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Molecular classification of hepatocellular carcinoma anno 2011

H. van Malenstein [a,*], *J. van Pelt* [a], *C. Verslype* [a,b]

[a] *Department of Hepatology, University Hospital Gasthuisberg Leuven, Belgium*
[b] *Department of Digestive Oncology, University Hospital Gasthuisberg Leuven, Belgium*

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ABSTRACT

Hepatocellular carcinoma has an increasing incidence and high mortality. Treatment options are limited if the disease is not diagnosed in its early stage. The natural course of the disease is aggressive but not always predictable. Molecular profiling is a promising tool for classification in order to optimize prognosis prediction and treatment for an individual patient. In the last decade a large amount of studies has been conducted to better classify hepatocellular carcinomas. The focus of this review is on implications of molecular classification for prognosis and therapeutic decision making in HCC patients. Most studies used microarray technique for genome wide profiling, but other methods to detect genomic changes and microRNA are gaining interest. The whole genome profiling studies identified differences in affected signalling and tried to relate this to prognosis. Some common subgroups were identified, such as the *proliferation* cluster and the *beta-catenin* cluster. However, there is still little overlap between most studies. Better study design and bio-informatical analysis might help in this context.

1. Background

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and is the third most common cause of cancer related death.[1] It is an epithelial tumour of which the cells share some characteristics with normal hepatocytes and that originates either from mature hepatocytes or stem cells. In 80% of the cases HCC develops on the background of advanced fibrosis, due to e.g. viral hepatitis or alcohol.[2] In these cases hepatocellular carcinoma seems to develop following a multistep process, along dysplastic changes to invasive cancer.[3–7] Dysplastic nodules are present in around 15% of the cirrhotic livers and eventually about 30% of the high grade dysplastic nodules evolve to HCC. In a small percentage of cases HCC can develop in a non-cirrhotic liver either from liver adenomas, in patients with hepatitis B virus infection[8] or after high aflatoxin B1 exposure.[9]

The exact pathogenesis is poorly understood, since the dysplastic changes vary between different patients. This is also reflected in the natural course of the disease, which is very unpredictable.[10] Currently, for an individual patient the prognosis can be estimated and treatment can be determined using a clinical classification scheme such as the Barcelona Classification (BCLC).[11] This classification system, like other prognostic scores, takes into account tumour size and number, macroscopic vascular invasion, remaining liver function and performance status. However, patients within the same BCLC stage still have a variable course of their disease. Different attempts have been made to better classify these tumours on a molecular basis in order to optimize treatment. Especially with the development of new drugs, such as tyrosine kinase inhibitors, it seems promising to profile these tumours molecularly and aim for patient directed therapy. This review will highlight the current advances in the molecular classification

* *Corresponding author:* Address: Herestraat 49, Bus 703, 3000 Leuven, Belgium. Tel.: +32 16 345845; fax: +32 16 345846.
E-mail address: Hannah.vanmalenstein@med.kuleuven.be (H. van Malenstein).
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of HCCs. The focus of this review is on implications of molecular classification for prognosis and therapeutic decision making in HCC patients. Molecular classification has already proven its value in the differentiation between benign and malignant lesions and in the classification of hepatocellular adenomas.[12–15] Different techniques can be used for classification, this review primarily looks at classification based on RNA expression and microarray studies. With the development of the microarray technique HCCs can be classified on their RNA expression levels. Other methods, such as comparative genomic hybridisation (CGH)- and single nucleotide polymorphism (SNP)-arrays, identify chromosomal alterations and recently there is gaining interest in microRNA profiling.

2. Traditional classification

2.1. *Tumoursuppressor or oncogene mutations in HCC*

The first attempts to classify HCC were based on oncogene or tumoursuppressor activity. Already in cirrhotic nodules there are chromosomal aberrations in about half of the cases, especially in nodules displaying dysplasia. But in HCC there are also mutations in specific cancer related genes, more specific; activating oncogene mutations or inactivating tumoursuppressor mutations (Table 1). The most common mutation is found in the tumoursuppressor TP53 which leads to inactivation of this gene. TP53 mutations are observed in up to 50% of HCC cases.[16] A clear relation was found between aflatoxin B1 exposure and a point mutation at codon 249 of TP53.[17] However, the finding of such specific mutations is a rare phenomenon.

The second most common mutation of a specific gene is activation of the oncogene β-catenin, found in 20–40% of HCC.[18,19] Mutations lead to the accumulation of β-catenin, both in the cytoplasm and in the nucleus.[20] Beta-catenin activates Wnt-signalling, which plays a role in differentiation, proliferation, epithelial–mesenchymal transition and stem-cell renewal. In a small proportion of cases β-catenin accumulates in another fashion. Bi-allelic mutations of the AXIN1 gene lead to blockage of β-catenin phosphorylation and, therefore, prevent degradation.[21] Other mutations that are rarely found concern the tumoursuppressors retinoblastoma 1 (RB1), p16 (CDKN2A), insulin-like growth-factor-2 receptor (IGF2R) and PTEN and the oncogene KRAS2.[18,22–28]

