Identifying Clonal Origin of Multifocal Hepatocellular Carcinoma and Its Clinical Implications

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Hepatocellular carcinoma (HCC) is characterized by high prevalence of multifocality. Multifocal HCC can arise synchronously or metachronously either from intrahepatic metastasis (IM) or multicentric occurrence (MO). To date, there have been no established criteria to accurately distinguish whether multifocal HCC originates from IM or MO. Histopathological features remain the most convenient strategy but with subjectivity and limited accuracy. Various molecular biological techniques involving assessment of TP53 mutation status, hepatitis B virus integration sites, and chromosomal alterations have been applied to determine the clonal origin. The introduction of next-generation sequencing facilitates a more comprehensive annotation of intertumor heterogeneity, resulting in more sensitive and accurate clonal discrimination. Generally, MO-HCC has better overall survival than IM-HCC after curative resection. Adjuvant antiviral treatment has been proved to decrease post-treatment recurrence probably by reducing MO-HCC recurrence, whereas adjuvant sorafenib treatment targeting prior micrometastasis failed to reduce IM-HCC recurrence. Recent studies recommended transcatheter arterial chemoembolization (TACE) and traditional Chinese medicine Huaier granule as effective adjuvant treatments probably by preventing IM and both types of recurrences respectively. Immunotherapy that inhibits immune checkpoint interaction may be an optimal choice for both MO- and IM-HCC. In the future, effective personalized therapy against multifocal HCC may be achieved.

INTRODUCTION

Hepatocellular carcinoma (HCC) ranks the fifth most common and second most lethal malignancy worldwide (1). Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is the leading cause of HCC (2). Compared to other gastrointestinal cancer, multifocality of HCC still remains a big challenge in the treatment of HCC. Multifocal HCCs can arise synchronously or metachronously either from intrahepatic metastasis (IM) of the primary tumor or multicentric occurrence (MO) caused by *de novo* carcinogenesis (Figure 1). Approximately 41% to 75% of patients are initially diagnosed with multifocal HCCs (3–6). According to a recent study, 35%–43% of patients with a single tumor on preoperative imaging have occult multifocality identified on explanted liver, indicating a higher actual incidence of multifocal HCC (7). Even after curative resection, postoperative recurrences could reach a high rate of 70%–80% within 5 years (8,9). Etiologically, MO-HCC tends to be more related with liver background factors, whereas IM-HCC is more dependent on tumor factors (10). Multifocal HCC based on HCV background with worse liver function is more likely derived from MO as compared to HBV background, whereas IM-HCC by itself tends to be more poorly differentiated and more aggressive (11,12). Notably, neither mechanism is mutually exclusive and both factors can contribute to multifocal HCC. Although MO-HCC

responds well to regional therapy under the premise of adequate hepatic functional reserve, IM-HCC tends to recur early with a grim prognosis despite aggressive treatment interventions (13,14). Because treatment algorithm and prognosis vary between the two different types, exact assessment of the clonality of individual tumors is required. Herein, we briefly review the current strategies of discriminating between MO- and IM-HCC, and introduce their potential clinical implications.

CLINOPATHOLOGIC FEATURES OF IM/MO HCC

Conventionally, the diagnosis of MO was based on the histopathological criteria established by the Liver Cancer Study Group of Japan: (i) all nodules were well differentiated; (ii) recurrent nodules were moderately or well-differentiated in different segments from the primary poorly differentiated HCC; (iii) "nodule-in-nodule" detailed as moderately or poorly differentiated HCC embraced by well-differentiated margin; and (iv) nodules contain adenomatous hyperplasia or dysplastic nodules in the peripheral region (15,16). However, IM-HCC was diagnosed as nodules growing in contiguity with portal vein thrombi or satellite nodules surrounding a large main tumor. Based on pathological criteria alone, approximately 27.5% and 59.4% of patients in a cohort of 160 cases with multifocal HCC were identified as MO- and IM-HCC respectively (17). Poor histological grade at initial

