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Molecular classification and novel targets in hepatocellular carcinoma: recent advancements

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Abstract

Hepatocellular carcinoma (HCC) is one of most lethal cancers worldwide. Strategic decisions for the advancement of molecular therapies in this neoplasm require a clear understanding of its molecular classification. Studies indicate aberrant activation of signaling pathways involved in cellular proliferation (e.g., epidermal growth factor and RAS/mitogen-activated protein kinase pathways), survival (e.g., AKT/mechanistic target of rapamycin pathway), differentiation (e.g., WNT and Hedgehog pathways), and angiogenesis (e.g., vascular endothelial growth factor and platelet-derived growth factor), which is heterogeneously presented in each tumor. Integrative analysis of accumulated genomic datasets has revealed global scheme of molecular classification of HCC tumors observed across diverse etiological factors and geographic locations. Such framework will allow systematic understanding of the frequently co-occurring molecular aberrations to design treatment strategy for each specific subclass of tumors. Accompanied with growing number of clinical trials of molecular targeted drugs, diagnostic and prognostic biomarker development will be facilitated with special attention on study design and with new assay technologies specialized for archived fixed tissues. New class of genomic information, microRNA dysregulation and epigenetic alterations, will provide insight for more precise understanding of disease mechanism and expand the opportunity of biomarker/therapeutic target discovery. These efforts will eventually enable personalized management of HCC.

Keywords

Hepatocellular carcinoma; molecular classification; meta-analysis; signaling pathway

Introduction

Primary liver cancer, predominantly hepatocellular carcinoma (HCC), is a major health problem being the third most common cause of cancer death worldwide[1]. While more than

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80% of the cases occur in sub-Saharan Africa and Eastern Asia, the incidence has been increasing in Western countries[2]. In the U.S., liver cancer is the most rapidly increasing cause of cancer death, which is ranked at third in men ages 40 to 60, suggesting its large socioeconomic impact[2, 3]. Potentially curative therapies like surgical resection, transplantation and percutaneous ablation are only available to patients with limited disease which represent around one third of the cases[3].

Majority of patients with HCC have underlying chronic liver diseases. Eighty-percent of the patients are accompanied with liver cirrhosis caused by either of chronic infection of hepatitis B virus (HBV) or hepatitis C virus (HCV), excessive alcohol intake, inherent or lifestyle-related metabolic disorders, exposure to dietary hepatocarcinogen like aflatoxin, etc[4]. Distribution of these risk factors varies across geographic regions: Chronic HBV infection is the major cause in developing countries in Asia. HCV is the dominant etiology in developed countries in the West and Japan. Food contamination with aflatoxin is seen in Southeast Asia[2].

In the clinical management of HCC, disease staging system is a vital component to classify patients into prognostic subgroups connected to adequate treatment option[4]. Clinical variables associated with prognosis reflect extent of tumor spread (tumor size, number of tumor nodules, vascular invasion, distant metastasis, etc) and severity of liver damage (protein synthesis and detoxification function, symptom of hepatic decompensation, etc.)[5], and some of these variables have been incorporated in clinical staging systems. Recent successful implementation of tumor surveillance program targeting cirrhotic patients has increased the proportion of patients at early stage HCC in developed countries[6]. This shift towards early stage disease has posed a challenge in prognostic risk assessment because such patient population is characterized by lack of known risk factors of poor prognosis. Hence, there is a pressing need for a genome-based classification to refine clinical staging system for this specific subgroup of patients[7–9]. In addition, recent success of molecular targeted agent, a multikinase inhibitor sorafenib, strongly encourages molecular information-based patient classification in view of personalized treatment of HCC[10].

In this review, we summarize previous efforts to establish a molecular classification of HCC performed on diverse patient populations across the world utilizing wide variety of DNA microarray platforms, and discuss unified global overview of molecular classification. We also overview rapidly increasing evidences on new class of genomic information, microRNA dysregulations and epigenetic alterations, and provide updated knowledge of relevant molecular pathways targeted by experimental therapies under development.

Genome-based molecular classification of HCC

Preceding efforts have suggested that molecular abnormalities involved in HCC pathogenesis include (1) cell cycle dysregulation caused by somatic mutations or loss of heterozygosity in *TP53*, silencing of *CDKN2A* or *RB1*, or *CCND1* overexpression, (2) increased angiogenesis accompanied with overexpression or amplification of *VEGF, PDGF*, or *ANGPT2*, (3) evasion of apoptosis as a result of activation of survival signals like nuclear factor-kappa B (NF-kB) pathway, and (4) reactivation of *TERT* with the process of carcinogenesis[11–16]. Besides these biological processes, which seem to be affected in majority of HCC tumors, rapidly evolving genome-based assays have revealed that there is huge molecular heterogeneity across HCC tumors.

Recent development of genomics technologies has enabled to obtain multiple layer of comprehensive molecular landscape directly from clinical tissue specimens. High-density single nucleotide polymorphism array and array-based comparative genomic hybridization have been employed to study structural chromosomal alterations, and depicted enormous

complexity and heterogeneity of genetic aberrations that occur throughout the genome, likely attributable to the accumulation of chromosomal rearrangements resulting from decades of chronic hepatitis and cirrhosis. Multiple studies showed that there are certain frequently affected regions, including gain in chromosome 1q, 6p, 8q, 17q, and 20q, and loss in 4q, 8p, 13q, 16q, and 17p[17]. However, hugely diverse alterations beyond these loci make it difficult to fish out "driver" events promoting HCC development and progression as opposed to "passenger" events. Several efforts have been tried to clarify potentially functional and clinically relevant alterations[18, 19].

Genome-wide gene-expression profiling has been applied as an alternative approach, which is assumed to directly capture functional dysregulation of molecular pathways observed in each tumor. This approach is expected to be more powerful because down stream effect of any molecular perturbations can be captured as altered gene-expression pattern ("signature") in the transcriptome space even if the gene or pathway component itself is not obviously changed at its transcript level[20, 21].

Dozens of genome-wide gene-expression studies have been conducted to date and reported potential roles of specific genes and molecular pathways in pathogenesis of HCC, and some of them proposed molecular classification of HCC (Table 1). An early study profiling a relatively large collection of HCC tumors obtained from mostly HBV-infected Chinese patients revealed the presence of two distinct subclasses associated with either poor or good prognosis[22]. The poor-prognosis-associated subclass signature was similar to that of HCC in *Myc/Tgfa* transgenic mice[23] as well as Met-regulated gene-expression in mouse liver[24]. The good-prognosis-associated subclass showed similarity to HCC in *E2f1, Myc*, or *Myc/E2f1* transgenic mice[23]. The investigators further identified a subset of the poor-prognosis tumors expressing hepatic progenitor cell-like (hepatoblast)[25] and transforming growth factor (TGF)-beta target gene signatures[26], which showed extremely poor prognosis.