These gene mutations play an important role in the hepatocarcinogenesis, however, they are inadequate to accurately classify the tumours. The prognostic impact of the different mutated genes remains unclear and hitherto unexplored as a therapeutic target. Therefore, there is gaining interest in the role of mutations in the oncogene PIK3CA, since there are PI3 kinase inhibitors. PIK3CA is an oncogene which is involved in several molecular processes, such as proliferation and is mutated in several types of cancer. In a series of 73 HCCs mutations in PIK3CA were reported in 35.6%.[29] PIK3CA is also thought to play a role in epithelial-to-mesenchymal transition (EMT).[30] The occurrence of EMT is a hallmark of aggressive cancer, since cancer cells with a mesenchymal phenotype have an increased invasive potential.[31] However, other studies suggest that the frequency of PIK3CA mutations is overestimated. In two studies no mutations were found in this oncogene, although these studies recognise the over activation of downstream targets such as mTOR and p70 S6.[32,33]

3. Microarray studies

3.1. *General classification and prognostic studies*

With the development of the microarray technique it became feasible to study HCC on a genome wide basis. The enormous amount of data may uncover new candidate genes and new significant targets. And in consequence, it became possible to study altered pathways more than single genes. Nowadays a large number of studies has been conducted. All these studies have a slightly different focus and can be divided into classification studies, prognostic studies and studies searching for therapeutic targets (Table 2).

The first study to identify subgroups based on molecular classification was conducted by Lee et al.[34] in 90 patients from China and Leuven (Belgium), where the majority was infected with hepatitis B virus (HBV). Based on the expression of 406 genes, patients could be clustered into groups with good or poor prognosis ($p < 0.001$). A subsequent analysis by the same group in 2006 in 139 patients led to an even stronger division between good and poor prognosis.[35] Patients could be classified as having a hepatocyte-like – or a foetal hepatoblast-like genotype, based on 941 genes that showed correlation with either gene expression of adult rat hepatocytes or rat foetal hepatoblasts. Patients with a hepatoblast-like gene expression had a significantly worse outcome.

Two other important studies identified clusters of HCC that were related to genomic or functional pathways.[36,37]

Table 1 – Mutation frequency of tumoursuppressor genes and oncogenes in hepatocellular carcinoma.

Gene	Full name	Role	Mutation rate in HCC (%)
TP53	Tumour protein p53	Tumoursuppressor	>50
CTNNB1	Beta-catenin 1	Oncogene	20–40
AXIN1	Axin 1	Oncogene	9
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	Oncogene	0–35
RB1	Retinoblastoma 1	Tumoursuppressor	0–14
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)	Tumoursuppressor	6–30
IGF2R	Insulin-like growth factor 2 receptor	Tumoursuppressor	18
KRAS2	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Oncogene	<2
PTEN	Phosphatase and tensin homolog	Tumoursuppressor	3

Table 2a – Classification studies of hepatocellular carcinoma.

Study	HCC type $n_{(HCC)}$	Subgroups
Boyault/Laurent-Puig[36,58]	All $n = 120$	Two groups (with each three subgroups): G1–3: High proliferation, chromosomal instability, activation of IGF or Akt/mTOR signalling – G2–3: Frequent p53 mutations G4–6: Chromosomal stability – G5–6: Activation of Wnt signalling, high prevalence of CTNNB1 mutations
Chiang[37]	HCV induced $n = 103$	Five subgroups (no association with prognosis) – CTNNB – Proliferation (IGF/mTOR upregulation and high AFP) – IFN related – Polysomy chromosome 7 (c-Met) – Unannotated
Wurmbach[86]	HCV induced $n = 35$	Dyplasia Early HCC (Pathways affected – up: Wnt, down: Jak/stat, insulin, TGF-b, Toll-like receptor Advanced HCC (cell cycle – and DNA repair genes)
Breuhahn[38]	All $n = 34$	Two subgroups (A) Upregulation of IFN-related genes (better prognosis) (B) Downregulation of IFN-related genes and apoptosis – B1 overexpression IGF2 – B2 Downregulation IGF2, FAF1, TNIP1
Katoh[71] (Array based Comparative Genomic Hybridisation)	All $n = 87$	Two groups (with each three subgroups based on chromosomal alterations) A1–3: Poor prognosis (intrahepatic metastasis, high AFP, HBV infection) – Gains in 1q, 6p, 8q and loss in 8p B1–3: Good prognosis (low frequency of chromosomal alterations) A3 + B2: RPS6KB1 (mTOR-pathway) A2: c-myc overexpression

Abbreviations: HCV, hepatitis C virus.

Interestingly, there was some overlap between the subgroups. In both cohorts there was a subclass of patients with increased cellular *proliferation*, with activation of Akt, RPS6 and IGF. The activation of the mTOR/Akt pathway in the proliferation subclass was confirmed by Villanueva et al.[32] Another shared subclass encompasses activation of the *β-catenin* pathway and nuclear accumulation of the β-catenin protein. Other subgroups were associated with AXIN1 mutations, CDKN2A methylation, polysomy of chromosome 7 and interferon (IFN) related genes. Immune related genes were also reported in another study, with a better prognosis for patients with upregulation of IFN-related genes.[38]

Several prognostic studies revealed genes or gene signatures that were associated with recurrence. A 12 gene set including downregulation of vimentin, CCND2 and PDGFRA was associated with an early recurrence in 60 patients.[39] Whereas another gene set associated with a recurrence in 80 patients comprised 57 genes, with for example dysregulation of the cytoskeleton related genes RACGAP1 and CDC42SE1.[40] A third study found 628 genes that were associated with recurrence in 65 HBV-positive HCC patients. Patients with high risk of recurrence showed upregulation of CD24.[41] Another gene that was reported to be associated with vascular invasion and metastasis in a series of HBV patients was osteopontin.[42]

3.2. *Mechanism-driven analysis*

Some studies applied a different approach to identify important genes, pathways and new therapeutic targets. Known or suspected oncogenic pathways were studied using *in vitro* models. These models try to control other confounding experimental variables and, therefore, a selection of relevant genes, solely concerning the mechanism of interest, can be identified. The advantage of this approach is that the number of genes is more in relation to the number of patients studied, which

Table 2b – Prognostic studies of hepatocellular carcinoma.

