

event in the early stage of hepatocarcinogenesis.<sup>(19,20)</sup> Moreover, some studies show that a *p16* deficiency does not fully restore the self-renewal capability of *Bmi-1*. In addition, reduced stem cell frequency occurs in *Bmi-1*-deficient neural stem cells, even when p16Ink4a and p19Arf are not expressed.<sup>(4,29,30)</sup> These studies indicate that there are additional downstream pathways that might mediate the effect of *Bmi-1* on self-renewal and cell proliferation.

From our gene expression analysis, we found that induction of *Bmi-1* in bone marrow cells resulted in an upregulation of *ABCB1*.<sup>(6)</sup> *ABCB1* appears to be a consistent feature of mammalian cells displaying resistance to multiple anticancer drugs, and has been postulated to mediate drug resistance.<sup>(31,32)</sup> Interestingly, recent findings also show expression of *ABCB1* in various stem cells,<sup>(33–35)</sup> which might make them less sensitive to cancer treatment. Increased expression of *ABCB1* was observed in HCC, particularly in early and well differentiated HCC, compared with the surrounding non-cancerous region. *ABCB1* expression decreases with the progression of HCC, suggesting a reflection of tumor dedifferentiation.<sup>(36)</sup> We showed here that *ABCB1* expression was clearly altered in parallel with *Bmi-1* expression. High expression of both *Bmi-1* and *ABCB1* was observed in the early stage of hepatocarcinogenesis, which suggests their collaboration in maintaining the cell's ability for self-renewal, proliferation, and increased resistance from apoptosis.

Although it is possible that *ABCB1* represents a novel downstream target for *Bmi-1*, further analysis is necessary to clarify the mechanism underlying the link between *Bmi-1* and *ABCB1* expression.

In summary, we evaluated the expression and involvement of the "stemness" gene, *Bmi-1*, in HCC, particularly in early stage hepatocarcinogenesis. The strong correlation observed between *Bmi-1* and *ABCB1* expression in HCC indicates a new regulatory pathway for *Bmi-1*, and reveals a potential novel target for enhancing future HCC treatment strategies.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** ATP-binding cassette transporter B1 (*ABCB1*) expression in hepatocellular carcinoma (HCC) cell lines and clinical samples. (a) Quantitative real-time PCR and Western blot of *ABCB1* in HCC cell lines. Expression of *ABCB1* was significantly higher in KIM-1 cells compared with the other cell lines. (b) *ABCB1* mRNA expression levels in HCC clinical cases. The relative mRNA expression levels in tumor tissues (black column, T) and corresponding non-cancerous, background liver tissues (gray column, N) (left panel). High levels of *ABCB1* expression were observed in well differentiated HCC. The average expression levels of *ABCB1* were higher in tumor tissues than in the non-cancerous background liver tissues (2.30 vs 1.23,  $P = 0.21$ ) (right panel). (c) Immunostaining of *ABCB1* in well differentiated HCC. An irregular and thicker form of canalicular pattern with cytoplasmic staining was observed in the tumor region compared with the non-cancerous background region (magnification,  $\times 100$ ). Black arrows outline the border between the non-cancerous background region (N) and the tumor region (T). (d) *ABCB1* expression in moderately differentiated HCC (magnification,  $\times 200$ ). Only an irregular canalicular pattern was observed (a, H&E stain; b, corresponding *ABCB1* staining).