Studies profiling mostly Caucasian patients with various etiologies[27] or solely HCV infection[28] reported that there is a subset of tumors characterized by increased cellular proliferation accompanied with phosphorylaton of *IGF, AKT*, and *RPS6*, indicative of kinase activation. These Western studies also highlighted the presence of a gene-expression subclass associated with somatic mutations in exon 3 in *CTNNB1* gene and nuclear accumulation of beta-catenin protein. Although this was assumed to indicate canonical WNT pathway activation, one of these researchers found that not canonical WNT targets like *CCND1* and *MYC*, but amino acid metabolism-related genes, *GLUL, LGR5*, and *SLC1A2*, were induced in this subclass[29]. This observation suggests that there is biological context-dependent diversity in activation of specific downstream path of complex WNT pathway cascade[30, 31].

Other molecular abnormalities observed in gene-expression-based subclasses of HCC tumors include overexpression of interferon (IFN)-related genes[28], mutually-exclusive expression of IFN-related genes and *IGF2*[32], polysomy of chromosome 7[28], and overexpression of *CD24*[33, 34]. Yamashita and colleagues proposed to classify HCC tumors in consideration of hypothetical cellular origin based on expression levels of *EPCAM* and alpha-fetoprotein (AFP)[35].

Other studies focused on gene-expression profiles correlated with clinical phenotypes of interest, including process of step-wise hepatocarcinogenesis[36–38], tumor recurrence after surgery[33, 39–44], and vascular invasion of HCC tumor[34, 42, 45]. Gene-expression patterns correlated with specific molecular events, e.g., *TP53* inactivation[45] and positivity of a progenitor marker *KRT19*[46], may help characterize each tumor. A gene-expression signature predictive of aggressive childhood hepatoblastoma[47] may also help classify HCC tumors,

given the hepatic progenitor cell-like (hepatoblast) signature presented prognostic relevance[25].

The wide variety of findings likely reflects the molecular heterogeneity of HCC pathogenesis, which may reflect highly diverse clinical background of the patients as well as DNA microarray platform-related measurement variation [48–50]. For these reasons, it has been unclear whether these pieces of molecular information are reproduced across different study populations. In addition, some studies highlighted difference in gene-expression pattern between HBV- and HCV-related HCC and chronic hepatitis[51–53], posing a question whether the viral etiology is dominant determinant of molecular characteristic of HCC tumor.

Nevertheless, multiple studies have suggested that HCC tumors can be roughly classified into two subgroups according to the extent of genetic instability exhibited as more frequent chromosomal alterations[54–57]. Higher genetic instability is associated with poor histological tumor differentiation[55, 58, 59], increased cellular proliferation and protein ubiquitination, suppression of apoptosis[22], and poor clinical outcome[22, 54, 55]. All of these findings seem to be linked to aggressive biological behavior of the tumors and strongly suggest that there is common molecular classification presented across diverse patient populations irrespective of the distribution of etiological factors, patient ethnicity, and disease stage. Such classification will serve as a global scheme to help unify the pieces of diverse molecular information reported in the literatures.

Common molecular subclasses of HCC

Many of the published microarray studies have made the datasets available in public domain. It has enabled systematic reanalysis to seek for common molecular classification across multiple studies[60–62]. A recent attempt of meta-analysis enrolling a total of 603 HCC patients has revealed that common transcriptome-based subclasses exist across multiple studies, supporting the idea that there is certain commonality in global molecular status of HCC tumors irrespective of the clinical heterogeneity in patient populations across the world[63].

As suspected from the previous studies, the distinction of aggressive and less-aggressive tumors was observed in all 9 independent datasets with striking similar gene-expression pattern with previously reported subclasses of aggressive and less-aggressive tumors (Figure 1). The subclasses of aggressive tumors (termed S1 and S2) were associated with larger tumor size and poorer histological differentiation. HBV-related tumors tend to be enriched in these subgroups in consistent with previous observation[27, 54, 55]. It is known that a subset of HBV-related HCC occurs before development of liver cirrhosis, assumedly due to direct carcinogenic effect of HBV, and often shows rapid disease progression. A body of evidence has suggested that there are certain HBV strains or host-related factors associated with this direct carcinogenic effect[64–67]. These factors may be correlated with the molecular classification.

The subclasses S1 and S2 showed distinct molecular aberrations, while sharing some common characteristics, e.g., activation of *E2F1* and inactivation of *TP53*. The subclass S1 was characterized by activation of TGF-beta pathway with significant similarity to previously reported TGF-beta-activated subclass[26]. Interestingly, this activation was accompanied with induction of experimentally-defined WNT target gene-expression signature[68], which was not associated with the *CTNNB1* mutations. This seems to support the idea of context-dependent diversity in downstream effect of WNT activation.

The subclass S2 is characterized by activation of *MYC* and *AKT*, overexpression of *AFP* and *IGF2*, and down-regulation of IFN-related genes. The mutually exclusive expression of *IGF2* and IFN-related genes is consistent with the previous observation[32]. Serum AFP level was also higher in this subclass. It is clinically known that just up to 60% of HCC tumors show elevated AFP[69]. The subclass may provide insight into underlying molecular property of this clinically well characterized subset of tumors. Yamashita's *EPCAM* signature[35] and Minguez's vascular invasion signature[34] are enriched in this subclass.

One interesting observation is that the hepatic progenitor cell-like signature[25] and the signature of aggressive hepatoblastoma[47] were mapped onto different subclasses, while *KRT19*-associated signature was similarly enriched in both of S1 and S2 subclasses. This may indicate that these signatures and markers thought to represent characteristic of the progenitor cells correspond to slightly different cell populations. Similarly, three gene-expression signatures predictive of early tumor recurrence were mapped onto either S1 or S2, suggesting there are multiple independent mechanisms of tumor cell dissemination in HCC.

The subclass of less-aggressive and more differentiated tumors (termed S3) characterized by retained normal liver function, i.e., relative up-regulation of genes and pathways involved in various metabolism, detoxification, and protein synthesis. Previously reported subclasses, "CTNNB1" [28], "polysomy of chromosome 7"[28] and Boyault's "G5,6"[27], showed moderate association, suggesting that these are subsets of S3 tumors. "CTNNB1" and "G5,6" subclasses were linked to *CTNNB1* gene mutations in previous studies. This is in agreement with the finding that beta-catenin activation resulting from *CTNNB1* gene mutations is seen in a subset of less-aggressive HCC tumors in integrative transcriptome analysis of human and mouse HCC[56]. Such molecular heterogeneity within this subclass may reflect multiple different paths of HCC development or difference in cellular origin.