Study	HCC type $n_{(HCC)}$	Subgroups
Lee[34,35]	All $n = 139$	Three subgroups A: Proliferation genes, high AFP; poor prognosis B: Opposite of A or smaller changes; good prognosis HB: Foetal features, genes associated with invasion; very poor prognosis
Kurokawa[87] PCR-based array	All $n = 100$	Ninety two genes expressed differently between patients with and without early recurrence Top 20 genes were strong predictors of recurrence (CDH1 among others)
Iizuka[39]	All $n = 60$	Genes associated with early (<1 yr) recurrence after 'curative' resection
Wang[40]	All $n = 80$	Cirrhosis and vascular invasion together predict early recurrence after surgery. One factor + gene signature predict too
Woo[41]	HBV $n = 65$	Genes associated with high risk of recurrence
Van Malenstein[46]	All $n = 320$	Two subgroups based on seven gene hypoxia score – High hypoxia score: poor prognosis – Low hypoxia score: good prognosis

Abbreviations: PCR, polymerase chain reaction; HBV, hepatitis B virus.

Table 2c – Phenotypic studies of hepatocellular carcinoma.

Study	HCC type $n_{(HCC)}$	Subgroups
Ye[42]	HBV induced $n = 67$	Two subgroups – Metastatic (overexpression of osteopontin) – Non-metastatic
Chen[88]	All $n = 102$	Sixty one genes were associated with the presence of vascular invasion. Of which MMP14 and ADAMTS1 are the most important
Korita[89] (IHC-based)	All $n = 125$	Overexpression of osteopontin (SPP1/OPN) is associated with vascular invasion
Okabe[90]	HBV + HCV $n = 20$	Significant difference between HBV- and HCV-induced HCC 151 genes have different expressions with vascular invasion

Abbreviations: IHC, immunohistochemistry; HBV, hepatitis B virus; HCV, hepatitis C virus.

improves the performance of the statistical tests used afterwards. Two studies used knock-out mice to identify gene expression related to either hepatocyte growth factor (HGF) and Met signalling or transforming growth factor beta (TGF-β) signalling.[43,44] HGF/Met signalling functions as a regulator of many cell functions, is upregulated after partial hepatectomy to promote regeneration and is associated with metastasis formation and angiogenesis. HGF/Met-related gene expression could predict survival in 79 independent HCC patients and metastastic disease in 103 HCCs. TGF-β signalling exerts a dual role, with on one hand tumour suppression but on the other hand oncogenic features in later stages of carcinogenesis. Based on microarray results, gene expression induced by TGF-β can be divided into an early and a late signature. The late TGF-β signature is associated with an aggressive phenotype of HCC, displaying shorter survival, higher recurrence rates and more invasive phenotype.

3.3. *Integrative analysis*

The overlap between the different microarray studies that are reported before is relatively poor. Previous results seem to depend on the patient population studied and the microarray platform used. With an integrative approach it may be possible to overcome these shortcomings. An analysis by Hoshida et al. combined eight available independent patient cohorts to reveal shared subclasses and affected pathways.[45] Furthermore they tried to combine expression data with the clinical phenotype of HCC. Finally, three robust subclasses were identified, with subclass 3 having the most favourable prognosis. Subclass 1 and 2 are characterised by upregulation of TGF-β and WNT signalling and encompass the hepatoblast-like phenotype,[35] the late TGF-β signature[44] and the proliferation subclass.[36,37] Subclass 3 on the other hand contains Lee's hepatocyte-like phenotype and the beta-catenin subgroup of Boyault and Chiang.

Although the classification looks promising, the major drawback of this analysis is the applicability. To determine the subclass to which a single patient belongs, it is still necessary to perform microarray on HCC tissue and subsequently complex bioinformatics analysis. Most microarray studies use relative gene expression and clustering compared to gene expression of large sets of HCC patients.

3.4. *Combination of in vitro and translational research*

Our laboratory recently identified a 7 gene set that is of prognostic importance based on *in vitro* and translational

research, thus a combination of a mechanism-driven and integrative analysis. We hypothesised that chronic exposure to hypoxia leads to an adaptive gene expression profile which influences the aggressive behaviour of the tumour cells. We determined the gene expression pattern for human HepG2 liver cells under chronic hypoxia by microarray. Differentially expressed genes were selected and their clinical value was assessed. In our integrative analysis we included four available independent microarray studies of patients with HCC in one single analysis. Three microarray studies were used as training sets to determine a minimal prognostic gene set and one additional study was used for validation. Using computational methods we identified 7 genes, out of 3592 differentially expressed under chronic hypoxia, that showed correlation with poor prognostic indicators in all training sets (272 patients) and this was validated in a 4th dataset (91 patients). Retrospectively the 7-gene set is associated with poor survival and early recurrence in 135 patients. Moreover, using a hypoxia score based on this 7-gene set we found that patients with a score >0.35 had a median survival of 307 days, whereas patients with a score ⩽0.35 had a significant longer median survival of 1602 days.[46] Another advantage of this study is the clinical applicability, since this method requires the determination of only 7 genes in contrast to most of the previous microarray studies that needed large cohorts for clustering and determining relative gene expression.