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## Molecular Diagnosis of Multistage Hepatocarcinogenesis

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Human hepatocellular carcinoma is recognized as a good model for multistage carcinogenesis, as the malignant steps from chronic liver disease through to advanced human hepatocellular carcinoma are relatively clear. We address the activation of different molecular pathways during hepatocarcinogenesis that is especially useful in the diagnosis of pathological multistage human hepatocellular carcinoma. In chronic liver disease, the gene-expression signature as well as the degree of liver fibrosis could help us to predict the development of human hepatocellular carcinoma or survival outcome after treatment for human hepatocellular carcinoma. Several genes, such as *HSP70*, *CAP2* and *GPC3*, have been identified as potential biomarkers for early human hepatocellular carcinoma. Classical oncogenes or tumor suppressor genes, such as *beta-catenin* and *p53*, are mutated during the progression from early to advanced human hepatocellular carcinoma. Also, the presence of hepatoblastic feature like CK19 in advanced human hepatocellular carcinoma can be used as a predictor of aggressive human hepatocellular carcinoma. Although many advances have been made in the diagnosis of multistage hepatocarcinogenesis, we still need further useful markers to more precisely evaluate each step of hepatocarcinogenesis for better treatment choices, and that will promote future molecular-targeted therapy.

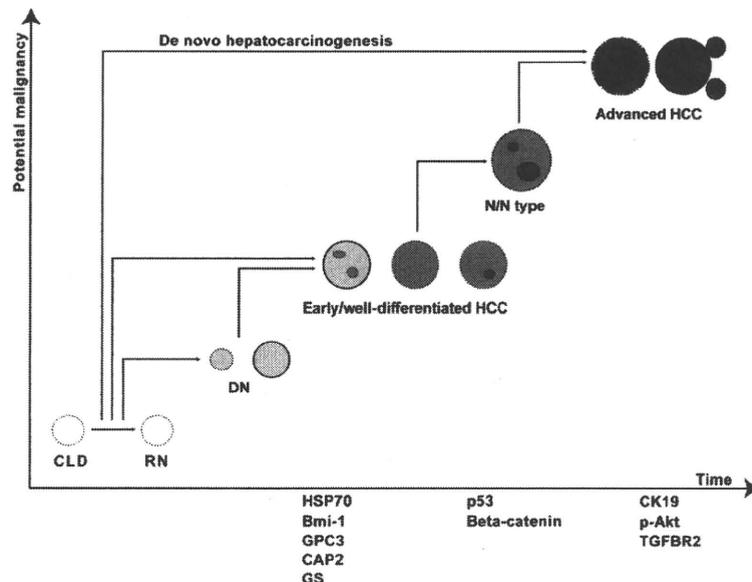
*Key words:* chronic hepatitis – early HCC – advanced HCC – hepatocarcinogenesis – molecular markers

### INTRODUCTION

Cancer is currently a major public health issue due to its high incidence and life-threatening nature. With careful and detailed follow-up of high-risk cases, we are now able to characterize and clarify the developmental process of human carcinogenesis, particularly in the early stages. In the case of human hepatocellular carcinoma (HCC), which still ranks as the third highest cause of cancer-related death globally (1), the malignant transformation step is well characterized. Through clinicopathological and molecular pathological observations, hepatocellular changes in HCC have been classified from chronic viral infection and liver cirrhosis, dysplastic nodules (DN) and early through to advanced HCC (Fig. 1). DN are usually found in chronic liver diseases and are classified as low grade or high grade (HGDN), depending on the degree of atypia. HGDN most often show increased cellularity, usually together with some cellular or structural atypia. However, these cellular or structural atypia are still indefinite for a diagnosis of HCC. In early HCC, the cell density increases compared with the surrounding non-cancerous

background liver tissue, and there is an increased nuclear/cytoplasmic ratio. Although cytological atypia are still mild, abnormal trabecular or pseudoglandular patterns as well as various degrees of diffuse fatty changes are frequently observed. Tumor cells appear as small hepatocyte-like cells that merge imperceptibly with the surrounding hepatic parenchyma, and this is sometimes difficult to recognize, both grossly and microscopically. Recently, an international consensus was achieved, indicating that the presence of stromal invasion is an important feature of early HCC and differentiates early HCC from HGDN (2). The transitional progress from an early to more advanced stage is histologically described as a nodule-in-nodule lesion, where moderately or poorly differentiated cancerous tissue grows within a well-differentiated cancer nodule. Advanced HCC often shows intrahepatic metastasis spread through portal vein invasion.

