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ABOUT COVER

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Transcriptome analysis creates a new era of precision medicine for managing recurrent hepatocellular carcinoma

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Abstract

The high incidence of hepatocellular carcinoma (HCC) recurrence negatively impacts outcomes of patients treated with curative intent despite advances in surgical techniques and other locoregional liver-targeting therapies. Over the past few decades, the emergence of transcriptome analysis tools, including real-time quantitative reverse transcription PCR, microarrays, and RNA sequencing, has not only largely contributed to our knowledge about the pathogenesis of recurrent HCC but also led to the development of outcome prediction models based on differentially expressed gene signatures. In recent years, the single-cell RNA sequencing technique has revolutionized our ability to study the complicated crosstalk between cancer cells and the immune environment, which may benefit further investigations on the role of different immune cells in HCC recurrence and the identification of potential therapeutic targets. In the present article, we summarized the major findings yielded with these transcriptome methods within the framework of a causal model consisting of three domains: primary cancer cells; carcinogenic stimuli; and tumor microenvironment. We provided a comprehensive review of the insights that transcriptome analyses have provided into diagnostics, surveillance, and treatment of HCC recurrence.

Key Words: Recurrent hepatocellular carcinoma; Microarrays; RNA sequencing; Single-cell RNA sequencing; Precision medicine; Tumor heterogeneity; Tumor microenviron-

ment

Core Tip: The high incidence of hepatocellular carcinoma (HCC) recurrence seriously threatens patient outcomes. This review detailed how various transcriptome profiling methods have contributed to our understanding of recurrent HCC with respect to the carcinogenicity of primary cancer cells, carcinogenic stimuli, and tumor microenvironments, which show great promise in improving the management of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 75%-85% of primary liver cancer caused by chronic liver injury[1]. The advance in surgical techniques and locoregional liver-directed therapies contributes to the prognosis of patients suffering from early HCC. However, high-relapse HCC remains a serious burden to patients treated with curative intent, as the annual recurrence rate of HCC following surgery is 50%-70% within 5 years[2-4]. Although recent progress in systemic treatments has led to the modification of treatment strategy for intermediate to advanced HCC[5], early detection of HCC recurrence can provide patients with more treatment options. It is therefore imperative to identify susceptible patients and offer regular monitoring.

Traditionally, post-treatment surveillance of HCC utilized periodic cross-sectional imaging and tumor markers for patient follow-up[6]. The major aim of post-treatment surveillance is early identification of diseases that might be amenable to subsequent local therapy[7]. However, multiphase, contrast-enhanced computed tomography and magnetic resonance imaging suffer from low per-lesion sensitivity[8], difficulty in assessing small HCCs[9], and post-locoregional therapy lesions[10,11]. Ancillary methods like alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin likewise suffer from high false positive and false negative results[12,13]. The imperfection of early HCC recurrence detection urges the need to seek a more reliable armamentarium.

In addition, owing to the inclination to the multifocal occurrence of HCC, it is often challenging to decipher whether the lesions observed after curative liver resection arise from primary HCC or multicentric origin. Differentiation of the two types of multifocal lesions is necessary since their distinct mechanisms may have different effects on the response to treatment[14,15]. Some researchers attempt to distinguish the two conditions based on temporality of tumor occurrence. Early intrahepatic recurrence (IHR), defined as recurrent tumors detected within fewer than 2 years after surgery, has been attributed to residual lesions or intrahepatic micrometastasis from the initial tumor, while late (more than 2 years after surgery) IHR is largely considered a newly developed primary lesion[16,17]. However, such a definition has limited diagnostic accuracy and has not been validated[14,18].

Early studies have analyzed the clonality of multiple HCCs by assessing DNA ploidy, hepatitis B virus (HBV) integration sites, or microsatellite aberration mainly involving loss of heterozygosity and copy number variations (CNV)[19]. The heterogeneity not only exists among multifocal tumors but has also been found within a single lesion[20]. The molecular technologies that have been utilized for these approaches, such as DNA fingerprinting and whole-exome sequencing, are beyond the scope of this review and therefore will not be discussed further. We aimed to emphasize the complexity and heterogeneity behind recurrence widely seen in clinical practice.

The incidence of IHR of HCC after curative resection may be influenced by central factors including the specifics or clonality of primary tumor cells, the microenvironment that offers a susceptible niche for tumor cells to metastasize, and the existence of distinct carcinogens[21,22]. Reviewing past literature, we propose a model of three causations to illustrate the interplay between the factors that determine the recurrence of HCC, analogous to the well-known epidemiologic triangle for infectious disease (Figure 1). The recurrence of HCC results from interactions between primary cancer cells, the tumor microenvironment, and carcinogenic stimuli.

In recent years, transcriptome analysis emerged as a powerful tool to investigate the expression of disease phenotype and its association with genotype[23-25]. The evolution of bench work and laboratory equipment enables assays to be more efficient, enjoy higher throughput, and be more cost-effective. Such advances in molecular biological technology have facilitated the investigation of three causative domains of HCC recurrence described above and yielded abundant results. In the current

Primary cancer cells
Tumor heterogeneity
Tumor clonal evolution
Cancer stem-like cells

Tumorigenesis
recurrence

Microenvironment
Infiltrating immune cells
Parenchymal cells
Stromal cells

Carcinogenic stimulants
Virus
Cirrhosis
Toxin, lipid, and others

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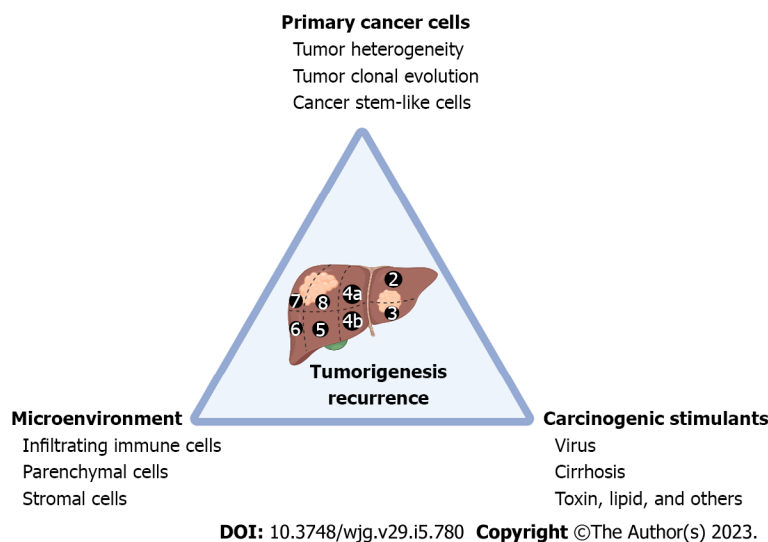
Figure 1 Causal model of hepatocellular carcinoma recurrence. The figure was created with BioRender.com.

review, we focused on describing the role of transcriptome analyses, including real-time quantitative reverse transcription (RT-q) PCR, microarrays, and RNA sequencing (RNA-seq), as well as the rapidly evolving single-cell transcriptome analysis, among the latest work on this topic. We summarized the major findings of studies that may provide us with a clearer picture of HCC recurrence and give us insight into potential diagnostic targets as well as therapeutic strategies.