Transcriptional profiling of a panel of hepatoma cell lines revealed that there are two distinct clusters based on genome-wide gene-expression pattern, which is tightly correlated with expression status of AFP[70, 71]. Interestingly, we found that these AFP-producing hepatoma cell lines presented the S2 subclass signature, while most of the rest presented the S1 subclass signature[63].

It has become obvious that each HCC tumor nodule simultaneously harbors dysregulation of multiple molecular pathways, and the involvement of each pathway is highly heterogeneous across tumors. Tumor classification based on genomic information helps identify recurrently co-occurring dysregulations. It will be informative to clarify whether these are coordinated events, cross talk between affected pathways, or mere co-occurrence of independent events with no specific interaction between pathways in designing personalized combination targeted therapy. It is known that HCC tumors often exhibit "nodule-in-nodule" structure, where undifferentiated subnodule present within differentiated nodule. It is possible that some of previously generated profiles reflect gene expression of mixture of these sub-nodules, which may be independently governed by distinct molecular mechanisms. Or the profiles may be mixture of heterogeneous cell populations even if it looks histologically homogeneous.

Furthermore, recent genomics studies suggest that gene expression in non-tumor liver, usually diseased with chronic hepatitis or cirrhosis, harbors information of risk of *de novo* cancers[44, 72], multicentric cancers[73], and intrahepatic metastasis[74], and anti-tumor inflammatory response[75, 76], which could be used for the purpose of outcome prediction. These findings may provide additional information onto current clinical staging system once replicated in multiple independent patient populations.

Clinical outcome and molecular classification

There is a need for refining current clinical systems with genomic variables able to capture biological characteristics relevant for understanding patient's outcome. There are several critical issues in generalizing molecular outcome prediction in HCC. First, genome-based prognostic markers are often invalid due to inappropriate study design[77–79]. Representation of clinical characteristics is often biased when the samples are retrospectively collected from tissue resource, which was archived without intension of the study at the time of acquisition. Small sample size often enhances the bias. Due to this frequently observed problem, signatures trained to predict the same outcome may capture different biological properties and therefore not be generalizable. In addition, validation is often not conducted appropriately in independent set of patients.

Second, HCC outcome prediction is more complicated compared to other cancer types because of the fact that most of the patients have two life-limiting diseases, HCC and cirrhosis[8]. More specifically, outcome of surgically treated HCC is determined by two distinct types of tumor recurrence: dissemination of primary tumor cells ("intrahepatic metastasis" or "early recurrence") and *de novo* tumors arisen from underlying diseased liver presenting carcinogenic "field effect" ("multicentric carcinogenesis" or "late recurrence")[80–85] (Figure 2A). That is, it is critical to know which type of recurrence is the major determinant of outcome in each study population[86]. Imamura and colleagues proposed a convenient way to enrich these two types of recurrence by using a cut-off of 2 years after the surgical resection[87]. This has enabled to define and assess clinical and molecular variables associated with prognosis driven by either type of recurrence[44, 88, 89].

The hazard of "early" and "late" recurrences could present differently according to stage of the tumor covered in each study (Figure 2B). It is worth to note that recurrence within 2 years can include both the dissemination and *de novo* carcinogenesis. In a patient series including relatively early stage diseases, tumors are more likely to be removed with less chance of dissemination, and thus it is assumed that "late" recurrence (i.e., *de novo* tumors) becomes more influential on patient prognosis. The hazard of "early" recurrence could even become negligible in patients with very early stage HCC increasing as a result of regular tumor surveillance[6, 44]. In this scenario, molecular signature trained for "recurrence within 2 years" may capture more aggressive *de novo* carcinogenesis arisen from the "field effect". In contrast, prognosis of patient series with advanced HCC is assumed to be mostly determined by "early" recurrence (i.e., primary tumor dissemination).

Increasing complexity of the molecular classification: miRNA and epigenetics

The complexity in defining the molecular classification of HCC has increased with the recent publications identifying specific miRNAs and epigenetic changes as relevant for predicting clinical outcomes. These findings also expand the armamentarium of potential targets for therapies in HCC.

microRNA dysregulation in HCC

microRNA (miRNA) is a class of small non-coding RNA that negatively regulates gene expression by targeting mRNA for translational repression or cleavage[90]. miRNA is involved in the regulation of a variety of cellular processes such as cell proliferation, cell differentiation, apoptosis, and stem cell maintenance, and has been reported to be relevant in various human diseases including cancer. Depending on the genes they target, miRNA can act as oncogene or tumor suppressor gene[91].

Recently, different studies reported aberrant expression of miRNA in HCC tumors compared with their normal counterparts, suggesting its involvement in hepatocarcinogenesis (Table 2). Among them, *MIR21* has been shown to be overexpressed also in a variety of cancers, e.g., breast, lung, ovarian carcinomas, and glioblastoma. *MIR21* directly inhibits the expression of *PTEN*, leading to abnormal activation of phosphoinositide-3-kinase/*AKT* pathway[92]. Similarly, *MIR221/MIR222* overexpression has been reported in pancreas, stomach and colon cancers. The cyclin-dependent inhibitors *CDKN1B* (p27) and *CDKN1C* (p57) were found to be regulated by *MIR221*[93]. *MIR143* is transcribed by NF-kB and associated with metastasis by repressing *FNDC3B*[94]. Other aberrantly overexpressed miRNA in HCC include *MIR224*[95], *MIR9*[96], and *MIR181*, which is expressed in *EPCAM*-positive tumors[97].

On the other hand, several miRNAs have been found significantly down-regulated in HCC. Members of *Let-7* family were suppressed in advanced stage HCC with poor prognosis, and their expression was found to be regulated by *LIN28*[98]. *Let-7* family includes tumor suppressor miRNAs that target oncogenes like *MYC, RAS*, and *HMGA2*. This may be the mechanism of up-regulation of some oncogenes in human HCC, in which gene mutations are not frequently observed[17, 99]. *MIR122* is a liver-specific regulator of cholesterol and fatty-acid metabolism, and was found to be down-regulated in more than 70% of HCC, suggesting that loss of tissue lineage-specific miRNA may be associated with dedifferentiation of the tumor[100]. *MIR122* was silenced by DNA methylation, and involved in tumourigenesis by activating *CCNG1*. *MIR1*, regulating *FOXP1, MET*, and *HDAC4*, was also silenced by gene methylation[101] Another study showed that loss of *MIR122*, transcriptionally controlled by *HNF1A, HNF3A* and *HNF3B*, was associated with metastatic potential of the tumors[102]. Epigenetic silencing also affects *MIR124* or *MIR203*, which target *CDK6, VIM, SMYD3*, and *IQGAP1* or *ABCE1*, respectively[103]. *MIR101* was also suppressed in HCC, and associated with regulation of an oncogene *FOS*[104] and an apoptosis-related gene *MCL1*[105]. Down-regulation of *MIR195*[106] and *MIR34A*[107] were reported to be associated with cell cycle regulation and cell migration/invasion via *MET*, respectively. Reduced *MIR26* expression was associated with regulation of NF-kB and interleukin-6 (IL6) pathways108.