Until now, many molecular mechanisms involved in multistep hepatocarcinogenesis have been reported. One of the prominent features of hepatocarcinogenesis is the increase in hypervascularity during the process of dedifferentiation and



**Figure 1.** Evolution stage of human hepatocellular carcinoma (HCC) with its related molecular diagnosis. Some variable patterns may be observed in addition to the typical multistage developmental process of hepatocarcinogenesis. Some cases may show a direct development to the advanced stage of HCC, known as de novo hepatocarcinogenesis. CLD, chronic liver disease; RN, regenerative nodule; DN, dysplastic nodule; N/N, nodule-in-nodule.

progression, indicating the importance of angiogenic switches for the progression from early to progressed HCC (3). Understanding the molecular mechanisms involved in the malignant transformation of HCC is an important step to help us identify molecular markers or signatures in HCC development. However, the usefulness of these molecular markers in clinical practice needs careful evaluation. In this review, we will focus on the progress of some of the molecular pathology that has been, or could be, used in the diagnosis of multistage hepatocarcinogenesis.

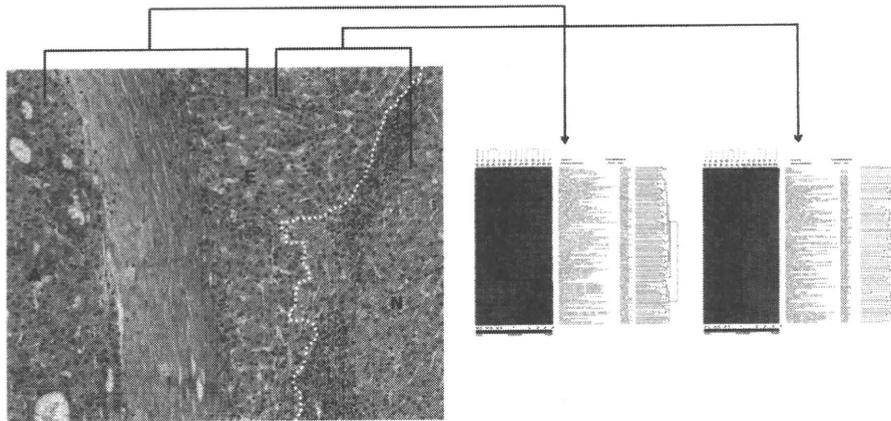
### RISK ASSESSMENT OF HCC DEVELOPMENT IN CHRONIC LIVER DISEASE

Hepatitis viral infection (hepatitis B virus (HBV), hepatitis C virus (HCV) or co-infection) is the most common cause for developing HCC worldwide. Almost all cases of HCC develop with the presence of chronic liver disease and if cirrhosis develops, the probability of HCC is increased. In HBV-infected individuals, the incidence of HCC is approximately 100 times higher compared with the uninfected population. HCV is still a major cause of HCC in Japan. HCV-associated HCC typically develops after 20–30 years after infection and is generally preceded by liver cirrhosis (4). Scoring the staging of chronic hepatitis obtained from liver biopsy specimens could be determined according to the internationally recognized degree of fibrosis scale (5). The risk of liver cancer development among individuals with HCV infection ranges between 1% and 7% per year. Another cytological change predictive for HCC development is steatosis, marked by clear vacuoles, due to fat accumulation within the hepatocytes. The presence of hepatic steatosis is associated with

increased frequency of HCC in patients with HCV-related cirrhosis (6,7). In addition to these histological findings, identifying molecular changes in liver cirrhosis associated with HCC will be necessary for the proper management of chronic liver diseases and prevention of HCC. The genome-wide expression-profiling method has been widely applied as a new approach, not only to discover molecules involved in a variety of tumor carcinogenesis (8–10), but also to identify molecules associated with precancerous conditions (Fig. 2). Around 186 signature genes were reported to be associated with the risk for HCC recurrence and could predict the survival prognosis after resection of HCC, as well as identify compensated cirrhotic patients at a high risk for HCC development (11).