QUANTITATIVE RT-QPCR ANALYSIS OF RECURRENT HCC

Kary Mullis invented PCR in 1984. Russel Higuchi and colleagues later exploited fluorescence technology, making it possible to monitor PCR results using fluorescent probes[26,27]. These advances, combined with reverse transcriptase, which had been discovered earlier in 1970, brought about the development of RT-qPCR[28]. Since the late 1990s, RT-qPCR has been widely utilized in the exploration of differential gene expression (DGE) in various diseases, including HCC.

Several studies have utilized RT-qPCR to analyze the DGE in HCC recurrence. To clarify the genes responsible for the hematogenous spreading of HCC cells, one study measuring the expression of matrix metalloproteinase 9 (*MMP9*) and vascular endothelial growth factor in pairs of non-tumor and tumor samples with RT-qPCR found that the expression of *MMP9* in tumors was related to recurrence, while the expression of vascular endothelial growth factor was not. The same study also examined *AFP* mRNA in blood samples and found that the level was associated with recurrence and could serve as a predictor of recurrence or metastasis of HCC[29]. Similarly, another study reported that the mRNA level of *AFP* in peripheral blood samples significantly correlated with postoperative extrahepatic metastasis and disease-free survival[30].

In RT-qPCR, the quantification of complementary DNA (cDNA) from genes of interest is typically compared to that of reference genes, also called housekeeping genes, to allow the normalization of differences seen in different samples. Common reference genes include beta-actin (*ACTB*), beta-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyl-transferase 1, and TATA box binding protein[31]. A suitable reference gene must have stable expression across different conditions of samples. It is noteworthy that both *ACTB* and *GAPDH*, two commonly used reference genes, have been reported to be highly expressed in HCC when compared with non-tumor tissues[32], while TATA box binding protein and hypoxanthine phosphoribosyltransferase 1 were reported to be more suitable reference genes in HCC[33]. However, many currently available data with detection of significant DGE of HCC still used *ACTB* and *GAPDH* as reference genes[34,35], and whether the two genes are reliable for RT-qPCR normalization in HCC specimens requires more investigation.

The step of cDNA amplification endows RT-qPCR with a wide dynamic window and relatively high sensitivity to detect genes expressed with low abundance such as cytokines, and RT-qPCR is regarded as the "gold standard" of transcriptome analysis[36]. However, RT-qPCR also carries several limitations. Technically, various factors may impact the amplification and cause deviation from the ideal mathematical model of PCR, including the RNA quality, the efficiency of RNA-to-cDNA conversion, the primer quality, operator technique, as well as the "Monte Carlo" effect, an inherently and unavoidably high variance in the results from PCR reactions with a low starting template concentration[37]. In terms of its application in transcriptome analysis of clinical diseases, it is relatively low-throughput and can only

test a limited number of genes of interest with known sequences[38].

These limitations largely confine its ability to discover novel DGE of disease status. Only when researchers already know which "suspect" genes or pathways to target can RT-qPCR efficiently identify the disease-related DGE. Without predefined genes of interest and sufficient biological plausibility, it is challenging to identify novel disease-specific DGE solely with RT-qPCR. To achieve the prospective profiling of the transcriptome of HCC and recurrence, a more high-throughput technology that is capable of screening massive numbers of genes with various degrees of probability to have a disease-specific expression in parallel is needed. Thus, in the research of HCC, RT-qPCR is mainly used as a validation tool to confirm the DGE identified by two other technologies that emerged later on: microarrays and RNA-seq. These two research tools have yielded abundant results in all three aspects of HCC recurrence in the causal model we propose (Figure 1).

MICROARRAY ANALYSIS OF RECURRENT HCC

First developed by Schena *et al*[39] at Stanford University in 1995, microarrays have been widely applied in medical research as a high-throughput tool to reveal gene expression in disease status[39]. Microarrays can be divided into two main categories: cDNA microarrays and oligonucleotide microarrays. The surfacing of commercial platforms, such as Human UniGene Set RZPD 1 clone set for cDNA microarrays and Affymetrix Human Genome U95Av2 array for oligonucleotide arrays, have made the technology for both types of arrays widely accessible[40]. Additionally, the oligonucleotide microarrays have further been developed to identify single nucleotide polymorphism (SNP), named SNP arrays, in which the probes designed for harboring the SNP positions are hybridized with fragmented DNA molecules to examine the specific alleles of all SNPs[41]. The advance in microarray technology allows researchers to screen tens of thousands of RNA transcripts simultaneously and makes it possible to identify new genes with DGE related to diseases or specific pathophysiological conditions of interest. In addition to its high-throughput applicability, it has other major advantages including the wide availability of uncomplicated bioinformatics tools, more manageable data, and relatively low cost[38].

Microarray analysis has long played a central role in the field of HCC research. Iizuka *et al*[18] conducted a comprehensive review of the abundant data yielded with this revolutionizing technology [18], in which the authors classified the microarray-based approaches into three groups based on the distinct objectives of the studies, class comparison, class discovery, and class prediction, as proposed by Simon *et al*[42]. Such classification, however, was mainly according to study methodologies but hardly compared findings in the context of biological mechanisms and pathogenesis of HCC. To organize the diverse findings of microarray analyses in an integrated manner and to provide insights with pathobiological plausibility, we summarized the currently available data within the framework of our causal model of HCC, including the carcinogenic profile of primary cancer cells, carcinogenic stimuli, and the tumor microenvironment, in the following paragraphs.

Carcinogenicity of primary HCC cells

Lau *et al*[32] utilized cDNA microarrays to analyze the differential expression of mRNA of 4000 genes in paired HCC and noncancerous tissues[32]. They found that 211 genes were upregulated while 147 genes were downregulated, of which six genes were highly expressed and ten genes were downregulated in more than 30% of pairs. This was the first time when microarray technology was used in humans. Subsequently, either cDNA or oligonucleotide microarray studies, targeting either cell lines or patient-derived samples, have been widely conducted to discover the DGE related to the carcinogenic profile of primary HCC cells. The differentially expressed genes that have been identified include those associated with cell-cell interaction[43,44], transcription factors[18,43,45,46], apoptosis[43,45,47], cytokines[43,45], growth factors and/or growth suppression signals[43,45,48,49], cell proliferation[44,45,47,49,50], the cell cycle[43,45,49], tissue-specific expression proteins related to cell differentiation and development[45, 51], metabolism[49], angiogenesis[43,45,49], and stress-related response[35,50]. With the maturity and extensive application of microarrays, researchers further exploit this powerful technology to identify genes associated with HCC progression and prognosis. For instance, one study found that the upregulation of *ADAR, PSMD4, D9SVA, CCT3, GBAP, RDBP*, and *CSRP2* with downregulation of *IL7R* were associated with dedifferentiation of HCC[52]. Other studies focusing on metastasis and rapid progression of HCC identified differential expression in vimentin[53], granulin-epithelin precursor[54], ephrin-A1[53], and N-Myc downregulated gene 1[55].

