motility, and invasion, so its dysregulation contributes to carcinogenesis and metastasis; The Metastasis-associated in colon cancer-1 (MACC1) is a key regulator gene of hepatocyte growth factor-MET pathway that promotes angiogenesis and metastatic spread; therefore, over MACC1 expression was related to a higher risk of recurrence; moreover, genetic polymorphisms of MACC1 gene was assessed to be related to poor outcome in patients having HCC. Two single nucleotide polymorphisms rs1990172/rs975263 in the MACC1 gene may be potential biomarkers for predicting HCC recurrence in LT patients.[28,29] On the other hand, HCC recipients with rs9200 heterozygous GA variant undergoing OLT were shown to be associated with lower OS, reduced RFS and higher risk for recurrence, so indicating that rs9200 GA type may be an independent prognostic marker for HCC patients post-LT;




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however, for these records' validity, larger cohort studies with longer follow-ups and strict transplant standards selection are needed.[34] Regarding, circulating tumoural cells and tumoural cell-free nucleic acids in peripheral blood represent a non-invasive test that has been evaluated for early diagnosis, micro-metastasis prediction, treatment guidance and prognoses in HCC patients owing to ongoing improvements in cell separation and identification methods; however, their potential role as preoperative predictors of HCC recurrence after LT is still a controversial issue[44,45]. High circulating nucleic acids and AFP mRNA expressions in peripheral blood has been suggested to be released from apoptotic/necrotic cancerous cells, and were associated with poor prognosis, and so can be used as a surrogate of circulating micrometastasis and increased risk of HCC recurrence after LT. Moreover, two single nucleotide polymorphism (rs894151 and rs12438080) located in 8q22 and 15q26 were found in pre-transplant patients' plasma circulating DNA had shown to be associated with HCC recurrence after LT. However, larger multi-centre studies are needed with longer follow-up outcomes to consider these circulating tumour components as valuable markers in clinical practice and evidence of their association with HCC recurrence after LT.[30,35,46] As regards, the prognostic role of various genes involved in cell-cycle regulation expression has been taken into consideration to occur in tumourigeneses. The tumour suppressor retinoblastoma protein pRb expression was shown to be useful as a predictive factor of vascular invasion and recurrence in HCC transplanted patients.[31] Finally, altered (gain and/or loss) chromosomal DNA at tumour suppressor and/or oncogene loci play a vital role in carcinogenesis and spread, and so, molecular analysis of AI of MSs situated near these loci appears to reflect the inherent metastatic potential of HCC and could be used for HCC recurrence prediction post-LT.[42] One of the 9 significant MS in HCC, MYCL 5NT, is located within the known oncogene l-myc on chromosome 1p34, proved to have the strongest correlation with HCC recurrence. Moreover, the evaluation of loss of heterozygosity (LOH) at each MS locus can identify putative tumour suppressor genes (TSGs) and thus provide a variety of HCC molecular markers; LOH on chromosome 17p13.1 showed to be correlated to metastatic HCC recurrence, while LOH on 4q and 8p was found to be associated with HCC progression. Also, a panel of tumour suppressor gene markers of allelic loss (APC, CDKN2A, DCC, MET, MYC1, OGG1, p34, p53 and PTEN) together with other clinicopathological features using artificial neural network model could predict HCC recurrence after LT. In addition, a study reported a LOH in the PTEN loci (involved in the PI3K/AKT/mTOR signalling pathway) was markedly correlated with a lower risk of HCC recurrence, and it seemed to have a protective effect on the risk of HCC recurrence. Similarly, *Duilio Pagano et al.* analysed AI in 19 MSs and assessed the post-LT HCC recurrence risk concluding AI analysis could have prognostic value in the risk management of HCC recurrence after LT. They observed AI was associated with HCC recurrence in 3 main *loci* (D3S2303, D9S251 and D9S254).[32,33,39–41]

## SCORING OF MOLECULAR RISK FACTORS

Accumulation of genetic and epigenetic alterations is a hallmark of cancer genomes, including those in HCC. Various wide cancer-related gene aberrations have been reported to be related to HCC recurrence including cellular proliferation, invasion and spread promoting aggressive neoplastic behaviour. Hence, the evaluation of liver transplant cases with HCC recurrence showed that the molecular threats for tumour spread were involving TP53 mutation and the telomerase reverse transcriptase promoter mutations expression, wide chromosome aberration and global hypomethylation with CTNNB1 mutation absence and subclassified them to determine molecular characteristics related to tumour aggressiveness. A scoring model had been speculated based on the molecular alterations including CTNNB1 mutation, TP53 mutation, telomerase reverse transcriptase promoter mutation, TSG promoter hypermethylation, significant global hypomethylation and chromosomal alterations and other clinicopathological features of a cohort of LT HCC cases concluding that the previous molecular risks were proved to predict the metastatic recurrence after curative treatments and associated with shorter RFS, and also could be a marker for considering systemic therapy for HCC patients[36,37].

In summary, significant progress has been done in the molecular field of HCC. Several risk HCC drivers including genes, proteins and miRNAs have been evaluated as biomarkers and scoring models that could predict metastatic recurrence after curative treatments. However, further analysis with comparative evaluation should be performed even if it is costly and time-consuming. Moreover, the impact of HCC-related minor genetic/epigenetic aberrations on survival outcomes needs to be more clarified; also it is uncertain whether molecular changes detected with low clonality could affect the tumour's biological trait. Therefore, evidence is currently insufficient to recommend any hypothesis for future studies on larger scales to verify the positive results.

## AVAILABILITY OF DATA AND MATERIALS

The data were collected and analysed from several studies as mentioned in the references.



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## ETHICS APPROVAL AND CONSENT

This article does not contain any studies with human or animal subjects performed by the author.

## CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

N. B. collected, designed the research data and wrote the manuscript with critical final revision and editing. (Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing).

## CONFLICTS OF INTEREST

The author declares no competing interests

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