### MOLECULAR DIAGNOSIS OF EARLY HCC

Early HCC is characterized as pre-invasive or early invasive cancer with a mild deviation of the clinicopathological features from chronic liver disease. Thus, early HCC lacks the typical findings of advanced HCC, such as increased arterial supply, elevated common tumor markers ( $\alpha$ -fetoprotein (AFP), protein-induced by vitamin K absence or antagonist II (PIVKA II)) and obvious histological atypia. There have been several efforts to identify molecular signatures involved in early HCC, and some of them have been used as diagnostic markers. Heat shock protein 70 (*HSP70*), found by gene-expression profiling from approximately 12 600 analyzed genes, is abundantly upregulated in early HCC. Expression of *HSP70* increases under conditions of environmental cellular stress, thus the tumorigenesis process in early HCC may act as a stressful condition to stimulate *HSP70* expression (10). *HSP70* expression is not observed in



**Figure 2.** Two-way hierarchical clustering algorithm in multistep hepatocarcinogenesis. Comparison of expression profile between early HCC (E) components and their non-cancerous surrounding tissue (N), and between advanced HCC (A) and early HCC components. Using this method, we can identify upregulated and downregulated genes involved in multistep hepatocarcinogenesis.

non-malignant nodules or other benign nodular lesions, hepatocellular adenoma and focal nodular hyperplasia. This makes HSP70 a useful histological marker to distinguish early HCC from precancerous lesions and to differentiate benign and malignant liver nodules (12). Using the same profiling method, the cyclase-associated protein2 (*CAP2*) gene was also found to be upregulated in early HCC. *CAP* was originally identified in the budding yeast *Saccharomyces cerevisiae*, and at least two isoforms of *CAP*, *CAP1* and *CAP2* have been found in mammals (13–15). Using our originally raised polyclonal antibody against human *CAP2*, we found that *CAP2* was upregulated in a stepwise manner through the progression of HCC. Interestingly, stromal invasion, a characteristic feature of early HCC, was frequently found to be positive and clearly highlighted with *CAP2* (16). Our unpublished observation also supports the evidence of *CAP2* involvement in cancer cell invasion.

Other immunohistological markers commonly cited in early HCC are glypican-3 (*GPC3*) and glutamine synthetase (*GS*). *GPC3* is a heparan sulfate proteoglycan anchored to the plasma membrane and normally expressed in the fetal liver and placenta, but not in normal adult liver tissue. *GPC3* is widely recognized as an efficient serological and histochemical marker for early HCC (17–19). It is also reportedly helpful in distinguishing small focal lesions arising in liver cirrhosis and identifying some cirrhotic macronodules with malignant potential (20,21). However, *GPC3* has also found to be expressed in benign liver tissue with active inflammation, which urges caution when interpreting *GPC3* staining (22). Additional markers may thus be needed to boost the diagnosis. *GS* is an enzyme that catalyzes glutamate and ammonia to form glutamine in the mammalian liver (23). The final product from *GS* synthetase, glutamine, is reported to provide an energy source for tumor cells (24). *GS* is a target of the Wnt/beta-catenin pathway and is a good immunohistochemical marker of beta-catenin activation in HCC (25,26). Overexpression of *GS* increases with the

stepwise progress of hepatocarcinogenesis, indicating an important role of *GS* in promoting the metastatic potential of HCC (27,28). A combination of the three-marker panel of *HSP70*, *GPC3* and *GS* can raise the accuracy of detecting early HCC. A 72% sensitivity and 100% specificity, where the most sensitive combination was *HSP70* and *GPC3*, have been shown (29).