It has also been widely known that differential expression of certain genes in primary cancer cells is associated with early recurrence. One study identified the expression profile of claudin-10, along with the pathological tumor-node-metastasis (pTNM) stage, to be independent predictors for HCC recurrence, and the results were validated with RT-qPCR[56]. Another study compared DGE of patients with recurrence *vs* those without, and it was found that four HLA genes (*HLA-DRA, HLA-DRB1, HLA-DG*, and *HLA-DQA*) encoding major histocompatibility complex class II antigens had significantly lower expression in the early IHR group[57]. Furthermore, while the DGE detected by microarrays and RT-

qPCR, as well as pathological tumor-node-metastasis stage and venous invasion were all found to be associated with early IHR in the univariate association study, the multivariate study only identified DGE of *HLA* as an independent predictor for early IHR. Iizuka *et al*[58] compared the gene expression of tumor cells between patient groups with and without recurrence and found that cell adhesion-related genes, including *ITGA6* and *SPP1*, had higher expression levels in HCC with early IHR[58].

Given that portal vein invasion (PVI) is known as a major prognostic factor of HCC recurrence[16,59-61], many studies used microarrays to identify the DGE related to PVI. One study found that cell growth-related genes *TAF4B*, *SLC4A7*, *RAB38*, and *RYR1* were associated with PVI[62]. Another study discovered that upregulation of *MMP14* and downregulation of two cytochrome P450 enzyme (*CYP*) genes, *ADAMTS1*, and *ITGA7* were associated with PVI[63,64]. Moreover, one study identified DGE of 110 sequence tags, *RHOC*, and two small GTPase-related genes (*ARHGAP8* and *ARHGEF6*) to be PVI-associated[63,64]. Lastly, one of the studies listed above successfully used PVI-associated DGE data from the microarray analysis to predict recurrence after surgical resection of HCC[62]. Although not directly analyzing recurrence-associated DGE, these studies offered abundant insight into the carcino-genicity of primary cancer cells defined by PVI.

Knowledge established by microarray studies has commonly been combined and applied to predict the recurrence and outcome of HCC. A data mining study examining the DGE between patients with IHR and those without recurrence based on pre-existing microarray databases generated one set of four differentially expressed genes (*STC1, FOXK2, MMP1,* and *LOXL2*) that promoted either cell cycle advancement or histone modulation could predict the incidence of early recurrence[65]. Another study conducting microarray analysis in human primary HCC tumors developed a 172-gene molecular prediction system for early IHR and tested its performance in independent cases[66]. The value of the predictive system was found to be a significant prognostic factor according to multivariate Cox regression analysis. Thus, DGE related to early IHR can be designed to predict clinical outcomes.

In summary, identification of DGE using microarrays, either by directly comparing recurrence with non-recurrence groups or indirectly looking at surrogate predictors such as PVI, enables us to clarify the carcinogenicity and the propensity of recurrence in primary HCC.

Carcinogenic stimuli

Clinical association studies have identified various risk factors of HCC. Common risk factors for HCC include HBV and hepatitis C virus (HCV) infection, cirrhosis, alcoholic liver disease, and nonalcoholic fatty liver disease. Less common risk factors include exposure to environmental toxins, Wilson's disease, hereditary hemochromatosis, alpha1-antitrypsin deficiency, primary biliary cholangitis, and autoim-mune hepatitis[67]. In addition to the carcinogenicity of the primary HCC cells, these carcinogenic factors also play a decisive role in the development and recurrence of HCC. Microarray studies with clustering analysis based on some of these clinicopathological features have been widely performed to provide information about how risk factors contribute to HCC at a molecular level, with the greatest proportion of data coming from studies related to viral hepatitis.

Some studies compared the DGE of HCC cells to that of noncancerous liver tissues in HBV-positive and HCV-positive groups[67,68]. One study directly used oligonucleotide microarrays to compare transcriptomes in HBV-associated *vs* HCV-associated HCC, finding DGE in 83 genes, of which 31 and 52 genes showed increased expression in HBV and HCV-associated HCC, respectively[69]. The genes with DGE found in HBV-positive HCC mainly involved imprinted genes and genes associated with signal transduction, transcription, and metastasis, while in HCV-positive HCC the DGE was mainly found in genes related to detoxification or immune response. Such findings highlight the distinct mechanisms of viral carcinogenesis.

Another study used cassette ligation-mediated PCR to identify the human genome sequence next to the HBV DNA integration site and then conducted a microarray experiment to directly measure the characteristic expression of the affected genes[70]. In addition to viral hepatitis, certain toxins are also known as carcinogenic in favor of HCC, and microarray technology has been utilized to predict the carcinogenicity of chemicals by analyzing changes in gene expression in animal or cell culture models [71,72].

Finally, cirrhosis of any etiology is a major risk factor in the development of HCC. One study compared the gene expression profiles between HCC in patients with cirrhosis and without cirrhosis and identified several genes related to the regulation of inflammation, growth, and invasion of precan-cerous cells in cirrhotic liver, including C-C motif chemokine receptor and ligand (*CCR7* and *CCL5*), C-X-C motif chemokine ligand, and cytochrome P450 enzymes (*CYP2E1*, *CYP2C9*, and *CYP2A6*)[73].

In terms of recurrence, Kim *et al*[74] developed a risk scoring system with DGE of 65 genes identified with microarrays analyzing primary tumor cells in HBV-positive HCC patients and validated this system in another group of patients, finding that the classifiers successfully predicted early recurrence but failed to predict late recurrence[74]. The same author group further tried another approach by conducting a systemic analysis of gene expression from non-cancerous human liver tissue undergoing hepatic injury and regeneration. They identified a 233-gene signature that was significantly associated with late recurrence of HCC and validated the system in HBV-positive HCC patients who had received curative surgical treatment[75]. Network analysis of the gene signature identified signal transducer and activator of transcription 3/Notch signaling activation to be significantly related to late recurrence of

HCC. With prediction using microarray and multivariate logistic regression analysis, the authors scaled down the system to a four-gene (*RALGDS, IER3, CEBPD,* and *SLC2A3*) model that could successfully predict HCC recurrence. Interestingly, different models targeting cancerous or non-cancerous tissues could predict early and late recurrence of HCC, respectively, which may reflect the distinctive pathogenesis behind early and late recurrence of HCC in HBV-positive individuals.

Similarly, the recurrence of HCV-positive HCC has also been well studied. One study compared the DGE of noncancerous liver tissues from HCV-positive HCC patients with single nodular HCC recurrence and multicentric recurrence. Next, the authors developed a predictive system based on DGE in 36 genes, which was validated to successfully predict multicentric recurrence[76]. Also targeting HCV-positive HCC, another study found that the DGE profile observed in primary HCC biopsy or explant could not predict recurrence-free survival, while those yielded from noncancerous tissues could [77,78], which agreed with other studies on HBV-positive HCC[74,75]. Therefore, regardless of HBV or HCV-positive HCC, late recurrence was more likely to originate from a new clone of cells rather than the original HCC cells.

Another study focusing on genes associated with recurrence of HCC in HCV-positive patients awaiting liver transplantation identified the DGE profile of genes related to viral response as well as transcriptional network regulated by interferons, specifically interferon-α/β-inducible genes (signal transducer and activator of transcription 1, *OAS1*, and *MX1*), to be associated with recurrence-free survival[78]. The study also found that *FAIM3*, an anti-apoptotic gene, and *USP18*, a gene encoding an enzyme of the deubiquitinating protease family, were overexpressed in patients with recurrent HCC. Collectively, these studies exemplified how microarrays contribute to our understanding of carcinogenic stimuli in HCC recurrence.