Increasing evidences suggest that miRNA expression profiling can be used for cancer classification. An early study suggested that miRNA profiling may harbor tissue lineage-specific information more strongly compared to mRNA profiling[109]. This is in agreement with the hypothesis that miRNA directs tissue-specific developmental functions[110]. Similarly, Rosenfeld and colleagues showed that miRNA profiling correctly identified cancer tissue origin[111]. miRNA profile can also be used to distinguish cancer from normal tissues, supporting its potential value as diagnostic marker for early cancer[112–114]. Especially in HCC, miRNA profiling is expected as a source of additional information to understand the complex molecular heterogeneity and provide clues for new therapeutic targets. Unsupervised clustering of miRNA profiles revealed subclasses associated with histological and etiological factors as well as mutations in *CTNNB1* and *HIF1A* genes[115]. HCV-related HCC tumors were classified into distinct molecular subclasses associated with aggressive clinical phenotypes, *CTNNB1* mutations, etc.[116] HBV- or HCV-specific dysregulation was also reported[114].

Other studies reported prognostic relevance of miRNA expression. Overexpression of *MIR221* was associated with tumor multinodularity and higher risk of recurrence after surgery[117]. *MIR125B* expression was correlated with good survival[118]. Low *MIR26* level was correlated with poor survival but a better response to interferon therapy[108]. miRNA expression signature predictive of patient survival[119] and metastatic potential[120] were also reported.

These evidences clearly show that miRNA has a great potential in biomarker and therapeutic target discovery, providing rich molecular information. The relatively smaller number in the human genome (~1,000 for miRNA vs. ~30,000 for mRNA) may make expression profiling less complicated. The short nucleotide length (19–24bp) will ensure better preservation even in fixed tissues[121, 122], suggesting its high clinical applicability. It has become possible to modulate expression of specific miRNA by using chemically-modified oligonucleotides, locked nucleic acid (LNA), and miRNA mimics, which are currently evaluated *in vivo*[123]. A recent study showed that the replacement of only one miRNA, *MIR26A*, using an adenovirus-associated vector resulted in inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and protection from disease progression without toxicity in a mouse model of HCC[124].

Epigenetic alterations in HCC

There is increasing evidence that epigenetic changes play an important role in cancer pathogenesis. They seem to act predominantly in the initiation of carcinogenesis and complement genetic alterations in the development and progression of cancer[125]. These changes involve alterations in DNA methylation and histone modifications, two mechanisms that lead to heritable changes in gene expression that are not caused by alterations in the genomic DNA sequence. A common event resulting in the disruption of cell signaling pathways in cancer is the silencing of genes by hypermethylation of CpG islands within their promoter regions[126]. In HCC, tumor suppressor genes and genes involved in cell cylce, apoptosis, or DNA repair among many others have been reported to be hypermethylated and repressed[127–144] (Table 3). Interestingly, HCV infection seems to accelerate the methylation process in HCC[145]. DNA methyltransferases (DNMT) are enzymes responsible for DNA methylation and frequently overexpressed in cancer cells. Overexpression of *DNMT1* and *DNMT3B* were reported as early events during hepatocarcinogenesis[146]. The former is responsible for maintenance of genomic methylation status, while the latter acts mainly in *de novo* methylation. While hypomethylation of promoter regions can lead to activation of oncogenes like *MYC* or *RAS*[147, 148], genome-wide global hypomethylation is a much more common event in cancer leading to genomic instability, which was also described in HCC [149] as correlated with higher histological grade and larger size of the tumors[147].

Histones actively regulate gene expression through post-translational modifications including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation on their N-terminal tails. These can regulate gene expression either directly by the histone tail modification or indirectly through chromatin remodeling. Some genes regulated by histone modifications have been described in HCC: histone H3 lysine 27 methylation for *PGR* and *ESR1*, histone H3 Lysine 9 hypoacetylation and trimethylation for *TMS1*, and histone H3 lysines 4 and 9 tri-methylation for *JNK1*[150–152]. Hepatitis B-x antigen was shown to recruit *HDAC1* and transcriptionally repress expression of *IGFBP3*[153].

DNA methylation mapping in 12 genes revealed cancer-specific profiles of hypermethylated CpG islands (hypermethylomes) that were able to distinguish different tumor types[154]. Genome-wide DNA methylation profiling integrated with multiple genomic information revealed handful of core pathways in glioblastoma[155]. These results suggest that large-scale DNA methylation profiling may be used to classify HCC tumors. In fact, level of genome-wide hypomethylation correlated with HCC progression and genomic instability, and CpG hypermethylation of 105 tested tumor suppressor genes correlated with HCC developoment and progression[149]. Interestingly, more global hypomethylation, promoter hypermethylation, and genomic instability were correlated with previously described subclass of poor prognosis[22, 149]. A profiling of methylation status of ~6,500 CpG islands in HCC tumor and corresponding non-tumor tissues identified different methylation patterns specific to normal, preneopastic, and cancerous tissues as well as poorly differentiated tumors[156]. A study

showed variation in methylation pattern according to geographic location, suggesting the effect of environmental factors[132]. Change of methylation pattern was already observed in non-cancerous liver tissues in hereditary hemochromatosis[157].

In view of cancer treatment, one great potential of epigenetic aberrations is their reversibility, which may revert transformed malignant cells toward normal state[158]. Demethylating agents and histone deacetylase inhibitors (HDACi) are able to rescure epigenetically silenced gene expression either by demethylation of methylated promoter regions or by histone acetylation[159]. Demethylating agents like 5-aza-2'-deoxycytidine and 5-azacytidine have first been approved by FDA for the treatment of myelodysplastic syndrome and are currently under preclinical and clinical evaluation for several other hematological and solid cancers including HCC[160]. Vorinostat was approved for the treatment of cutaneous T-cell lymphoma, while several novel HDACis (MS-275, tributyrin, valproic acid) have been tested preclinically in HCC and other cancers, showing promising results mainly when used as a part of combination therapy. A pan-HDACi LBH589 was preclinically evaluated in other cancer types[161], and is now tested in combination with sorafenib in a phase I trial for advanced HCC.