Recently, we identified the overexpression of *Bmi-1*, a member of the Polycomb gene group, in early HCC. *Bmi-1* is indicated as one of the signaling pathways that results in the 'stemness feature' in cancer through its ability to immortalize cells by inducing telomerase activity. *Bmi-1* also promotes tumorigenesis by acting as a negative regulator for the well-known tumor suppressors genes, *p16* and *p19* (30,31). In our study, *Bmi-1* expression appeared as small dots inside the nuclear area that may reflect concentrated sites of *Bmi-1* activity. Elevated *Bmi-1* expression was particularly evident in early and well-differentiated HCC, compared with the surrounding, non-cancerous liver tissue (32). Investigations to explore the applicability of *Bmi-1* as a new diagnostic marker for early HCC are currently on going.

## MOLECULAR SUBCLASSIFICATION OF ADVANCED HCC

Advanced HCC shows a solid growth pattern with a decrease in the distinct trabecular pattern, and pleomorphisms are frequently observed. It commonly displays a variety of histological grading within its nodule; poorly differentiated tissue is located inside, surrounded by well-differentiated tissue. A transitional progress from early to advanced HCC is observed histologically as a nodule-in-nodule lesion. The presence of the *p53* mutation has been detected in the inner, advanced nodule, but not in the outer well surrounding nodule, suggesting an association between the *p53* gene mutation and the late events of HCC progression (33–35).

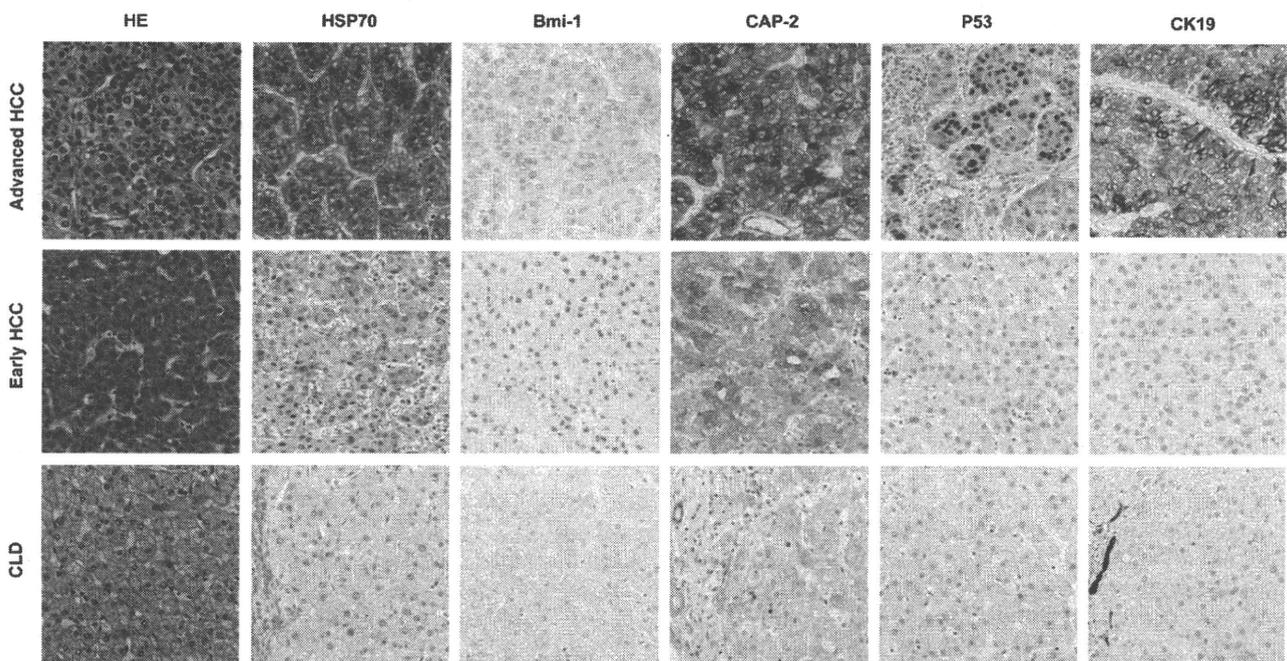
Nuclear accumulation of p53, detected by immunohistochemistry, is useful for the recognition of advanced HCC. Another mutation associated with the malignant progression of HCC is the *beta-catenin* mutation. Disruption of the Wnt/beta-catenin pathway causes accumulation of beta-catenin in the nucleus (36). However, the correlation of beta-catenin expression and HCC progression is not yet clear. Some studies report its association with a poor prognosis, whereas other studies associate it with a more favorable outcome (36–38). Additional signaling pathways associated with the aggressiveness of HCC include the TGF- $\beta$  and Akt pathways. The potential of TGF- $\beta$  gene expression to refine the diagnosis and prognostic predictions in HCC patients has also been reported. The outcome of TGF- $\beta$  signaling is highly contextual in different tissues and depends on the cellular microenvironment. TGF- $\beta$  signaling in carcinogenesis is intriguing due to its ability to act both as a tumor suppressor in the early stage, and a tumor promoter in the later stage. In HCC, changes in TGF- $\beta$  signaling are still controversial and unclear. Coulouarn et al. showed that patients with a late TGF- $\beta$  signature showed a significant decrease in mean survival time compared with patients with an early TGF- $\beta$  signature. A late TGF- $\beta$  signature predicted liver metastases and identified HCC cell lines according to their degree of invasiveness (39). We also found that poorly differentiated HCC showed a reduced expression of TGFBR2, and this correlated with intrahepatic metastasis and early recurrence (40). It will be interesting to further explore the implications of TGF- $\beta$  signaling in