Tumor microenvironment

Compared to the other two components in our causal model of HCC recurrence, microarray-based studies focusing on the tumor microenvironment and surrounding tissue suffer from less available data. This may be due to the limited ability of microarrays, as one of the "bulk" transcriptome analysis methods, to reveal the status of an individual cell. However, we found one study using cDNA microarrays to compare the gene expression profiles of noncancerous peripheral tissue from two HCC patient groups: those with primary HCC and venous metastases or confirmed extrahepatic metastases by follow-up, termed metastasis-inclined microenvironment samples; and those with HCC without detectable metastases, termed metastasis-averse microenvironment[79]. The authors found DGE in *HLA-DPA1*, *HLA-DRA* (antigen-presenting dendritic cells, B cells, epithelial cells), *PRG1*, and *ANXA1* to be associated with a metastatic phenotype, reflecting a T helper 2-predominant, anti-inflammatory cytokine profile, for which *CSF1* may be responsible. Interestingly, the increased expression of *HLA-DRA* in surrounding noncancerous tissue, as observed in this study, contrasts with one of the studies we cited above in which *HLA-DRA* genes showed lower expression in tumors[57]. The spatial distribution, migration of immune cells, and dynamic nature of the microenvironment may serve as plausible reasons for the discrepancy.

RNA-SEQ ANALYSIS OF RECURRENT HCC

Although microarray technology allows simultaneous measurement of numerous genes in one sample, it still has major drawbacks such as the limited dynamic range, high background noise, and an inability to detect novel transcripts. The advent of next-generation sequencing technology gave rise to a new technology, RNA-seq, that can address these limitations.

In 2008, RNA-seq was initially described by Nagalakshmi *et al*[80] as a new quantitative sequence-based method to map transcribed regions of the yeast genome[80]. Compared to microarray-based methods, RNA-seq enjoys higher genome coverage and better profiling of dynamic transcriptomes, providing information about alternative splicing, allele-specific expression, non-coding RNA, and SNPs. Unlike RT-qPCR and microarrays, which are largely limited by the requirement for *a priori* knowledge of the sequences being interrogated, RNA-seq is exclusively competent to discover novel transcripts [81]. As a result, despite disadvantages such as higher cost and large dataset generation, RNA-seq has been replacing microarrays over the past decade for transcriptome analyses in basic and translational research[82]. A comparison of RT-qPCR, microarrays, and RNA-seq are summarized in Table 1. The typical workflow for RNA-seq and microarray analysis in HCC translational research is illustrated in Figure 2.

Carcinogenicity of primary HCC Cells

RNA-seq enables us to identify specific DGE and expand our knowledge of the pathogenesis of HCC. One study performed pairwise DGE analysis between HCC and non-HCC tissues, finding upregulation of oxidative phosphorylation and higher expression of associated DNA damage-related signals in HCC compared to non-HCC samples. These findings suggest development of HCC may result from oxidative stress generated from overactive oxidative phosphorylation[83]. Another study analyzing poorly differ-
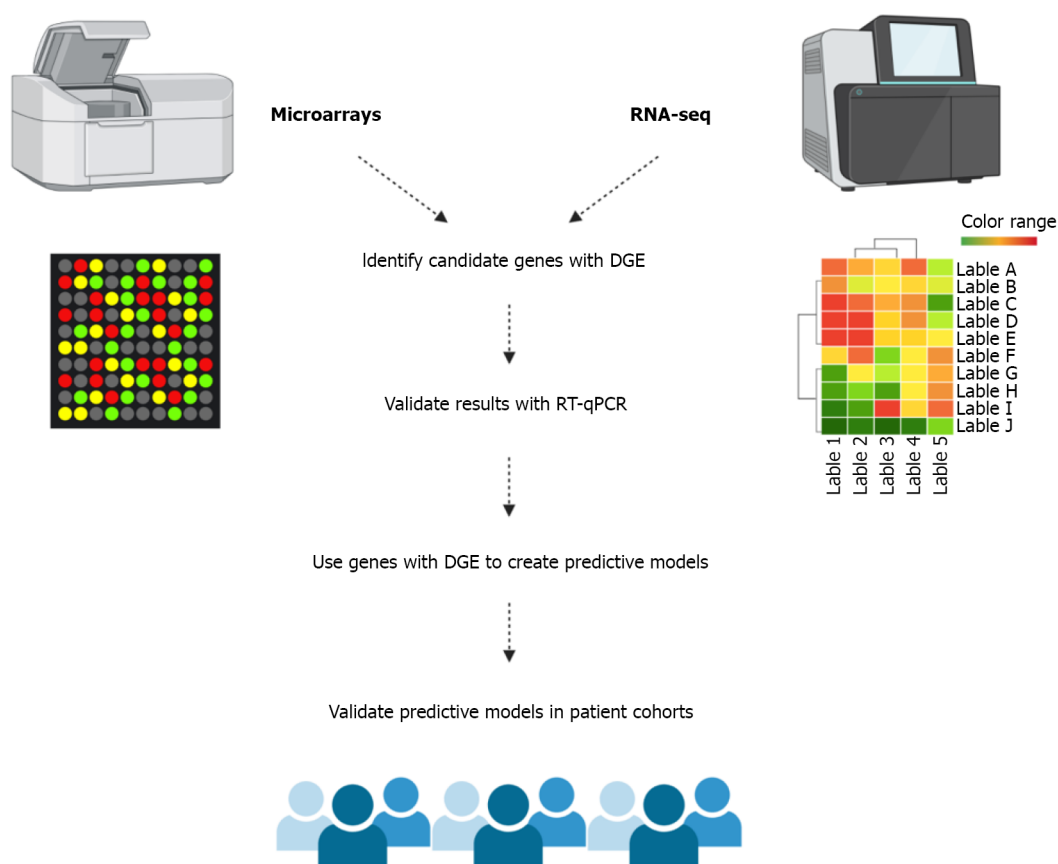
Table 1 Comparisons of real-time quantitative reverse transcription PCR, microarrays, and RNA-sequencing and their applications in hepatocellular carcinoma recurrence

	RT-qPCR	Microarrays	RNA-seq
Basic steps	RNA isolation, genome DNA removal	RNA isolation, mRNA extraction	RNA isolation, mRNA extraction
	cDNA preparation with RT	cDNA library preparation	Quality and quantity check
	Use of primers for amplification	Labeling with fluorescence	cDNA library preparation
	Data analysis	Hybridization with transcript probes on slides	Sequencing
		Scanning	Data analysis
		Image processing and data analysis	Validation
		Validation	
Throughput	Low	High	High
Dynamic range/sensitivity	Widest/high	Narrow/low	Wide (compared to microarrays)/high
Need for reference genome	No	No	Yes
Known sequences of genes of interest	Required	Required	Not required
Cost	Low	Low	High
Advantages	Low cost, simple	High throughput	Ability to detect novel differential transcripts
	Highest dynamic range	Relatively low cost	Splice junctions, SNP, non-coding RNA
	Gold standard	Good bioinformatics and statistical practices	
Downsides	Dependence on pre-existing knowledge of genes of interest	Difficulty to detect novel transcripts, non-coding RNA, splicing, or other dynamic natures of transcriptome	Large data storage
	Low throughput		High cost
		Need for designing probes	
		Low dynamic range	
Applications and main achievements in HCC recurrence-related research	Commonly used as a validation tool for confirming DGE results yielded from other high throughput analyses[56]	Providing abundant information on carcinogenicity of primary HCC cells and carcinogenic stimuli; laid the foundation for our current understanding of the pathogenesis of HCC recurrence[18]	Prospectively discovering DGE as potential novel classifiers for the carcinogenic profile of recurrent HCC cells; elucidating how HBV triggers HCC recurrence by interrupting the human genome[92,94,96]

cDNA: Complementary DNA; DGE: Differential gene expression; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; RNA-seq: RNA-sequencing; RT: Reverse transcription; RT-q: Real-time quantitative reverse transcription; SNP: Single nucleotide polymorphism.

entiated, moderately differentiated, and well-differentiated HCC with RNA-seq found DGE in poorly differentiated HCC to be mostly associated with cell metabolism, cell cycle, translation, and blood coagulation, of which the upregulation of *NOVA1*, *NSMCE2*, and *KIAA0196* and downregulation of *AQP9* were validated with RT-qPCR[84].