3.5. *Surrounding liver tissue*

Predicting the prognosis in cirrhotic patients with HCC is complicated by the nature of the disease. Cirrhotic livers, especially with the presence of dysplastic nodules, are 'livers at risk' for developing new tumours. Therefore, prognosis in HCC patients does not only depend on the tumour characteristics and its risk of recurrence but also on the risk of a second primary tumour. The gene expression in the surrounding liver is of additional value in prognosis prediction (Table 3). In a multivariate analysis a 186 gene signature from surrounding liver was the strongest predictor of late recurrence.[47] Late recurrence can be seen as the formation of a secondary primary tumour. The gene set was also a significant predictor for overall survival.

The surrounding liver tissue seems to be important for the development of intrahepatic metastases as well. A gene expression analysis comparing patient with and without hepatic metastases showed that immune-suppressive responses may promote the dissemination of the tumour.[48] A 17 gene set, including ten different interleukins and interferon-gamma, could predict venous metastases.

4. Revival of pathology in the classification of HCC

4.1. *Progenitor cell phenotype and cancer stem cells*

For many years it is known that fibrolamellar HCC behaves differently than true HCC. But even true hepatocellular carcinomas are heterogeneous tumours. Pathology differences in differentiation grade can be observed and in some cases cholangiocyte characteristics. As in other solid tumours, it is thought that HCC is a genetically generated disease originating from a cell population with stemness features, the so-called cancer stem cells.[49–51] In the liver, three types of cells are regarded as the source of malignant transformation being hepatocytes, cholangiocytes and progenitor cells. In the liver progenitor cells are located at the terminal branches of the biliary tree[52] and are activated in chronic liver disease.[53] Several studies suggest that HCC with stem cell features have a poor prognosis. As mentioned before, Lee et al. showed significant survival differences between HCC patients with a hepatocyte-like phenotype and a hepatoblast-like phenotype.[35] Other studies suggest that HCC positive for Keratin 19 (KRT19), which is a progenitor cell marker, has a higher rate of recurrence after resection or transplantation.[54–56] Other foetal markers, such as AFP and epithelial cell adhesion molecule (EpCAM) are of prognostic value as well, since EpCAM + AFP + HCC is associated with poor survival.[57]

5. Promising other genome wide approaches

5.1. *Genomic changes*

As in other tumours there is an accumulation of genomic alterations in HCC, and not only in gene expression measured by microarray. Chromosomal stability, DNA methylation and changes in copy number all influence gene expression and tumour behaviour. The abundant presence of these changes in HCC rendered them interesting for classification. The prognostic relevance of these changes is for example supported by the fact that fractional allelic loss (FAL) was an independent prognostic marker in resected or transplanted HCC.[58,59]

Table 3 – Surrounding liver tissues of hepatocellular carcinoma.

Study	HCC type $n_{(HCC)}$	Subgroups
Budhu[48]	96% HBV $n = 115$	Two subgroups – Metastatic (increase in Th2 cytokines, decrease in Th1 cytokines) – Non-metastatic
Hoshida[47] (DASL assay)	All $n = 307$	Two subgroups I. Good prognosis – Genes associated with normal liver function (ADH5/6/9A1, AKR1A1/D1, CYP2B6) II. Poor prognosis/high recurrence risk – Associated with inflammation (IFN signalling, NFkB, TNFa, IL6)

Abbreviations: HBV, hepatitis B virus; DASL, cDNA-mediated annealing, selection, extension, and ligation.

Chromosomal instability is associated with telomere shortening.[60,61] Telomere shortening has been described in chronic liver disease[62] and telomere dysfunction promoted HCC development in an animal model.[63] Telomere shortening is further induced by the activation of telomerase (TERT), in about 90% of HCC there are increased TERT mRNA levels. HBV has been shown to integrate in the TERT locus in human HCCs,[64] which might increase mRNA expression. The role of HBV in chromosomal instability is supported by Laurent-Puig et al., where they showed in 137 HCCs that HBV infected patients showed instability more frequently.[58]

Genomic instability is further associated with aberrant methylation and aberrations in mismatch repair genes. Methylation is important in both hepatocarcinogenesis as well as tumour progression. The role of methylation was demonstrated in liver cancer cell lines, where demethylating agents inhibit proliferation.[65] The most frequently reported site of hypermethylation is on the tumoursuppressor gene p16(INK4a).[25,66,67] Other aberrant methylation was reported on the Ras-pathway and genes involved in angiogenesis.[68]

Gains and losses of chromosomes are also common and can be detected with methods such as comparative genomic hybridisation (CGH). Several common alterations were detected, the most frequent were losses in the arms 17p, 8p, 16q, 16p, 4q, 9p, 13q, 1p and 6q and gains in the arms 1q, 7q, 8q and 17q.[18,69,70] In some HCC cases the focal gains are in the regions of a known cancer-related gene. For example, copy number changes have been described in the c-Myc region[71] and in the VEGF region.[37] The counterpart of these chromosomal gains is the loss of heterozygosity (LOH). In HCC tumoursuppressor genes are in some cases targeted by LOH, for example TP53, AXIN1 and RB1.