Up-dated advancements in signaling pathways and targeted therapies

Intracellular signaling pathways regulate cellular functions, including cell differentiation, proliferation, apoptosis, metabolism, etc. Studies have demonstrated aberrant activation of multiple signaling pathways in human cancer, including HCC. Their translational interest resides on being the substrate for many molecular targeted therapies ready for clinical evaluation. Erlotinib in lung cancer with *EGFR* overexpression/mutation [162], trastuzumab in breast cancer with *ERBB2* amplifications [163], and imatinib in chronic myeloid leukemia[164] are examples of the pathway-targeted strategy.

In HCC, accumulated evidences have showed dysregulation of pathways involved in cellular differentiation (e.g., WNT, Hedgehog), proliferation (e.g., *EGF, IGF, HGF*, RAS/mitogen-activated protein kinase), survival (e.g., AKT/mechanistic target of rapamycin (MTOR)), and angiogenesis (e.g., *VEGF, PDGF, FGF*), etc.[17, 165–169] It is known that there are functional and structural overlap and crosstalk between these pathways, which hamper their understanding. Some pathways are found to be associated with the molecular subclasses of HCC tumors. The subclass of aggressive tumors (e.g., Chiang's "proliferation", Lee's "cluster A", and Boyault's "G1~3") is accompanied with activation of pathways involved in cell proliferation and survival like insulin-like growth factor (IGF) and AKT/MTOR pathways. A subset of the less-aggressive tumors showed dysregulation of pathway potentially involved in cell differentiation (e.g., Chiang's "CTNNB1" and Boyault's "G5,6"). Some of them may indicate "oncogene addiction"[170] that can be utilized for targeted therapy, while others may represent the path of cancer initiation and progression each tumor went through.

Recent genomic studies showed that non-tumor liver tissue exhibits dysregulation of molecular pathways like NF-kB and IL6 pathways[44, 74, 171]. Case-control studies reported association of Epidermal growth factor (EGF) signaling pathways with the risk of HCC development in cirrhotic patients[172, 173]. These preliminary data may indicate relevance of these pathways as targets of chemopreventive therapy[174, 175].

The success of a multikinase inhibitor, sorafenib, has opened the door for molecular targeted therapy as practical treatment option in HCC[10, 176] and also presented an urgent need for unified framework to evaluate such molecular targeted agents in clinical trials. The American Association for the Study of Liver Diseases consensus panel recently recommended the use of robust clinical endpoints such as overall survival and time to

disease progression rather than less robust endpoints like progression free survival or response rate in HCC trials[177]. The positive trial identifying the survival benefits of sorafenib despite marginal response rates pointed to the need of assessing time to event end-points in phase II studies, and also to capture benefits with modified metrics, such as the AASLD-JNCI modification of RECIST criteria[177].

Following sorafenib, numerous compounds have been moved to later phases of clinical development (Table 4). Phase II trials of an EGF signaling inhibitor, erlotinib, showed median survival between 11 and 15 months alone or in combination with an anti-angiogenic drug bevacizumab[178–180]. Another multikinase inhibitor sunitib, targeting *VEGFR, PDGFR*, and *CKIT*, has also been under evaluation[181, 182], showing some anti-tumoral effect, although toxicity seems to be higher than sorafenib at the dosage of 50 mg. These results need to be confirmed in large phase III trials, some of them are currently ongoing. Finally other multikinase inhibitors such as brivanib or ABT-869 are moving to phase III in the HCC arena (see review in Llovet et al[11]). In the next 3–4 years, results of these trials may change current treatment guidelines for HCC[11, 183].

Conclusions

With the rapid development of genomics technologies, it has become feasible to comprehensively characterize multiple levels of molecular alterations and aberrantly activated signaling cascades involved in tumor development and progression. This is a powerful tool in novel biomarker and drug development in HCC. The global scheme of molecular classification will enable to summarize the highly complicated and fragmented pieces of information of molecular aberrations, and help link to specific treatment strategy. It would also help design more efficient clinical trials for molecular targeted agents[184]. Considering such compounds often target unexpected molecule depending on biological context[185], precise understanding of mechanism of action will be critical to maximize treatment efficacy, while minimizing adverse effect.

Association of molecular subclasses with clinical outcome is strongly affected by study design. Unless special attention is taken on this issue, even new high-throughput technologies like massively parallel sequencing (so-called next-generation sequencing) may merely increase the complexity of the data. By sharing clinical information together with genomic datasets, it may become possible to correct the problem with reanalysis of synthesized larger datasets. Recently emerging genome-based assays specialized for fixed tissue[44] will help solve this study design problem by allowing access to large collection of archived tissues associated with well-designed clinical studies conducted in the past.

Numerous lines of ongoing clinical trials of molecular targeted drugs will continue to drive the rapid progress of the field and eventually make personalized management of HCC patients a reality in the near future.

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Abbreviations

AFP	alpha-fetoprotein
DNMT	DNA methyltransferases
EGF	epidermal growth factor
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDACi	histone deacetylase inhibitors
IFN	interferon
IGF	insulin-like growth factor
IL6	interleukin-6
miRNA	microRNA
MTOR	mechanistic target of rapamycin
NF-kB	nuclear factor-kappa B
TGF	transforming growth factor