Since RNA-seq has a greater dynamic range and is more capable of detecting genes expressed with low abundance, another study analyzed blood samples taken from HCC and non-HCC patients in which the authors first identified 1578 dysregulated genes with RNA-seq and then validated them with RT-qPCR. Six genes (*SELENBP1, SLC4A1, SLC26A8, HSPA8P4, CALM1,* and *RPL7p24*) were differentially expressed, and the *CALM1* expression level was found to decrease along with tumor enlargement and thus had potential as a novel biomarker for tracking HCC[85]. While RT-qPCR also has a wide dynamic window and has been used to analyze peripheral blood during diagnosis[29,30], the ability of RNA-seq to scan many genes at a whole-genome scale makes the identification of candidate biomarkers more efficient. Moreover, RNA-seq is exclusively capable of analyzing non-coding RNA and has been utilized to identify long non-coding RNA (lncRNA) as well as small nucleolar RNA host genes. For instance, *SNHG4*, which is involved in the regulation of ribosomal RNA synthesis, RNA processing, and surveillance pathway, was found to be closely related to the tumorigenesis of HCC[86]. These studies

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Microarrays　　　　　　　　RNA-seq

Identify candidate genes with DGE

Validate results with RT-qPCR

Use genes with DGE to create predictive models

Validate predictive models in patient cohorts

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Figure 2 Typical workflow of bulk transcriptome analysis in translational hepatocellular carcinoma research. The figure was created with BioRender.com. DGE: Differential gene expression; RNA-seq: RNA-sequencing; RT-q: Real-time quantitative reverse transcription.

exemplified the unique advantages of RNA-seq in discovering cancer mechanisms.

RNA-seq has also been used to analyze the carcinogenicity of HCC cells specifically related to recurrence. One study performing transcriptome analysis of 128 post-liver transplant HCC recurrence tissue samples found that the DGE was mainly found in genes involved in DNA synthesis, chromatin segregation, and mitosis, which might facilitate DNA replication and the growth of cancer cells[87]. The authors also performed mutation analysis in this study. Interestingly, the expression of some well-known mutations previously identified in HCC, such as p53, beta-catenin 1, and telomerase reverse transcriptase, did not appear to be significantly associated with HCC recurrence or prognosis. One explanation the authors proposed was that tumors recur after circulating HCC cells present at the time of transplantation traverse through the circulation, survive the turbulent flow environment, proceed through the pulmonary circulation, and finally seed themselves within the new liver. Another possible explanation is that recurrence is influenced by a complex interplay between primary cancer cells and "extratumoral" factors such as neurotransmitters, metabolism, or other constituents. This explanation is consistent with previous results from microarray studies finding that DGE of primary tumor cells failed to predict later recurrence[74,78].

Exploiting the merits of RNA-seq for detecting SNP and CNV, one study comparing liver gene expression in transplant patients with and without recurrence found glutathione S-transferase A2 (*GSTA2*) expression to be associated with early phase systemic injury and reactive oxygen species levels. Moreover, *GSTA2* could serve as a predictor of recurrence. Further, the authors identified that the G335C SNP of the *GSTA2* coding sequence, corresponding to an S112T amino acid substitution, was associated with HCC recurrence and survival[88]. Another study analyzing pairwise DGE analysis between primary and recurrent HCC found SNP variants of *GOLGB1* and *SF3B3* to be significantly related to more aggressive phenotypes[89].

Over the past decade, the popularity of high-throughput sequencing technology has not only made RNA-seq widely available but also brought about a publicly accessible comprehensive repository for genome-wide gene expression data such as the Gene Expression Omnibus and ArrayExpress. The Cancer Genome Atlas (TCGA) collects the results of cancer-related research, and mining of these shared databases provides insights by integrating results from different studies[90]. For example, Wang *et al* [91] identified a 77-gene signature associated with early HCC recurrence by conducting microarray experiments and cross-referencing the results with RNA-seq data from TCGA[91]. Combining data from

an ensemble of transcriptome profiling tools enables researchers to validate and complement results.

Carcinogenic stimuli

Thanks to its ability to capture the dynamic nature of transcriptome profiles, RNA-seq offers a unique advantage in investigating how carcinogenic factors alter transcriptome patterns in HCC development. It is known that HBV DNA can be integrated into the human genome and may result in somatic mutations[92]. One study found DGE of ten matched pairs in HBV-related HCC and non-HCC tissues to be mostly related to cell growth, metabolism, and immune-related pathways, which were significantly enriched at 8q21.3-24.3[93]. Moreover, the authors found a highly upregulated exon-exon junction at the *ATAD2* gene, an important protein that acts as a cofactor for Myc proteins, androgen receptor, and estrogen receptor-alpha. Also using RNA-seq, another study identified contrasting genomic and transcriptomic alterations such as HBV integration, somatic mutation, and CNV by comparing tumor with non-tumor samples[94]. For nonviral carcinogenic factors, one study identified 747 mRNAs and 8 lncRNAs with DGE between HCC and non-HCC cirrhotic tissues, narrowing down the results to 15 hub genes based on an association study with AFP levels in blood samples. Of these, *SPX*, *AFP*, and *ADGRE1* were validated in an independent HCC cohort[95].

With respect to recurrence, one study performed RNA-seq in HBV-related HCC patients to compare tumor and non-tumor tissues with various degrees of fibrosis[96]. HBV host genes overlapped with pathogenic SNPs in tumor suppressor genes of non-tumor tissues. Overlap was more significant in non-tumor tissues among recurrent cases, suggesting that tumor recurrence was highly associated with the integration of HBV genomes into precancerous tumor suppressor genes. Additionally, the difference in pathogenic SNP count between recurrent and non-recurrent patients was much larger in the low fibrosis group compared to the high fibrosis group, indicating that different recurrence risk models are needed for patients with low and high fibrosis. Taken together, these studies show the strength of RNA-seq in investigating the molecular genetic basis of HCC recurrence.