HCC. Akt phosphorylation (p-Akt) has been identified as a significant risk factor for early disease recurrence and poor prognosis in HCC through its correlation with anchorage-independent growth malignant behavior (41).

In addition, some biliary or hepatic progenitor markers, such as CK7 or CK19, are frequently found in poorly differentiated cells (42). The prevalence of intrahepatic metastases was found to be significantly higher in patients with CK7-positive HCC, than in those with CK7-negative HCC. Patients with CK19 positivity had a significantly higher incidence of early recurrence suggesting a worse prognosis than CK19-negative HCC patients (42–45). HCC is postulated to be generated through carcinogenesis from hepatic progenitor cells (HPC) that are capable of differentiating into both hepatocytes and biliary epithelial cells (43,46). However, whether the hepatoblastic feature origin occurs via a de-differentiation progress, or originates from HPC, is still a matter of debate. Another hepatic progenitor cell marker, identified as epithelial cell adhesion molecule (EpCAM), when combined together with the embryonic liver marker, AFP, could help to define the clinicopathological characteristics of HCC subtypes. These HCC subtypes may resemble certain stages of hepatic lineages and enable prognostic assessment of HCC patients (47).

## CONCLUSION

We have briefly reviewed the development and application of the molecular diagnosis of multistage hepatocarcinogenesis (Fig. 3). We need to standardize the histological



**Figure 3.** Panel of histological markers in HCC clinical specimens. The use of HSP70, Bmi-1 and CAP2 was helpful to recognize early stage of HCC. HSP70 and CAP2 expressions were getting increased following the progression of HCC, whereas Bmi-1 was particularly highly expressed in early- or well-differentiated HCC. In advanced stage of HCC, positive p53 and CK19 specimens were frequently observed. Their expressions were also related to a worse prognosis of HCC.

molecular markers used in the diagnosis of HCC, as the developmental process of hepatocarcinogenesis itself may not be simple and differs from patient to patient. We also need to detail the molecular characteristics and histological patterns that reflect the malignant potency in each step of hepatocarcinogenesis to determine the accurate staging of HCC. A combination diagnostic approach using biomarkers besides histological markers, such as serum markers, genetic markers or imaging contrast, will also greatly enhance our ability to overcome the highly malignant HCC. This will further establish prevention, as well as promote better treatment choice in the new era of molecular-targeted therapy and individualized cancer treatment.

### Conflict of interest statement

None declared.

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