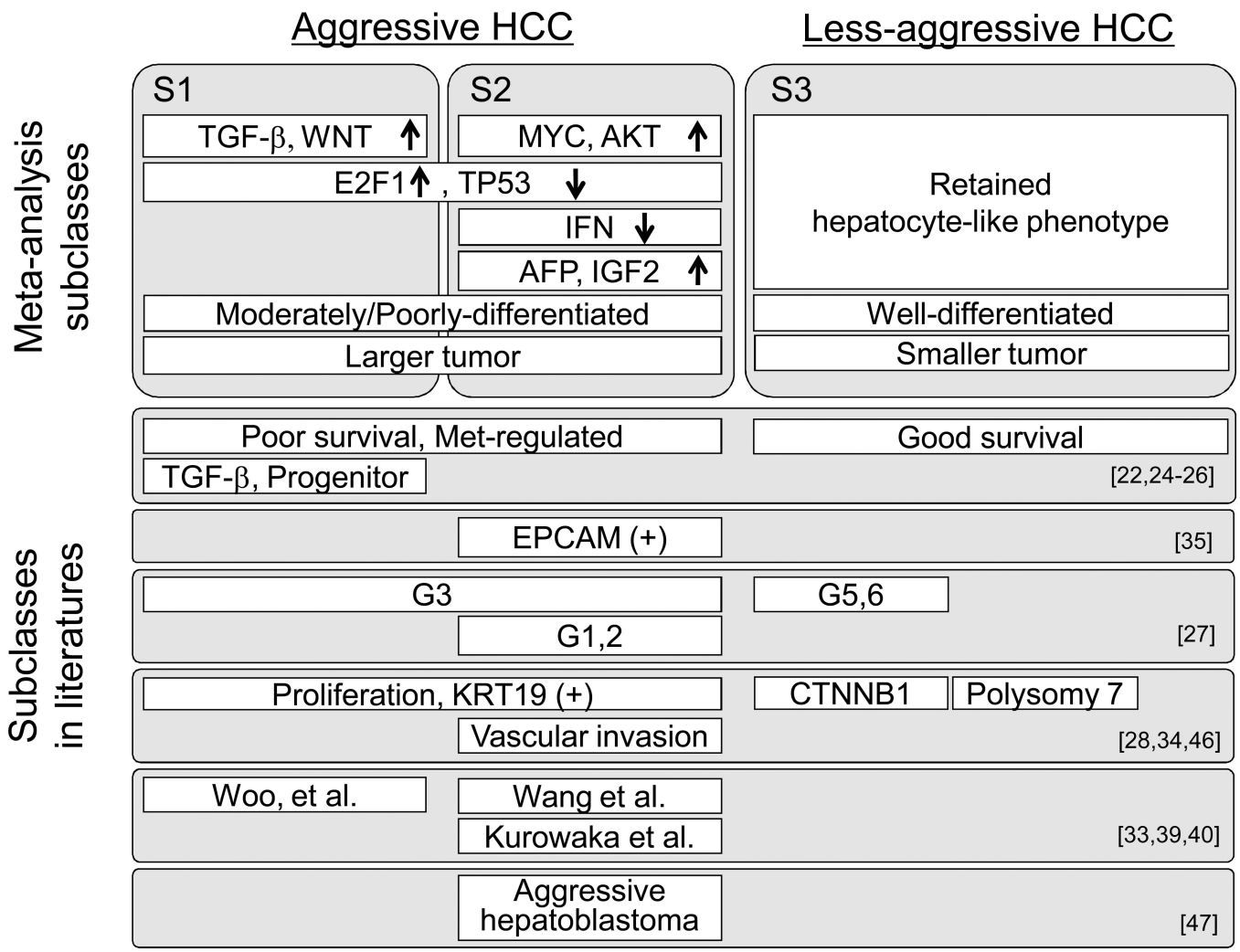

Figure 1.

Global overview of molecular classification of HCC. Correspondence between subclasses defined by a meta-analysis[63] (upper panel) and subclasses/signatures in literatures (lower panel, see Table 1 for the details) was evaluated using Gene Set Enrichment Analysis[20] with a significance threshold of false discovery rate <0.25.

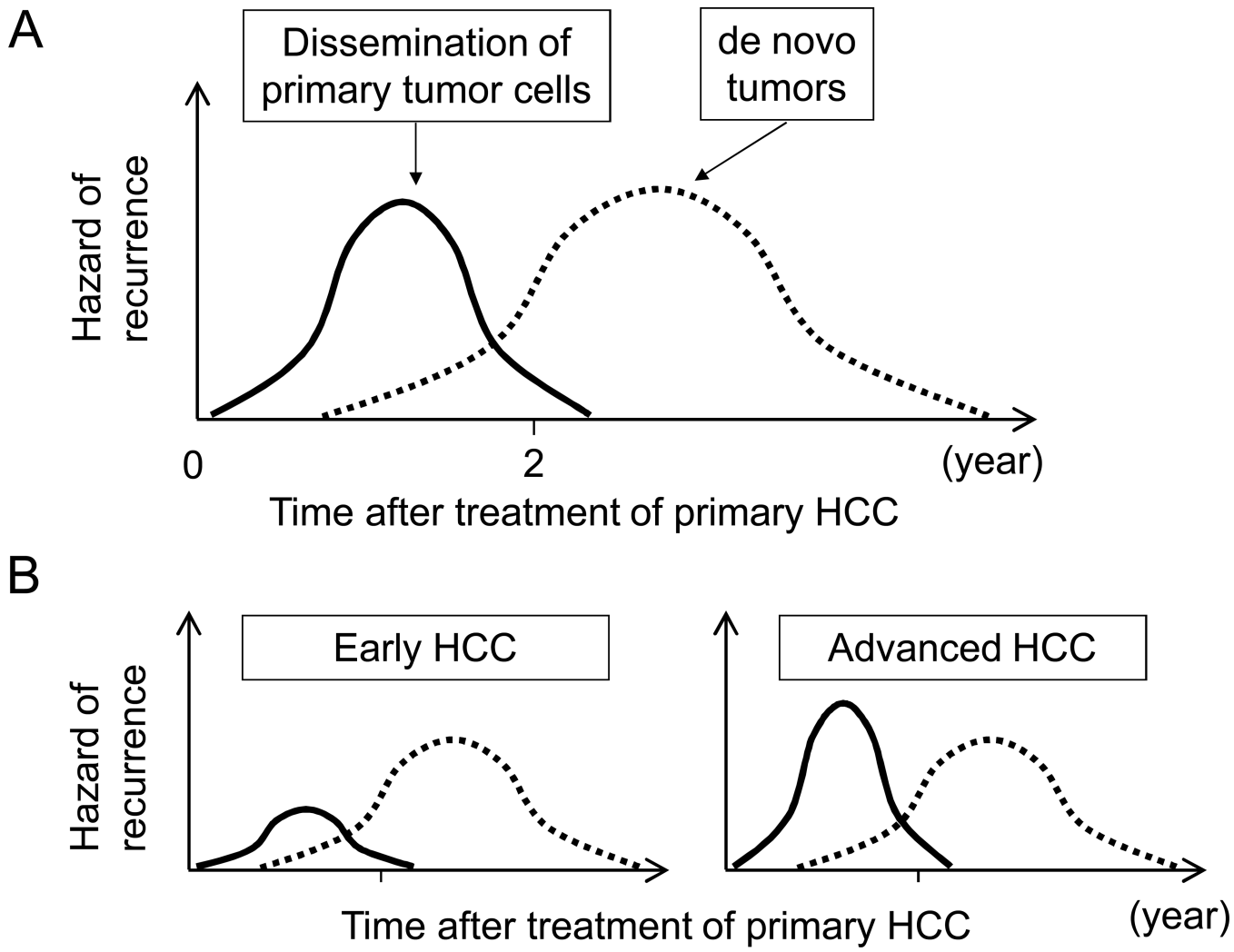

Figure 2.
(A) Hazard of HCC recurrence according to the mechanism. (B) Hazard of HCC recurrence in early stage (left panel) and advanced stage (right panel) HCC.
Solid line indicates the risk of tumor sell dissemination from the primary tumors. Dashed line indicates the risk of de novo cancers arisen from the carcinogenic “field effect”.