Genomic instability shows correlation with the proliferation subclass of HCC. However, classification purely based on these genomic alterations is difficult. As mentioned before for oncogenes and tumoursuppressor genes, the prognostic impact of the different chromosomal changes remains unclear and hitherto there are no therapeutic options directed to these changes. Another disadvantage of these technologies is to extract *driver* events promoting HCC development and progression as opposed to *passenger* events in the hugely diverse alterations, especially since there are several candidate genes in every chromosomal region. To overcome this shortcoming, some studies combined CGH and microarray analysis. Patil et al. correlated the expression of 48 genes with the copy number of chromosome 8q in 49 HCC samples and found a positive correlation for COPS5.[72] COPS5 regulates E3 ubiquitin ligases and overexpression increases proliferation in Hep3B cells, suggesting its role as an oncogene. Another study by Villanueva et al. showed chromosomal gains in the RICTOR region and activation of the mTOR pathway.[32]

5.2. *MicroRNA*

The latest advance in genome wide studies is microRNA (miRNA) profiling. miRNA are small non-protein-coding RNA molecules, they play an important role in different biological processes, such as proliferation, differentiation and apoptosis by regulating gene expression. One single miRNA consists of only ~22 nucleotides but targets around 200 genes.[73] miRNAs can act as tumoursuppressors or oncogenes and some miRNA dysregulation was found in specific tumour subtypes, suggesting they could be used for classification.

Dysregulation of several miRNAs have been reported in HCC, such as miR-195,[74] miR-221,[75] miR-122[76] and miR-101.[77] One study found a correlation between miRNA expression and underlying aetiology or oncogene/tumoursuppressor gene mutations. miR-126 underexpression was associated with alcohol intake, miR-96 overexpression with HBV infection and miR-375 dysregulation was associated with beta-catenin mutation.[78] Another study could predict venous metastases based on the expression of 20 miRs.[79]

The poor overlap between different studies is again striking. Furthermore, there is a need to better characterise the target genes of every miRNA and its exact role in the biological processes, especially to relate miRNA expression to new therapeutic targets.

6. Linking classification with therapy: targeted agents

One of the final goals of molecular classification is the identification of novel therapeutic targets. Targeted therapy was recently boosted by the success of sorafenib treatment. Sorafenib is a multikinase inhibitor that targets the serine-threonine kinases Raf-1 and B-Raf, the vascular endothelial growth factor (VEGF) receptors and the platelet-derived growth factor beta (PDGFB) receptor. Sorafenib exerts its effect on tumour cells as well as endothelial cells and pericytes.[80] Sorafenib was the first agent with positive results; the phase III SHARP trial and the Asian-Pacific trial demonstrated an overall survival benefit of three months compared to a placebo in patients with advanced HCC.[81,82] Whether sorafenib is beneficial in earlier stages is currently under investigation. Randomised trials with sorafenib as adjuvant treatment in combination with resection and sorafenib in combination with transarterial chemoembolisation are running.

Different pathways are dysregulated in HCC and could be used as targets. For example, mTOR/Akt – and insulin-like growth factor pathways seem to be activated in different aggressive subclasses, such as the cluster A in Lee et al., the proliferation subclass by Chiang et al. and the G1-3 subtype by Boyault et al. These data suggest a role for mTOR inhibition. Already in cell lines and in a mouse model an additive effect was shown for the combination of sorafenib with the mTOR inhibitor rapamycin (RAD001).[83] Phase III trials with RAD001 in HCC patients are ongoing. Other compounds studied are for example erlotinib (targets EGFR) and brivanib (targets VEGFR2 and FGFR). These studies are conducted in patients with advanced HCC, for example in those patients that showed progression under sorafenib. The rationale to use a target such as the fibroblast growth factor (FGF) receptor is that the FGF pathway can be activated to induce angiogenesis in an alternative manner in patients under anti-angiogenic therapy directed to VEGF.[84] Another tumour promoting factor is c-KIT. However, recently a phase III trial with Sunitinib (targets VEGFR, PDGFR and c-KIT) was stopped early because of toxicity and lack of benefit compared to sorafenib.

Based on the molecular classification as it is known to date, some frequent activated pathways are still untargeted. Especially for the overactivation of Wnt-signalling (activated by β-catenin or Axin 1 mutations) and to a lesser extent for the TGF-β pathway, there are currently no drugs available.