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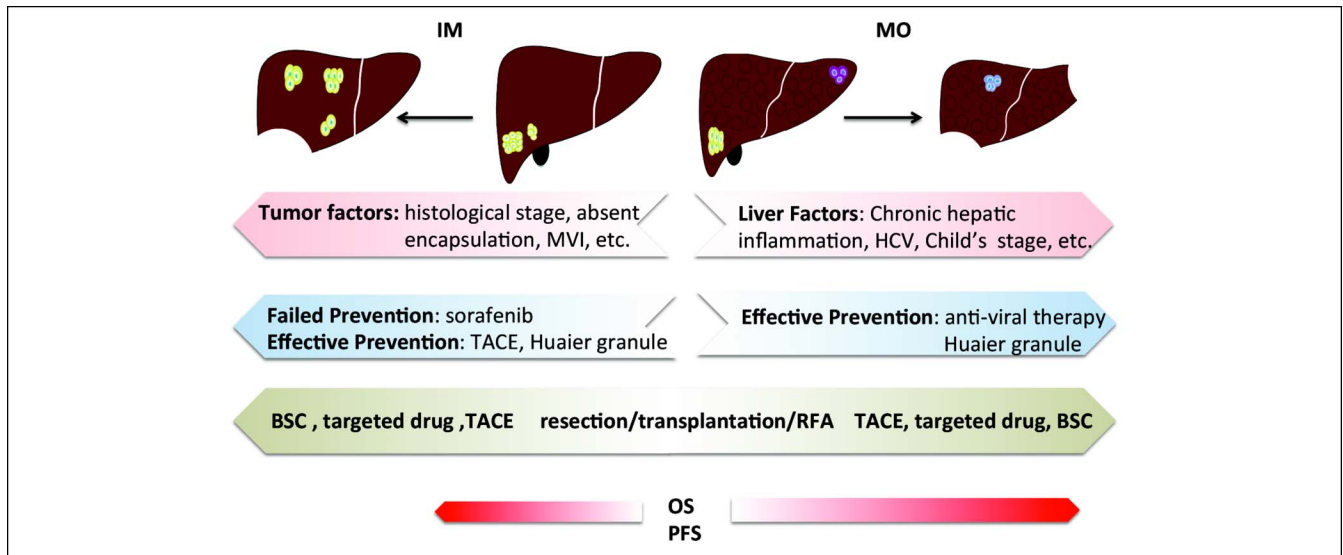
Figure 1. Intrahepatic metastasis/multicentric occurrence formation and clinical significances. BSC, best supportive care; IM, intrahepatic metastasis; HCV, hepatitis C virus; MO, multicentric occurrence; MVI, microvascular invasion; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization.

resection, absent tumor capsule at initial resection, and short recurrence-free survival (RFS) time were regarded as independent clinical factors to differentiate between IM and MO recurrences through pathologic identification (18). Other factors that might impact IM and MO differentiation included portal vein and/or microvascular tumor thrombus and Child's stage (17,19–21). Notably, the pathological criteria disregard the possibility of metastasis of well-differentiated HCC and rapid dedifferentiation of MO-HCC. Additionally, pathological criteria alone are inadequate to discriminate the clonality of all the lesions. In extreme cases, MO and IM may be simultaneously detected within the liver, making pathological differentiation even more difficult (22,23).

Noninvasive imaging examinations facilitate identification of IM-HCC when multifocal HCC exhibits typical distribution as satellite nodules surrounding a large main tumor or nodules growing along the portal vein thrombi. Otherwise, the discrimination between IM- and MO-HCC is confusing. Despite similar patterns in tumor locations and mean sizes of synchronous small and multiple recurrent HCCs between patients with IM and MO in a previous study, MO group showed different images in at least one imaging techniques including ultrasonography, computed tomography (CT) during arterial portography, and CT arteriography, whereas most patients with IM displayed similar images by the above 2 or 3 imaging techniques (24). The diagnosis of MO occurrence was more accurate when the patients had a hepatitis activity index score of noncancerous region ≥8. Another study identified approximately 23% of postoperative HCC nodules as MO recurrences according to the incompatibility of CT findings between primary and recurrent lesions (25). Artificial intelligence–based medical imaging adept at identifying differences among nodules may have the potential to make IM/MO discrimination in the future.