Table 1

Molecular classification of HCC

References	N*	Dominant etiology	Dominant patient ethnicity	Analyzed molecular information	Subclass (trained phenotype)	Molecular characteristecs	Clinical characteristics
Molecular information derived from tumor tissue							
Lee [22]	90	HBV, HCV	Asian, Caucasian	Gene expression	Cluster A (vs. B)	Increased cell proliferation, ubiquitination. Induction of Met-regulated genes in mice	Poor survival
Lee [25], Kaposi-Novak [24], Coulouam [26]	61 + 78	HBV, alcohol, HCV	Asian, Caucasian	Gene expression	Cluster A-HB (subset of cluster A)	Hepatic progenitor cell-like gene expression (AP1 targets e.g., FOS, JUN, and oval cell markers, e.g., KRT7, KRT19, and VIM), TGF-beta activation	Very poor survival
Boyault [27]	57 + 63	Alcohol, HBV, HCV	Caucasian	Gene expression, gene mutation, promoter methylation, LOH, HBV copy number	G1, G2, G3, G4, G5, G6	Activation of AKT (G2), increased cell cycle (G3), promoter methylation in CDKN2A (G2) and CDH1 (G5,6), chromosomal instability (G1,2,3), mutations in AXIN1 (G1,2), TP53 (G3), and CTNNB1 (G5,6), higher WNT activation (G6)	Women, African origin, young, high AFP (G1), high copy of HBV (G2), relatively poor survival (G3), satellite nodules (G6)
Chiang [28]	91	HCV	Caucasian	Gene expression, DNA copy number, gene mutation, immunostaining	Proliferation, CTNNB1, interferon, polysomy 7	Phosphorylation of IGF1R, AKT, and RPS6 (proliferation), CTNNB1 mutations (CTNNB1), polysomy of chromosome 7 (polysomy 7)	Recurrence (within 2 years, polysomy 7)
Yamashita [35]	40 + 238	HBV	Asian	Gene expression	EPCAM+AFP+ (hepatic stem cell-like), EPCAM+AFP- (bile duch epithelium-like), EPCAM-AFP+ (hepatocytic progenitor-like), EPCAM-AFP- (mature hepatocyte-like)	Up-regulation of stem/ progenitor markers (KRT19, KIT, and EPCAM) and WNT pathway activation in EPCAM+ tumors	Poor survival (EPCAM-AFP+, EPCAM+AFP+)
Chen [45]	102 (82 patients)	HBV	Asian	Gene expression	Right cluster (vs. left cluster) in Fig.3A in Chen et al.[45]	Up-regulation of genes in "proliferation", "stromal", "lymphocyte" clusters, down-regulation of genes in "liver specific" cluster	Not described
Breuhahn [32]	43 (39 patients)	HCV, HBV, alcohol	Not described	Gene expression	Group A (vs. B)	Up-regulation of interferon-related genes, down-regulation of IGF2 (IGF2 is upregulated in subcluster B1)	Not described

References	N*	Dominant etiology	Dominant patient ethnicity	Analyzed molecular information	Subclass (trained phenotype)	Molecular characteristecs	Clinical characteristics
Villanueva [46]	73 + 164	HCV, HBV	Caucasian	Gene expression	KRT19-positive	Gene expression correlated with CK19 positivity	Recurrence (within 2 years, KRT19+ tumors)
Minguez [34]	79 + 135	HCV, HBV	Caucasian	Gene expression	Vascular invasion	Up-regulation of CD24, CDKN3, YY1AP1	Vascular invasion
Ye [42]	20 + 20	HBV	Asian	Gene expression	Portal vein tumor thrombus	Up-regulation of osteopontin (SPP1)	Intrahepatic metastasis, poor survival
Iizuka [43]	33 + 27	HCV, HBV	Asian	Gene expression	Recurrence (within 1 year)	Up-regulation of SEMA3F, down-regulation of immune response-related genes (TNFAIP3, TRIM22) and metastasis-related genes (VIM, CCND2)	Recurrence (within 1 year)
Kurokawa [39]	60 + 40	HCV, HBV	Asian	Gene expression	Recurrence (within 2 years)	Up-regulation of ALCAM, CDH1, KRT8, down-regulation of IGF2R, RB1	Recurrence (within 2 years)
Woo [33]	65 + 139 (from Lee [25])	HBV	Asian	Gene expression	Recurrence	Up-regulation of CD24, down-regulation of PPAR-alpha target genes	Recurrence (within 1 year)
Wang [40]	23 + 25	HBV	Asian	Gene expression	Recurrence	Up-regulation of RACGAP1, KCNK1, SMURF2, USH1C, GSTM2, down-regulation of CNGA1, INSIG1	Recurrence
Yoshioka [41]	42 + 97	HCV, HBV	Asian	Gene expression	Recurrence (within 2 years)	Cancer progression and carcinogenesis-related genes	Recurrence (within 2 years)
Katoh [54]	87	HCV, HBV	Asian	DNA copy number, gene mutation	Cluster A (vs. B)	Chromosomal instability, mTOR activation (subcluster B2)	Poor survival, more HBV + tumors
Laurent-Puig [55]	137	Alcohol, HCV, HBV	Caucasian	LOH, gene mutation	Chromosomal instability (vs. chromosomal stability)	More mutations in TP53 and AXIN1, less mutations in CTNNB1	Poor survival, more vascular invasion, HBV+ tumors, high AFP
Luo [57]	37 (HCC)	Unkwon, HCV (no HBV)	Not described	Gene expression, DNA copy number	Cluster A (vs. B)	Up-regulation of CYP/ADH family genes, PEG10, IRS1, ERBB3, down-regulation of HIF1A, PPBP, RGS2, SOCS3, CRP, TIMP1, IGFBP5, IGFBP7, COL4, COL6, instable genomic regions common to all HCC, trend of accumulation of HCV in cluster B	No identifiable difference in tumor histology and size