Tumor microenvironment

In addition to primary tumor cells and carcinogenic stimulus, researchers use this powerful tool to investigate the impacts of tumor microenvironment on recurrence. One study performing RNA-seq to analyze DGE between HCC tumors and surrounding cirrhotic tissue showed a gradual suppression of local tumor immunity coinciding with disease progression. In addition, the authors divided tumors into T cell-infiltrated and T cell-excluded based on the localization of CD8+ cytotoxic T lymphocytes visualized by immunohistochemistry staining and performed RNA-seq to examine the DGE between two groups. Twenty-three identified genes were associated with fibrosis and potentially modulated by transforming growth factor beta, platelet-derived growth factors, sonic hedgehog protein, or Notch pathways[97]. Given the emerging evidence suggesting that lncRNAs participate in cancer immunity, another study used RNA-seq to look at immune-related lncRNAs and related mechanisms from the TCGA database, identifying nine immune-related lncRNAs associated with HCC recurrence *via* Cox regression analysis[98]. The authors also created a recurrence prediction model based on their findings that was validated in an independent patient cohort.

The literature reviewed above shows how RNA-seq could be used to reveal molecular features of tumor immune biology in HCC progression and recurrence. However, most of the studies are limited to the discovery of DGE in bulk tissue samples, which may not be able to reflect the highly complicated tumor milieu and immune diversity. Although one study did consider tissue compartmentalization and tried to cluster cell populations to correlate DGE results with histology and immunohistochemistry staining[97], the approach was inefficient. The advent of single-cell RNA-seq (scRNA-seq) could address this issue and has improved our understanding of transcriptomes during cell-cell interaction. ScRNA-seq holds particular promise in research focusing on the tumor microenvironment, as described in the next section.

SINGLE-CELL TRANSCRIPTOME ANALYSIS OF RECURRENT HCC

Conventional bulk RNA-seq inherits the drawback of "averaging out" the data and does not have adequate resolution to delineate cell trajectory and cell-cell interactions. However, HCC is a heterogeneous disease with complex etiologies and tumor milieu[99,100]. There is thus a need to study the heterogeneity of tumoral cells and their ecosystem, particularly the immune cells.

To achieve a high resolution of cell subpopulations in malignant tissue, various single-cell isolation techniques have been developed. Generally, the isolation methods can be categorized into either cell marker-based selection or size-based selection, including fluorescence-activated cell sorting, laser microdissection, manual cell picking, serial dilution, magnetic-activated cell sorting, microfluidics, and CellSearch system[101,102]. When combining single-cell isolation with sequencing techniques, these technologies become promising tools to study intertumoral and intratumoral heterogeneity both spatially and temporally. Since mRNA transcriptome sequencing in a single cell was first reported in 2009[103], advances in sequencing techniques and single-cell selection methods have driven different

applications in the field of cancer biology. In recent years, droplet-based systems for high-throughput scRNA-seq such as inDrop, Drop-Seq, and 10X Genomics[104] have gained attention. Although previous studies used them to profile early HCC and its tumor microenvironment[105-107], studies for recurrent HCC are still limited.

Tumor heterogeneity, including intertumor and intratumor heterogeneity, is responsible for the recurrence of HCC[108]. Previously, researchers stressed the genomic profiling and molecular subclassification of intertumor heterogeneity[109-111]. Nevertheless, cancer cell adaptation, drug resistance, and tumor microenvironment are more closely related to intratumor heterogeneity[108]. To correlate the gene expression landscape of intratumor heterogeneity with HCC patient outcome, Losic *et al*[112] characterized a gene signature composed of 363 genes in the TCGA-HCC database[112]. The gene signature was associated with worse survival and was able to compete with other pre-existing single-biopsy prognostic signatures. The gene signature was also correlated with early tumor recurrence in the Heptromic Cohort as well as with higher levels of the prognostic biomarker AFP. At the single-cell level, the authors found transcriptional factor heterogeneity in the gene regulatory network by analyzing cis-regulatory sequence motifs from seven different locations in two HCC patients.

Another study found cellular heterogeneity in primary tumors similar to portal vein tumor thrombus and metastatic lymph nodes *via* scRNA-seq[113]. Additionally, authors focused on intratumoral T cells, in which they found CD8+ T cell clusters to be more enriched in HBV/HCV-related tumors compared to HBV/HCV-unrelated HCCs, concluding that chronic HBV/HCV infection may lead to CD8+ T cell exhaustion in HCC tumors. This phenomenon reflects the immune checkpoint blockade efficacy of viral-associated HCC in clinical scenarios, as the high checkpoint blockade response rate is related to CD8+ T cell density and programmed cell death protein 1 expression[114-116]. Furthermore, the authors found ligands highly expressed in protumorigenic and prometastatic hepatocytes related to inflammation (*e.g.*, C-X-C motif chemokine ligand 10/CXCR3) and immunosuppression (*e.g.*, macrophage-migration inhibitory factor/CD74), respectively. Distinct functions among malignant hepatocytes shape the immune microenvironment of HCC and provide hints to both tumor progression and immunotherapy.

Tumor recurrence and treatment resistance are partially determined by cancer stem-like cells (CSCs), which consist of a special subset of cells with stemness features and dictate cellular hierarchy and traits of dormancy and plasticity[117-119]. In a previous study, Zheng *et al*[120] combined transcriptome and functional analysis of HCC cells at the single-cell level to assess the degree of CSC heterogeneity as well as relationship to patient prognosis[120]. Discrete CSC subpopulations identified using single-cell surface markers all had a higher self-renewal ability compared to marker-negative cells but demonstrated appreciable biological differences in cell division and response to hypoxic stress in between. In addition, the authors found a 286-gene signature linked to CD133 and epithelial cellular adhesion molecule (EpCAM) are independent predictors of HCC patient survival. Moreover, HCC CSCs display an altered pattern of self-renewal heterogeneity when cultured under normoxia or hypoxia, suggesting a biological plasticity to these cells. Another article utilized scRNA-seq to identify two main HCC populations characterized by differential EpCAM expression[121]. Notably, a CD24+CD44+-enriched subclone within the EpCAM+ population exhibited a specific oncogenic expression signature and indicated the stemness of HCC. These findings were further confirmed by *in vitro* knockdown and *in vivo* tumorigenicity studies.

Sun *et al*[122] combined RNA-seq with single-cell profiling in paired samples from tumor and non-tumor regions of primary or recurrent HCC to unveil the unique immune ecosystem of recurrent HCC. The authors observed decreased regulatory T cells (Treg) and T cell proliferation with an increased proportion of CD8+ T cells and dendritic cells[122]. In addition, the authors concluded that CD8+ T cells in primary tumor and recurrent tumor samples showed the same transition trajectories but displayed considerably different immune and transcriptional states, suggesting that different immune therapy strategies should be considered for the treatment of primary and recurrent HCC. Specifically, CD8+ T cells in recurrent tumor samples, characterized by overexpression of *KLRB1*, revealed an innate dysfunctional state with low cytotoxicity and immunosuppressive phenotypes, which differed from the exhaustion state observed in primary tumor samples. The authors thus provided a model in which CD8+ T cell clones reside in a low proliferative and unresponsive state in the recurrent tumor due in part to tumor selection, suggesting that those cells are unable to recognize and eliminate recurrent tumor cells displaying subclonal neoantigens. In summary, data from this study indicated that malignant cells in the recurrent tumor demonstrated strengthened immune evasion capacities and reduced immune cell proliferation. Recurrent malignant cells could impair antigen presentation in dendritic cells *via* the programmed death-ligand 1-CD80 and cytotoxic T lymphocyte-associated protein 4-CD80 axes. Malignant cells may also recruit innate-like CD161+ CD8+ T cells *via* the CCL20-CCR6 axis, which could compromise anti-tumor immunity in early-relapse HCC.