7. Conclusion

The development of the genomic techniques made it possible to generate a huge amount of data. With this data it has become feasible to characterise molecular alterations and dysregulation of signalling pathways. Molecular classification can be a very powerful tool for biomarker development and to optimise prognosis prediction and patient directed therapy. Currently, there are two subgroups identified in different studies; the *proliferation* subgroup and the *beta-catenin* subgroup. Furthermore, several signalling pathways and target genes have been identified and need to be further tested. But although great progress has been made, there is room for improvement. Microarray studies for example have some important drawbacks that limit the usefulness in clinical practice to date. Consistency in study results is lacking and the association with clinical phenotype is not always strong. This might be due to the varying aetiologies in a single analysis and the small cohort size. Furthermore, the poor overlap is also due to different microarray techniques, different mathematical models and experimental models.[85] In the future these drawbacks should be taken into account with the design of new studies. Ideally, analyses should be performed with sufficient patients from the same stage of the disease. Moreover, additional information can be found when the genomic changes are analysed over time to better characterise the molecular aberrations in tumour development and progression. One step further is the clinical application of the molecular markers. It will be interesting to further develop PCR-based techniques for genes of interest. These techniques seem more robust, but are only practical in a limited number of genes.

Conflict of interest statement

None declared.

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REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;**55**(2):74–108.
2. Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004;**127**(5 Suppl. 1):S5–S16.
3. Libbrecht L, Desmet V, Roskams T. Preneoplastic lesions in human hepatocarcinogenesis. *Liver Int* 2005;**25**(1):16–27.
4. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002;**31**(4):339–46.
5. Nam SW, Park JY, Ramasamy A, et al. Molecular changes from dysplastic nodule to hepatocellular carcinoma through gene expression profiling. *Hepatology* 2005;**42**(4):809–18.
6. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer* 2006;**6**(9):674–87.
7. Okuda K. Hepatocellular carcinoma. *J Hepatol* 2000;**32** (1 Suppl.):225–37.
8. Bralet MP, Regimbeau JM, Pineau P, et al. Hepatocellular carcinoma occurring in nonfibrotic liver: epidemiologic and histopathologic analysis of 80 French cases. *Hepatology* 2000;**32**(2):200–4.
9. Ozturk M. P53 mutation in hepatocellular carcinoma after aflatoxin exposure. *Lancet* 1991;**338**(8779):1356–9.
10. Llovet JM, Bustamante J, Castells A, et al. Natural history of untreated nonsurgical hepatocellular carcinoma: rationale for the design and evaluation of therapeutic trials. *Hepatology* 1999;**29**(1):62–7.
11. Llovet JM, Di Bisceglie AM, Bruix J, et al. Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 2008;**100**(10):698–711.
12. Di Tommaso L, Destro A, Seok JY, et al. The application of markers (HSP70 GPC3 and GS) in liver biopsies is useful for detection of hepatocellular carcinoma. *J Hepatol* 2009;**50**(4):746–54.
13. Bioulac-Sage P, Rebouissou S, Thomas C, et al. Hepatocellular adenoma subtype classification using molecular markers and immunohistochemistry. *Hepatology* 2007;**46**(3):740–8.
14. Zucman-Rossi J, Jeannot E, Nhieu JT, et al. Genotype-phenotype correlation in hepatocellular adenoma: new classification and relationship with HCC. *Hepatology* 2006;**43**(3):515–24.
15. Rebouissou S, Amessou M, Couchy G, et al. Frequent in-frame somatic deletions activate gp130 in inflammatory hepatocellular tumours. *Nature* 2009;**457**(7226):200–4.
16. Minouchi K, Kaneko S, Kobayashi K. Mutation of p53 gene in regenerative nodules in cirrhotic liver. *J Hepatol* 2002;**37**(2):231–9.
17. Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* 1991;**350**(6317):429–31.
18. Laurent-Puig P, Zucman-Rossi J. Genetics of hepatocellular tumors. *Oncogene* 2006;**25**(27):3778–86.
19. Miyoshi Y, Iwao K, Nagasawa Y, et al. Activation of the beta-catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. *Cancer Res* 1998;**58**(12):2524–7.
20. Ishizaki Y, Ikeda S, Fujimori M, et al. Immunohistochemical analysis and mutational analyses of beta-catenin, Axin family and APC genes in hepatocellular carcinomas. *Int J Oncol* 2004;**24**(5):1077–83.
21. Satoh S, Daigo Y, Furukawa Y, et al. AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat Genet* 2000;**24**(3):245–50.
22. Yao YJ, Ping XL, Zhang H, et al. PTEN/MMAC1 mutations in hepatocellular carcinomas. *Oncogene* 1999;**18**(20):3181–5.
23. Weihrauch M, Benicke M, Lehnert G, et al. Frequent k-ras-2 mutations and p16(INK4A)methylation in hepatocellular carcinomas in workers exposed to vinyl chloride. *Br J Cancer* 2001;**84**(7):982–9.