MOLECULAR STRATEGIES FOR IM/MO DISCRIMINATION

Every cancer develops as a clone from a single cell origin (26). During the expansion of the neoplastic cell population, individual HCC cells acquire genetic and phenotypic differences from each other and the more aggressive ones metastasize to form intrahepatic subclonal clusters. Multifocal HCCs sharing the same dominant genetic aberration during neoplastic evolution or the same molecular markers existing even before carcinogenesis are of the same clone. On the other hand, MO-HCC displays different truncating genetic aberrations or molecular changes happening before neoplastic evolution. To overcome the limitations of IM/MO discrimination based on pathological features, a variety of molecular strategies have been used to deduce the lineage of multiple lesions, including assessment of TP53 mutation status, HBV integration sites, microsatellite aberration mainly involving loss of heterozygosity (LOH), and copy number variations (CNVs) through comparative genomic hybridization as shown in Table 1 (27).

Gene mutation assessment

Approximately 30% of HCCs bear mutations in tumor suppressor gene TP53 (28). Nodules sharing the same TP53 mutation pattern are supposed to be from the same clone. Although some groups successfully identified multifocal HCC with the same TP53 point mutations as IM-HCC by directly sequencing exon 4 to exon 9, many others failed to make the differentiation by sequencing exons 5,7,8 or by combined technique of high-resolution melting and subsequent sequencing (22–31). The reason may lie in the fact that TP53 mutations with a relatively low prevalence in white populations usually occur late in hepatocarcinogenesis (32). In another study, comparisons of mutations in the TERT promoter region by Sanger sequencing and entire coding regions of 7 well-characterized HCC driver genes (ARID1A, ARID2, AXIN1, CTNNB1, TERT, and TP53) by targeted next-generation sequencing (NGS) enabled clonal discrimination in 58% of the patients with multifocal lesions (33). Besides nuclear DNA, mitochondrial DNA (mtDNA) mutations within D-loop region also served as a useful molecular tool for the determination of clonality in multifocal HCC (20,34). Owing to the lack of effective DNA repair system, damage to mtDNA is more frequent and serious than to nuclear DNA, resulting in increased sensitivity by direct sequencing of mtDNA D-loop to make discrimination.

Table 1. Discrimination of IM/MO hepatocellular carcinoma based on molecular strategies

Molecular strategies	Virus (%)	Synchronous/ metachronous	Total cases	Tumors	IM cases	MO cases	IM + MO cases	Undetermined cases	References
CGH, *TP 53* mutation, HBV integration pattern	HBV (100)	Synchronous	6	22	5	1	0	0	Cheung et al. (29)
CGH, HBV integration, LOH (17 markers)	HBV (90.9), NBNC (19.1)	Synchronous	11	25	7	3	1	0	Ng et al. (39)
LOH (4 marker), *TP 53* mutation, X chromosome inactivation	HCV (50), NBNC (50)	Synchronous	12	31	8	2	0	2	Hodges et al. (30)
Array CGH; QMSP; *TP53* mutation	HBV (26.7), HCV (66.7), NBNC (6.6)	Synchronous	6	12	2	1	0	3	Taniguchi et al. (31)
		Metachronous	9	18	4	3	0	2	
Methylation-specific PCR	HBV (15.8), HCV (68.4), NBNC (15.8)	Synchronous	13	27	9	3	1	0	Nomoto et al. (43)
		Metachronous	6	15	5	1	0	0	
LOH (15 markers)	—	Synchronous	9	19	2	2	0	5	Morimoto et al. (41)
		Metachronous	19	38	5	10	0	4	
CGH, HBV integration, X chromosome inactivation	HBV (61.3), HCV (19.4), NBNC (29.3)	Metachronous	31	62	12	12	0	7	Chen et al. (36)
LOH (15 markers)	HBV (97.5), NBNC (2.5)	Metachronous	40	100	28	10	2	0	Wang et al. (13)
mtDNA D-loop mutations	HBV (92.9), NBNC (7.1)	Synchronous	42	112	22	20	0	0	Li et al. (20)
LOH (10 markers)	—	Metachronous	160	—	102	48	4	6	Li et al. (17)