Differentially expressed genes and pathway enrichment found in single-cell transcriptomes can be further applied to discover candidate drugs for the prevention of HCC recurrence as well as to the study of immune cell-cell communication. To predict the disease-free survival time and postoperative recurrence of HCC, Fu and Lei[123] constructed a risk score based on three immune cell types (effector memory CD8+ T cells, Treg cells, and follicular helper T cells) from the TCGA-HCC database called the T cell risk score[123]. Next, the authors used scRNA-seq data from 12 primary and 6 relapsed HCC samples to identify 645 genes with differential expression in three T cell types. After survival analysis,

the authors established a gene risk score by 15 prognostic genes (*AP000866.1, ATIC, CAPN10, EDC3, EID3, NCKIPSD, OXLD1, PHOSPHO2, POLE2, POLR3G, SEPHS1, SRXN1, TIMM9, ZNF487, and ZSCAN9*), which showed consistency with the T cell risk score in disease-free survival and immune characteristics. The results indicated these 15 hub genes may play a role in the process of immune cells affecting disease-free survival.

Subsequently, these hub genes were screened with CellMiner, a web tool based on the NCI-60 cell line set for identifying potential therapeutic drugs[124]. Pearson correlations between the 15 hub genes and the half-maximal inhibitory concentration of targeted drugs were analyzed, and the studies suggested that postoperative treatment of these drugs, such as imexon, irofulven, and nelarabine, may delay HCC recurrence. Moreover, the authors explored immune cell-cell interactions, finding the strongest communication among these three cell types was from effector memory CD8+ T cells to themselves *via* the granzyme A-coagulation factor 2 receptor signaling pathway as well as effector memory CD8+ T cells to follicular helper T cells and Treg cells *via* the CCL5-CCR4 signaling pathway. These findings illuminate crosstalk among such cell types, which is beneficial in future investigation of effector memory CD8+ T cells, Treg cells, and follicular helper T cells in disease-free survival time and recurrence prevention for patients with HCC.

LIMITATIONS AND FUTURE DIRECTIONS

The current healthcare system is transitioning from the time of evidence medicine towards the era of precision medicine. The omics technologies are evolving quickly and have built up tremendous results. Admittedly, there is still a gap to be bridged between the bench and bedside for translating these technologies.

First, the development of other levels of omics research (*e.g.*, epigenetics, proteomics, and metabolomics) expands cancer research including HCC to a multitude of data. How to incorporate and harness this huge amount of information and confirm its clinical importance (and not just an association) remains a challenge. With the advancement of computing power and artificial intelligence, we have the opportunity to store data and deal with multi-omics in parallel. Researchers can thereby extract significant data, confirm it with mechanistic studies in the laboratory, and translate it into clinical trials.

Second, studies showed that physicians who had lower confidence in genomic or transcriptomic technologies would like to ask for guidelines or training support[125-127]. To popularize transcriptome analysis into clinical practice, we require more physicians who understand the concepts of both omics technologies and tumor biology to stand out. Thus, we can improve medical education, design relevant clinical trials, and formulate health guidelines and policies. In fact, it is not merely a portion of people's responsibility. Multidisciplinary networks to share the collection of patient samples, clinical data, the standard of techniques, and genetic counseling are all indispensable to making medical decisions.

Last but not least, the accessibility of these technologies, especially the cost, is still a burden to patients. We hope in the future that these technologies can be applied more widely, and the price can be affordable for people by following "Moore's law" (*i.e.* higher throughput and lower cost) as in the computer industry (genome.gov/sequencingcosts; accessed November 19, 2022). Meanwhile, health policymakers should recognize the need for providing patients with transparent information and protecting their privacy. Therefore, we can benefit most patients by achieving the P4 discipline (preventive, predictive, personalized, and participatory) in precision medicine[128].

CONCLUSION

The high recurrence rate of HCC remains a serious burden to patients undergoing curative treatments and a major challenge to patient outcome. Over the past few decades, the emergence and evolution of transcriptome profiling methods have benefited the discovery of disease mechanisms, diagnosis, and treatment of HCC recurrence. Our current understanding of HCC pathophysiology is largely based on the fruitful results yielded by transcriptome analysis technology. We found that these abundant studies can be categorized based on the three domains of tumorigenesis, which include carcinogenic profile of primary cancer cells, carcinogenic stimuli, and tumor microenvironment.

We herein summarized the major findings of RT-qPCR, microarray, RNA-seq, and scRNA-seq research under the framework of these three domains (Table 2) and in doing so revealed the strengths and limitations of each technique. Being low-throughput, RT-qPCR is limited in identifying new DGE and is mainly used to validate findings yielded by other high-throughput techniques. Microarrays and RNA-seq have yielded great achievements in the study of carcinogenicity among primary cancer cells and carcinogenic stimuli. Moreover, these technologies contributed largely to our current knowledge on HCC recurrence pathogenesis. To date, a workflow consisting of microarray/RNA-seq search for differential transcripts, RT-qPCR confirmation, predictive model generation, and independent patient cohort validation has become a standard approach in basic HCC science. However, current diagnostic and

Table 2 Representative transcriptomic studies in recurrence of hepatocellular carcinoma