24. Murakami Y, Hayashi K, Hirohashi S, Sekiya T. Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. *Cancer Res* 1991;**51**(20):5520–5.
25. Liew CT, Li HM, Lo KW, et al. High frequency of p16INK4A gene alterations in hepatocellular carcinoma. *Oncogene* 1999;**18**(3):789–95.
26. De Souza AT, Hankins GR, Washington MK, Orton TC, Jirtle RL. M6P/IGF2R gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. *Nat Genet* 1995;**11**(4):447–9.
27. Martins C, Kedda MA, Kew MC. Characterization of six tumor suppressor genes and microsatellite instability in hepatocellular carcinoma in southern African blacks. *World J Gastroenterol* 1999;**5**(6):470–6.
28. Jin M, Piao Z, Kim NG, et al. P16 is a major inactivation target in hepatocellular carcinoma. *Cancer* 2000;**89**(1):60–8.
29. Lee JW, Soung YH, Kim SY, et al. PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. *Oncogene* 2005;**24**(8):1477–80.
30. Yan W, Fu Y, Tian D, et al. PI3 kinase/Akt signaling mediates epithelial–mesenchymal transition in hypoxic hepatocellular carcinoma cells. *Biochem Biophys Res Commun* 2009;**382**(3):631–6.
31. Thiery JP. Epithelial–mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002;**2**(6):442–54.
32. Villanueva A, Chiang DY, Newell P, et al. Pivotal role of mTOR signaling in hepatocellular carcinoma. *Gastroenterology* 2008;**135**(6):1972–83. 1983 e1–e11.
33. Tanaka Y, Kanai F, Tada M, et al. Absence of PIK3CA hotspot mutations in hepatocellular carcinoma in Japanese patients. *Oncogene* 2006;**25**(20):2950–2.
34. Lee JS, Chu IS, Heo J, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology* 2004;**40**(3):667–76.
35. Lee JS, Heo J, Libbrecht L, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* 2006;**12**(4):410–6.
36. Boyault S, Rickman DS, de Reynies A, et al. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. *Hepatology* 2007;**45**(1):42–52.
37. Chiang DY, Villanueva A, Hoshida Y, et al. Focal gains of VEGFA and molecular classification of hepatocellular carcinoma. *Cancer Res* 2008;**68**(16):6779–88.
38. Breuhahn K, Vreden S, Haddad R, et al. Molecular profiling of human hepatocellular carcinoma defines mutually exclusive interferon regulation and insulin-like growth factor II overexpression. *Cancer Res* 2004;**64**(17):6058–64.
39. Iizuka N, Oka M, Yamada-Okabe H, et al. Oligonucleotide microarray for prediction of early intrahepatic recurrence of hepatocellular carcinoma after curative resection. *Lancet* 2003;**361**(9361):923–9.
40. Wang SM, Ooi LL, Hui KM. Identification and validation of a novel gene signature associated with the recurrence of human hepatocellular carcinoma. *Clin Cancer Res* 2007;**13**(21):6275–83.
41. Woo HG, Park ES, Cheon JH, et al. Gene expression-based recurrence prediction of hepatitis B virus-related human hepatocellular carcinoma. *Clin Cancer Res* 2008;**14**(7):2056–64.
42. Ye QH, Qin LX, Forgues M, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat Med* 2003;**9**(4):416–23.
43. Kaposi-Novak P, Lee JS, Gomez-Quiroz L, et al. Met-regulated expression signature defines a subset of human hepatocellular carcinomas with poor prognosis and aggressive phenotype. *J Clin Invest* 2006;**116**(6):1582–95.
44. Coulouarn C, Factor VM, Thorgeirsson SS. Transforming growth factor-beta gene expression signature in mouse hepatocytes predicts clinical outcome in human cancer. *Hepatology* 2008;**47**(6):2059–67.
45. Hoshida Y, Nijman SM, Kobayashi M, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res* 2009;**69**(18):7385–92.
46. van Malenstein H, Gevaert O, Libbrecht L, et al. A seven-gene set associated with chronic hypoxia of prognostic importance in hepatocellular carcinoma. *Clin Cancer Res* 2010;**16**(16):4278–88.
47. Hoshida Y, Villanueva A, Kobayashi M, et al. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med* 2008;**359**(19):1995–2004.
48. Budhu A, Forgues M, Ye QH, et al. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer Cell* 2006;**10**(2):99–111.
49. Roskams T. Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. *Oncogene* 2006;**25**(27):3818–22.
50. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001;**414**(6859): 105–11.
51. Campbell LL, Polyak K. Breast tumor heterogeneity: cancer stem cells or clonal evolution? *Cell Cycle* 2007;**6**(19) :2332–8.
52. Roskams TA, Theise ND, Balabaud C, et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology* 2004;**39**(6):1739–45.
53. Bird TG, Lorenzini S, Forbes SJ. Activation of stem cells in hepatic diseases. *Cell Tissue Res* 2008;**331**(1):283–300.
54. Durnez A, Verslype C, Nevens F, et al. The clinicopathological and prognostic relevance of cytokeratin 7 and 19 expression in hepatocellular carcinoma. A possible progenitor cell origin. *Histopathology* 2006;**49**(2):138–51.
55. Uenishi T, Kubo S, Yamamoto T, et al. Cytokeratin 19 expression in hepatocellular carcinoma predicts early postoperative recurrence. *Cancer Sci* 2003;**94**(10):851–7.
56. Yang XR, Xu Y, Shi GM, et al. Cytokeratin 10 and cytokeratin 19: predictive markers for poor prognosis in hepatocellular carcinoma patients after curative resection. *Clin Cancer Res* 2008;**14**(12):3850–9.
57. Yamashita T, Forgues M, Wang W, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. *Cancer Res* 2008;**68**(5):1451–61.
58. Laurent-Puig P, Legoix P, Bluteau O, et al. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. *Gastroenterology* 2001;**120**(7):1763–73.
59. Dvorchik I, Schwartz M, Fiel MI, Finkelstein SD, Marsh JW. Fractional allelic imbalance could allow for the development of an equitable transplant selection policy for patients with hepatocellular carcinoma. *Liver Transpl* 2008;**14**(4):443–50.
60. Plentz RR, Caselitz M, Bleck JS, et al. Hepatocellular telomere shortening correlates with chromosomal instability and the development of human hepatoma. *Hepatology* 2004;**40**(1):80–6.
61. Plentz RR, Schlegelberger B, Flemming P, et al. Telomere shortening correlates with increasing aneuploidy of chromosome 8 in human hepatocellular carcinoma. *Hepatology* 2005;**42**(3):522–6.
62. Urabe Y, Nouso K, Higashi T, et al. Telomere length in human liver diseases. *Liver* 1996;**16**(5):293–7.
63. Farazi PA, Glickman J, Jiang S, et al. Differential impact of telomere dysfunction on initiation and progression of hepatocellular carcinoma. *Cancer Res* 2003;**63**(16):5021–7.