CGH, comparative genomic hybridization; HBV, hepatitis B virus; HCV, hepatitis C virus; IM, intrahepatic metastasis; LOH, loss of heterozygosity; MO, multicentric occurrence; mtDNA, mitochondrial DNA; NBNC, non-HBV and non-HCV; QMSP, quantitative methylation-specific PCR.

Chromosome analysis

Analysis of X chromosome inactivation in female subjects is based on random lyonization of 2 X chromosomes during embryogenesis long before tumorigenesis (35). Tumors from the same patients with alternate allelic X chromosomal inactivation are supposed to be of independent origin (30,36). However, tumors with the identical allelic inactivation patterns cannot fully prove to be monoclonal (37). For patients with HBV infection, HBV DNA integrates into the host genome at random sites also long before hepatocarcinogenesis, making the junctional information specific to each tumor clone (38). As such, tumors with the same HBV DNA integration site are supposed to be of metastatic nature (29,35,39). Notably, the initial integration site may be lost because of occurrence of genetic rearrangement during tumor progression, so at least one junction has to be maintained to make differentiation. Frequent occurrences of LOH in HCC have also been used to trace the tumor lineage (40). Although microsatellite markers used for LOH analysis differed among various studies, the majority resides in chromosomes 1, 4, 6, 8, 9, 13, 16, and 17 (13,17,30,39,41). Lesions demonstrating identical allelic loss patterns of microsatellite markers support a common origin, whereas tumors with more than 30% of different LOH alleles suggest a different clone. The threshold is set with regard to high intratumor subclonal heterogeneity (41). LOH assay shows advantage over other molecular methods in that it is applicable to samples from liver biopsy and formalin-fixed, paraffin-embedded material besides freshly frozen tumor tissues (42). When DNA fingerprinting comparative genomic hybridization was applied to compare CNVs among HCC nodules, the criteria to determine clonality differed among each report, either by calculating correlation values according to statistical models or by comparison of major vs rare chromosomal alterations (29,31,36,39). For examples, tumors with clonal relationship (CR) value >0.95 were considered to be of MO origin, whereas CR value <0.2 was deemed as IM nature (36). On the other hand, tumors that shared similar major chromosomal changes like +6q, +8q, +13q, +17q, −4q, −5q, −7q, −9q, −11q, and −16q and identical rare chromosomal alteration like +10p were supposed to develop through IM (39). An obvious benefit for LOH and GCH strategies is their extensive applicability beyond female patients and patients with HBV infection. Nevertheless, analysis of HBV integration pattern or X chromosome inactivation can help to determine the clonality when a low percentage of different LOH patterns or CR values was observed (39). Considering the limitations of each technique, a combination of different molecular strategies is always required to make an optimal clonal discrimination.

DNA methylation analysis

Beyond genetic alterations, methylation status of multiple tumor suppressor genes was also used to discriminate MO-HCC from IM-HCC. According to the study conducted by Nomoto et al. (43), subsequent tumors that gained promotor hypermethylation in a gene previously undetected in the primary tumor were regarded as metastatic tumors, whereas tumors with reduced hypermethylated genes were determined as *de novo* malignancies. However, which lesion to be chosen as a reference for methylation comparison is not well established, especially among synchronous multifocal HCC. Taniguchi et al. (31) performed quantitative methylation-specific polymerase chain reaction (PCR) but failed to make clonal differentiation by quantitative methylation-specific PCR value because methylation patterns of multiple

tumors in the same patients were highly comparable. Unlike genetic changes, epigenetic changes reflecting the environmental factors are not necessarily irreversible.