Ref.	Method	Sample comparison	Major findings	Featured research domain
Jiang *et al*[29], 2000	RT-qPCR	Nontumorous liver *vs* tumor samples; peripheral blood from HCC patients	*MMP9* in tumors was related to recurrence. mRNA of *AFP* in blood samples was associated with recurrence	Primary cancer cells
Morimoto *et al*[30], 2005	RT-qPCR	Peripheral blood and bone marrow samples from patients with HCC *vs* benign diseases	*AFP* mRNA level in blood, but not bone marrow, could be useful for predicting postoperative tumor recurrence	Primary cancer cells
Cheung *et al*[56], 2005	Microarray	HCC tumors from patients with post-OP recurrence *vs* without recurrence	*CLDN10*, along with the pTNM stage, were independent predictors for HCC recurrence	Primary cancer cells
Matoba *et al*[57], 2005	Microarray	HCC tumors from patients with *vs* without post-OP early (< 1 yr) recurrence	*HLA-DRA*, *HLA-DRB1*, *HLA-DG*, and *HLA-DQA* had significantly lower expression in the early IHR group	Primary cancer cells
Iizuka *et al*[58], 2006	Microarray	HCC tumors from patients with post-OP IHR *vs* EHR	46 cell adhesion-related genes, including *ITGA6* and *SPP1*, had higher expression levels in HCC with early IHR	Primary cancer cells
Ho *et al*[62], 2006	Microarray	HCC tumors from patients with *vs* without PVI	Differential expression of 14 genes related to the human melanoma gene family, cell growth, DNA glycosylation, and thrombin inhibitors, can be used to predict recurrence	Primary cancer cells
Chen *et al*[63], 2002	Microarray	HCC tumor and corresponding nontumorous tissue with *vs* without PVI	*ARHGAP8* and *ARHGEF6* were PVI-associated.	Primary cancer cells
Okabe *et al*[64], 2001	Microarray	HCC tumor from patients with *vs* without PVI	Upregulation of *MMP14* and downregulation of two *CYP* genes, *ADAMTS1*, and *ITGA7* were associated with PVI	Primary cancer cells
Okamoto *et al*[76], 2006	Microarray	Multicentric *vs* single nodular recurrent HCV-related HCC	36 marker genes were associated with multicentric recurrence and were used to develop a predictive scoring system	Carcinogenic stimulants
Mas *et al*[78], 2007	Microarray	HCV-related HCC from patients with *vs* without disease progression	Upregulation of *FAIM3* and *USP18*, and downregulation of *TFP1*, *HIST1H4E*, and *NRG1* were related to disease-free survival after curative treatment	Carcinogenic stimulants
Nagalakshmi *et al* [80], 2008	Microarray	MIM *vs* MAM	*HLA-DPA1*, *HLA-DRA*, *PRG1*, and *ANXA1* were associated with a metastatic phenotype (Th2-predominant), for which *CSF1* may be responsible	Microenvironment
Yoshioka *et al*[66], 2009	Microarray	HCC tumors from patients with multiple early (< 2 yr) IHR *vs* with DFS > 3 yr	Informative gene sets including *PPARBP*, *RREB-1*, *BCL2*, *HDAC1*, and *BIRC5* were yielded and used for a predictive model, which was validated in independent cases	Primary cancer cells
Kim *et al*[74], 2012	Predictive model construction using microarray database	DGE in 65 genes from pre-existing databases were used for a predictive model for early HCC recurrence and validated in independent HBV-related HCC cohorts	A risk scoring system with 65 differentially expressed genes identified from microarray data successfully predicted overall survival < 3 yr post-OP	Carcinogenic stimulants
Kim *et al*[75], 2014	Predictive model construction using microarray database	DGE of 233 HIR-related genes from preexisting databases were used for a predictive model for late HCC recurrence and validated in independent HBV-related HCC cohorts	Genes related to STAT3/Notch signaling activation were related to late (> 1 yr) recurrence of HCC. *RALGDS*, *IER3*, *CEBPD*, and *SLC2A3* were independent predictors of recurrence.	Carcinogenic stimulants
Nakagawa *et al* [65], 2021	Predictive model construction using microarray database	Validation of intrahepatic metastasis risk signatures created based on a preexisting microarray database in an independent patient cohort	*STC1*, *FOXK2*, *MMP1*, and *LOXL2* that promote either cell cycle advancement or histone modulation could predict the incidence of early recurrence	Primary cancer cells
Liu *et al*[87], 2022	RNA-seq	HCC tumors from patients with *vs* without recurrence	Most altered expression genes are related to DNA synthesis (*MCM8*, *MCM6*, *TOP2A*, and *CDC7*), chromatin segregation (*BUB1* and *CDC6*), and mitosis (*NDC80* and *PPP2R3C*)	Primary cancer cells

Ng *et al*[88], 2021	RNA-seq	Paired tumor tissues *vs* nontumorous tissues from HCC patients	*GSTA2* expression was associated with early-phase systemic injury and reactive oxygen species levels and could serve as a predictor of recurrence	Primary cancer cells
Lachmann *et al* [90], 2018	RNA-seq	Paired primary *vs* recurrent HCC tumor tissues	Mutations of *GOLGB1* and *SF3B3* are potential key drivers for the aggressive phenotype in recurrent HCC	Primary cancer cells
Okrah *et al*[97], 2018	RNA-seq	HBV-related HCC tumor *vs* distant nontumorous liver tissues	More HBV gene integrations correlated with a higher recurrence rate	Carcinogenic stimulants
Wang *et al*[98], 2021	Validation of RNA-seq database	HCC tumors *vs* matched cirrhotic tissues; CD8+ CTL-infiltrated *vs* T cell-excluded tumor tissues	Local tumor immunosuppression coincided with disease progression. Association was found between elevated fibrosis and the T cell-excluded immune phenotype	Microenvironment
Ho *et al*[99], 2021	Predictive model construction using RNA-seq database	Validation of recurrence-associated lncRNAs identified by regression analysis of TCGA database	9 immune-related lncRNAs were tightly associated with recurrence	Microenvironment
Zheng *et al*[120], 2018	scRNA-seq	CSC *vs* non-CSC populations defined by triple+ or triple− surface expression of CD133, CD24, EpCAM	286 signature genes linked to triple+ CSC could predict tumor recurrence in 240 HCC cases with multivariable Cox regression survival risk prediction analysis	Primary cancer cells
Sun *et al*[122], 2021	scRNA-seq	Tumors from primary *vs* early-relapse HCC patients	Decreased Treg and T cell proliferation with an increased proportion of CD8+- T cells and DC were found in early-relapse tumors compared to primary tumors. CD8+ T cells with overexpression of *KLRB1* revealed an innate dysfunctional state with immunosuppressive phenotypes in recurrent tumors	Microenvironment
Fu and Lei[123], 2022	scRNA-seq	Primary *vs* early-relapsed HCC samples	ScRNA-seq analysis of primary *vs* relapsed HCC identified 645 genes with DGE across three T cell types. Univariate and multivariate analysis identified 15 prognostic genes (*AP000866.1*, *ATIC*, *CAPN10*, *EDC3*, *EID3*, *NCKIPSD*, *OXLD1*, *PHOSPHO2*, *POLE2*, *POLR3G*, *SEPHS1*, *SRXN1*, *TIMM9*, *ZNF487*, and *ZSCAN9*)	Microenvironment

AFP: Alpha-fetoprotein; CSC: Cancer stem cell; CTL: Cytotoxic T lymphocyte; *CYP*: Cytochrome P450; DC: Dendritic cell; DFS: Disease-free survival; DGE: Differential gene expression; EHR: Extrahepatic recurrence; EpCAM: Epithelial cellular adhesion molecule; *GSTA2*: Glutathione S-transferase A2; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HIR: Hepatic injury and regeneration; IHR: Intrahepatic recurrence; lncRNA: Long non-coding RNA; MAM: Metastasis-averse microenvironment; MIM: Metastasis-inclined microenvironment; *MMP9*: Matrix metalloproteinase 9; post-OP: Postoperative; pTNM: Pathological tumor-node-metastasis; PVI: Portal vein invasion; RNA-seq: RNA-sequencing; RT-qPCR: Real-time quantitative reverse transcription; scRNA-seq: Single-cell RNA sequencing; STAT3: Signal transducer and activator of transcription 3; TCGA: The Cancer Genome Atlas; Th2: T helper 2 cell; Treg: T-regulatory cell.

monitoring guidelines are still based on conventional AFP and imaging assessments, having yet to incorporate data from transcriptome analysis.

Whether these gene classifiers can be applied clinically to improve diagnostic accuracy and help identify high-risk patient groups needs further confirmation from population-based studies in groups more representative of the patient population than a limited research cohort. ScRNA-seq, with its remarkably high resolution, outperforms other tools in exploring cell-cell interactions and the tumor microenvironment. Although currently available studies are limited compared to other transcriptome techniques, the high resolution and high-throughput features of scRNA-seq make it a powerful tool with great potential in investigating the tumor environment. In an era when immunotherapy is rapidly advancing, the prospect of being able to decipher the cancer immune ecology serves as a continuous incentive for future scRNA-seq studies in HCC recurrence, which may help us to verify and optimize the efficacy of novel treatments as well as facilitate the inclusion of precision medicine in managing HCC recurrence.

FOOTNOTES

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