References	N*	Dominant etiology	Dominant patient ethnicity	Analyzed molecular information	Subclass (trained phenotype)	Molecular characteristecs	Clinical characteristics
Molecular information derived from non-tumor liver tissue							
Hoshida [44]	82 + 225	HCV, HBV	Asian, Caucasian	Gene expression	Poor-prognosis (vs. non-poor-prognosis)	Activation of IFN, NFkB, IL6 pathways, response to oxidative stress and viral infection, down-regulation of liver metabolism-related genes	Poor survival, recurrence (after 2 years)
Budhu [74]	20 + 95	HBV	Asian	Gene expression	Metastasis-inclined (vs. metastasis-averse) microenvironment	Up-regulation of Th2 cytokines (IL4, IL10, IL15, IL8, IL5, PRG1, HLA-DRA, HLA-DPA1, CSF1), down-regulation of Th1 cytokines (IL2, IL1A, IL1B, IL12A, IL12B, IFNG, TNF)	Recurrence, poor survival
Okamoto [73]	40	HCV	Asian	Gene expression	Multicentric (vs. single nodule) HCC	Up-regulation of FES, STMN1	Multicentric recurrence
Kim [72]	59 + 103 (from Chen [45])	HBV, HCV, hemochromatosis, Wilson's disease, alcohol, primary biliary cirrhosis, autoimmune hepatitis	Caucasian, Asian	Gene expression	HBV, HCV, hemochromatosis, Wilson's disease (vs. rest) overlapped with dysregulated genes in HCC	Up-regulation of EPCAM, MDK, down-regulation of C9, CD5L, CPB2, IL1RAP, MT1B	Similar gene-expression pattern in HCC

HBV: hepatitis B virus, HCV: hepatitis C virus, AFP: alpha-fetoprotein, LOH: loss of heterozygosity

*No. samples used to define classifiaction (+ no. samples used for validation)

Table 2

Dysregulated microRNA in HCC

Expression	microRNA	References
Up-regulated	MIR21	[92]
	MIR221	[93, 117]
	MIR181	[97]
	MIR224	[95, 113, 115]
	MIR9	[96]
	MIR143	[94]
	MIR18	[113, 119]
Down-regulated	let-7 family	[98]
	MIR1	[101]
	MIR26	[108, 124]
	MIR122	[100, 102]
	MIR124	[103]
	MIR203	[103]
	MIR195	[106, 113]
	MIR101	[104, 105]
	MIR34A	[107]
	MIR125A	[113]
	MIR199A,B	[113]
	MIR200A	[113]

Table 3

Epigenetic Alterations in Hepatocellular Carcinoma

Functional category	Gene	Gene Expression Cange	Epigenetic Alteration	Frequency	References
Cell cycle / Apoptosis	CDKN2A (p16)	Decreased	Hypermethylation	56–83%	[127–145, 150]
	PSMD9 (p27)	Decreased	Hypermethylation	48%	[128]
	CDKN2B (p15)	Decreased	Hypermethylation	43–49%	[128–130, 133, 135]
	TP53	Decreased	Hypermethylation	14%	[128, 129]
	CDKN2A (p14)	Decreased	Hypermethylation	8–20%, 25–30%	[128, 130]
	CASP8	Decreased	Hypermethylation	72%, 43–50%	[134, 145]
Proliferation/differentiation	CDH1 (E-Cadherin)	Decreased	Hypermethylation	33–67%	[130, 135]
	MYC	Increased	Hypomethylation	30%	[129, 136]
	APC	Decreased	Hypermethylation	45–77%, 78–91%	[127, 131, 135, 145]
	CDH13 (T-Cadherin)	Decreased	Hypermethylation / Histone Deacetylation		[137, 138]
	RAS	Increased	Hypomehylation		[136]
	SFRP1	Decreased	Hypermethylation	53–75%	[127, 139]
	SFRP2	Decreased	Hypermethylation	11–30%	[127, 139, 145]
	SFRP5	Decreased	Hypermethylation	29%	[127, 139]
Growth Factors / Receptors	IGFBP-3	Decreased	Hypermethylation	70%	[140]
	RASSF1	Decreased	Hypermethylation	85%, 54–95%	[129, 133, 134, 141, 145, 150]
	RASSF2	Decreased	Hypermethylation	6–48%	[145]
Other	SOCS1	Decreased	Hypermethylation	53–65%, 66–67%	[127, 135, 141, 145]
	SOCS3	Decreased	Hypermethylation	33%	[133, 141]
	CDH1	Decreased	Hypermethylation	39–46%	[133, 145]
	PRDM2	Decreased	Hypermethylation	5–33%, 64%	[142]
	HIC1	Decreased	Hypermethylation	78–86%	[133, 145]
	DCC	Decreased	Hypermethylation	6–9%	[145]
	RPRM	Decreased	Hypermethylation	6–30%	[145]
	CACNA1G	Decreased	Hypermethylation	3–89%	[145]
	RUNX3	Decreased	Hypermethylation	41–47%, 39–82%	[127, 145]
	PTGS2	Decreased	Hypermethylation	17–46%	[132, 145]
	DLC1	Decreased	Hypermethylation	24%	[143]

Functional category	Gene	Gene Expression Cange	Epigenetic Alteration	Frequency	References
	PGR	Decreased	H3-K27 methylation		[150]
	ESR1 (ER-alpha)	Decreased	H3-K27 methylation		[150]
	PYCARD	Decreased	H3-K9 hypoacetylation and trimethylation		[151]
	JNK1	Decreased	H3 lysines 4 and 9 tri-methylation		[152]
DNA repair	MGMT	Decreased	Hypermethylation	22–39%	[127, 131, 144]
	GSTP1	Decreased	Hypermethylation	41–86%	[127, 131, 135, 145]

Table 4

Molecular targeted drugs for HCC in phase II/III Clinical Trials

Drug	Molecular target
Phase III	
Brivanib	VEGFR2, FGFR
Erlotinib	EGFR
Linifanib	VEGFR, PDGFR
PI-88	FGF, VEGF
Rapamycin	mTOR
Sorefenib	BRAF, VEGFR, PDGFR
Sunitinib	VEGF
Phase II	
AMG-386	Angiopoietin 1/2
AVE-1642	IGF-1R
AZD-2171	VEGF
AZD-6244	MEK1/2
BIBF-1120	VEGF, PDGF, FGF
BIIB-022	IGFR1
Bortezomib	26S proteasome
CT-011	PD-1/2
Dasatinib	BCR/ABL
E-7080	VEGF, FGF, SCF
IMC-1121B	VEGFR 2
IMC-A12	IGFR1
Ispinesib	Kinesin spindle protein
Licartin	Fab'2 Fragment
LY-2181308	Survivin
Mapatumumab	TRAIL
Oblimersen	Bcl-2
RAD-001	mTOR
TSU-68	VEGF, FGF, PDGF
XL-184	MET, RET, VEFGR2
XL-647	EGFR, Her2, VEGFR2
ZD-6474	VEGF

www.ClicalTrial.gov accessed Nov. 2009