NEXT-GENERATION SEQUENCING FOR IM/MO DISCRIMINATION

The sensitivity in the molecular discrimination of IM and MO was limited because of a small portion of genetic alterations analyzed. The introduction of NGS including whole genome sequencing (WGS), whole exome sequencing (WES), and whole transcriptome sequencing (WTS) to clonal discrimination facilitates more integral identification of intertumoral heterogeneity in mutations, CNVs, and structural variations (SVs), as shown in Table 2 (22,23,44–50).

Gene mutation and phylogenetic analysis

According to the analysis of WGS or WES, different lesions with high level of ubiquitous nonsynonymous point mutations indicate metastatic nature, whereas those with nearly no common mutations suggest independent tumor origins. Various NGS-based studies have revealed that the actual shared mutation rates ranged from 8% to 97% in IM tumors. Furuta et al. (23) proposed that the cut-off value of 5% for shared mutation rate from WGS data was highly sensitive for IM discrimination. Furthermore, CR can be inferred from phylogenetic trees constructed according to mutation distributions (51). In phylogenetic trees, metastatic tumors reside in close proximity, whereas MO lesions are distant from each other (45,47). Similar somatic substitution patterns albeit distinct mutation landscapes were identified by principal

component analysis of 2 pairs of MO-HCC, indicating a major influence of etiological backgrounds on substitution patterns (44). Nevertheless, another study performed the same principal-component analysis but found no significant similarity in the substitution patterns among 4 of 8 MO pairs, probably suggesting that the substitution patterns were not strictly regulated by their liver backgrounds (23). It is difficult to determine whether multiple lesions without any shared mutations were actually MO when only a small number of mutations were identified because of low variant allele frequency.

Chromosome structural and copy number analysis

The presence of shared SV breakpoints further helps to make discrimination (23). Validation of shared SV breakpoints among multifocal tumors suggested the same origin, whereas totally different SV breakpoints indicated multiple occurrences. In contrast, shared CNVs have been observed among MO tumors (23,45). For example, the percentage of ubiquitous CNVs ranged from 22.2% to 100% in IM-HCC but it also reached as high as 46.7% in MO-HCC (45). Single nucleus genome sequencing showed that CNVs occurred relatively early in tumor evolution and remained highly stable during the mass expansion, whereas somatic mutations evolved gradually to generate clonal diversity, which might explain the discordance between shared mutation rate and shared CNV rate in MO-HCC (52,53). Nevertheless, it is still controversial whether different mutational clones with similar CNAs represent early divergent evolutions of the same clone or parallel phenotypic evolution of completely different clones.

Table 2. Discrimination of IM/MO hepatocellular carcinoma based on next-generation sequencing