64. Ferber MJ, Montoya DP, Yu C, et al. Integrations of the hepatitis B virus (HBV) and human papillomavirus (HPV) into the human telomerase reverse transcriptase (hTERT) gene in liver and cervical cancers. *Oncogene* 2003;**22**(24):3813–20.
65. Maeta Y, Shiota G, Okano J, Murawaki Y. Effect of promoter methylation of the p16 gene on phosphorylation of retinoblastoma gene product and growth of hepatocellular carcinoma cells. *Tumour Biol* 2005;**26**(6):300–5.
66. Wong IH, Lo YM, Zhang J, et al. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. *Cancer Res* 1999;**59**(1):71–3.
67. Matsuda Y, Ichida T, Matsuzawa J, Sugimura K, Asakura H. P16(INK4) is inactivated by extensive CpG methylation in human hepatocellular carcinoma. *Gastroenterology* 1999;**116**(2):394–400.
68. Calvisi DF, Ladu S, Gorden A, et al. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. *J Clin Invest* 2007;**117**(9):2713–22.
69. Steinemann D, Skawran B, Becker T, et al. Assessment of differentiation and progression of hepatic tumors using array-based comparative genomic hybridization. *Clin Gastroenterol Hepatol* 2006;**4**(10):1283–91.
70. Moinzadeh P, Breuhahn K, Stutzer H, Schirmacher P. Chromosome alterations in human hepatocellular carcinomas correlate with aetiology and histological grade – results of an explorative CGH meta-analysis. *Br J Cancer* 2005;**92**(5):935–41.
71. Katoh H, Ojima H, Kokubu A, et al. Genetically distinct and clinically relevant classification of hepatocellular carcinoma: putative therapeutic targets. *Gastroenterology* 2007;**133**(5):1475–86.
72. Patil MA, Gutgemann I, Zhang J, et al. Array-based comparative genomic hybridization reveals recurrent chromosomal aberrations and Jab1 as a potential target for 8q gain in hepatocellular carcinoma. *Carcinogenesis* 2005;**26**(12):2050–7.
73. Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. *Nat Rev Cancer* 2006;**6**(4):259–69.
74. Xu T, Zhu Y, Xiong Y, et al. MicroRNA-195 suppresses tumorigenicity and regulates G1/S transition of human hepatocellular carcinoma cells. *Hepatology* 2009;**50**(1): 113–21.
75. Gramantieri L, Fornari F, Callegari E, et al. MicroRNA involvement in hepatocellular carcinoma. *J Cell Mol Med* 2008;**12**(6A):2189–204.
76. Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. *Oncogene* 2009;**28**(40):3526–36.
77. Su H, Yang JR, Xu T, et al. MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. *Cancer Res* 2009;**69**(3):1135–42.
78. Ladeiro Y, Couchy G, Balabaud C, et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology* 2008;**47**(6):1955–63.
79. Budhu A, Jia HL, Forgues M, et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology* 2008;**47**(3):897–907.
80. Wilhelm SM, Carter C, Tang L, et al. BAY 43–9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004;**64**(19):7099–109.
81. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;**359**(4):378–90.
82. Cheng AL, Kang YK, Chen Z, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009;**10**(1):25–34.
83. Newell P, Toffanin S, Villanueva A, et al. Ras pathway activation in hepatocellular carcinoma and anti-tumoral effect of combined sorafenib and rapamycin in vivo. *J Hepatol* 2009;**51**(4):725–33.
84. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* 2005;**438**(7070):932–6.
85. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005;**102**(43):15545–50.
86. Wurmbach E, Chen YB, Khitrov G, et al. Genome-wide molecular profiles of HCV-induced dysplasia and hepatocellular carcinoma. *Hepatology* 2007;**45**(4):938–47.
87. Kurokawa Y, Matoba R, Takemasa I, et al. Molecular-based prediction of early recurrence in hepatocellular carcinoma. *J Hepatol* 2004;**41**(2):284–91.
88. Chen X, Cheung ST, So S, et al. Gene expression patterns in human liver cancers. *Mol Biol Cell* 2002;**13**(6):1929–39.
89. Korita PV, Wakai T, Shirai Y, et al. Overexpression of osteopontin independently correlates with vascular invasion and poor prognosis in patients with hepatocellular carcinoma. *Hum Pathol* 2008;**39**(12):1777–83.
90. Okabe H, Satoh S, Kato T, et al. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. *Cancer Res* 2001;**61**(5):2129–37.