Analysis platform	Samples	Virus	MO/IM diagnosis	Cases	Synchronous/ metachronous	Tumors	Shared mutations (%)	Shared CNVs (%)	Shared SVs (%)	References
WGS, WES	T, SN	HBV	IM	1	Metachronous	3	95.8	95.4	—	Tao et al. (49)
WES	T, DN, B	HBV	MO	1	Synchronous	2	0.8	—	—	Jiang et al. (46)
WES	T, B, NT	HBV	MO + IM	1	Synchronous	2	2.6	0	—	Shi et al. (22)
					Metachronous	2	57.1	86.7	—	
WGS	T, B	HCV	MO	2	Synchronous	4	0; 1.8	—	—	Fujimoto et al. (44)
WGS, WTS	T, SN, B, NT, PVTT	HBV	MO	1	Synchronous	2	1.0	0	0	Miao et al. (47)
			IM	1	Synchronous	4	65.4	100.0	80.0	
WES	T, NT	HCV	MO	1	Synchronous	2	0	—	—	Ikeda et al. (48)
			IM	2	Synchronous	4	24.3; 64.0	—	—	
WGS, WES	T, B, SN, NT, PVTT, BDTT	HBV	MO	1	Synchronous	2	0	46.7	—	Xue et al. (45)
			IM	9	Synchronous	26	8.0-97.0	22.2-100.0	—	
WGS, RNA-seq	T, B	HCV (75%) HBV (15%) NBNC (10%)	MO	6	Synchronous	13	0	0-9.3	0	Furuta et al. (23)
				6	Metachronous	12				
			IM	1	Synchronous	2	0[a]	0	5.1	
				6	Metachronous	13	40.0-80.9	5.3-100	1.4-63.3	
			MO + IM	1	Synchronous	3	0[a]	0	0	

B, blood; BDTT, bile duct tumor thrombus; CNV, copy number variation; DN, dysplastic nodule; HBV, hepatitis B virus; HCV, hepatitis C virus; IM, intrahepatic metastasis; MO, multicentric occurrence; NBNC, non-HBV and non-HCV; NT, adjacent noncancerous tissue; PVTT, portal vein tumor thrombus; RNA-seq, RNA sequencing; SN, satellite nodule; SV, structural variation; T, tumors; WES, whole exome sequencing; WGS, whole genome sequencing; WTS, whole transcriptome sequencing.
[a]The low sensitivity of shared mutations between IM pairs was due to low tumor content and low variant allele frequency.

Function analysis

To expand on the genomic studies, Miao et al. (47) performed WTS and found that the majority of functional changes were comparable among metastatic lesions but quite distinct among MO tumors. Upregulations of metastasis-related genes involved in cytoskeletal remodeling and extracellular matrix organization were exclusively observed in IM-HCC (47).

Besides the presentation of comprehensive genetic landscapes, another benefit for NGS is the ability to detect new biomarkers. In one patient with HCC with synchronous MO and metachronous IM, *FAT4* was detected by WES as the only gene shared in all the lesions (22). Its tumor suppressor function and clinical relevance were further validated *in vitro* and among patient population. Likewise, recurrently mutated ZNF717 in both monoclonal and polyclonal HCC has also been identified as a tumor suppressor in a recent single-cell genome sequencing study (54). *UBE3C* mutations were observed by WES as the only commonly mutated gene in an MO pair and a DN nodule from one HCC case with HBV background, and further experiments showed that UBE3C could promote HCC progression by regulating tumor epithelial–mesenchymal transition (46). *TTK*, as one of the most frequently expressed genes in MO pairs, proved to be negatively correlated with tumor grade (47). In another study, *LEPR* was found to harbor protein-altering mutations in both multifocal MO tumors and matched HCV-related cirrhotic liver tissues by WES and additional deep sequencing of selected exons (48). Importantly, *LEPR* dysfunction was proved to enhance susceptibility to hepatocarcinogenesis by a genetically engineered mouse model, indicating that a damaged liver was a heavy contributor to MO occurrence. Besides, a recent study verified *TERT*, *TP53*, and *CTNNB1* as trunk mutations in HCC, partly because of their ubiquitous distribution among intrahepatic metastases in most IM cases (55).

Analysis of WGS and WES is currently the best way to assess tumor clonality. However, NGS is available for only a small population because of relatively high expenses. Few studies are currently available analyzing a large number of patients with HCC. Furuta et al. (23) conducted NGS on 20 cases of multifocal HCC and found 6 cases with diagnostic discrepancies between the pathological and genetic criteria. All the 6 cases were indeed IM by WGS analysis but misdiagnosed as MO according to pathological features. When conflicting results exist, NGS analysis exerts more accuracy in the judgment of IM/MO HCC and should be given priority. Nevertheless, clinopathological criteria are still widely used because of their accessibility.

CLINICAL IMPLICATIONS FOR IM/MO DISCRIMINATION IN HCC

Like in HCC, synchronous multifocal lesion is also relatively common in a variety of other human cancers, including approximately 20.9% of non–small cell lung cancer (56), 28.7% of papillary thyroid cancer (57), and 80% of prostate cancer (58). Genomic analyses of some of these cancers also revealed a complex pattern of intertumor heterogeneity among the multifocal tumors as summarized in Table 3 (58–65). Of note, a high prevalence of multicentric origin has also been identified in multiple synchronous lung cancer (MSLC) and multiple papillary thyroid cancer (58–63). Unlike HCC, shared targetable dominant driver mutations, such as *BRAF* V600E mutation in multiple papillary thyroid cancer and *EGFR* L858R mutation in MSLC, are simultaneously detected in different clones within a case, facilitating effective targeted therapy despite wide SNV variations among MO lesions (59,60). Even in polyclonal MSLC, the co-existence of genomic heterogeneity and phenotypic convergence enables prediction of their biological consequence and selection of potential effective targeted therapy. However, such convergent evolution has not been identified in multicentric HCC (60).

Table 3. Discrimination of IM/MO in synchronous multifocal non-hepatocellular carcinoma tumors based on next-generation sequencing

Tumor type	Analysis platform	MO/IM diagnosis	Cases	Tumors	Shared mutations (%)	Shared CNVs (%)	Shared SVs (%)	References
MSLC (AD)	WGS, WES, CGH	MO	6	15	0	0	0	Liu et al. (59)
	WES	MO	4	11	0	—	—	Ma et al. (60)
MSLC (AD, SQCC)	Mate-paired sequencing	MO	7	14	—	—	0	Murphy et al. (61)
		IM	4	8	—	—	46%–89%	
MPTC	WES	MO	5	10	0%–7.1%	—	—	Lu et al. (62)
		IM	2	4	85.7%–100%	—	—	
		IM + MO	1	7	0%–62.5%	—	—	
	WGS, WES	MO	2	5	5.8%–11.1%	0	0	Xia et al. (63)
		IM	1	3	100%	100%	0	
Prostate cancer	WES	MO	2	4	0	—	—	Lindberg et al. (58)
UCC	WES	MO	3	6	0	0	—	Du et al. (64)
		IM	1	2	—	—	—	
		IM + MO	1	5	—	—	—	
MFBC	Targeted sequencing	MO	24	52	0	—	—	Desmedt et al. (65)
		IM	12	29	23.1%–77.8%	—	—	

AD, adenocarcinomas; CGH, comparative genomic hybridization; CNV, copy number variation; IM, intrahepatic metastasis; MFBC, multifocal breast cancer; MO, multicentric occurrence; MPTC, multifocal papillary thyroid carcinoma; MSLC, multiple synchronous lung cancer; RNA-seq, RNA sequencing; SQCC, squamous cell carcinomas; SV, structural variation; UCC, urothelial cell carcinoma; WES, whole exome sequencing; WGS, whole genome sequencing; WTS, whole transcriptome sequencing.

Moreover, the so-called "field cancerization" also hinder the radical treatment of multiclonal lesions in HCC (66). As shown by the genetic phylogeny studies, dysplastic nodules and even morphologically normal tissues with a high level of mutations highlight potential transformations to malignancies and independent clonal expansions (55,67). On the other hand, efforts to block the "seed and soil" dependence result in reduced MO recurrences. A typical example is antiviral treatment in HCC (70–72). Sustained anti-HBV or anti-HCV viral response after curative therapy of HCC successfully prolonged RFS (68–70). The benefits may result from alleviation of pro-oncogenic field by downregulation of hepatic inflammation and related signaling pathways associated with neoplastic transformation. Efforts to eliminate micrometastases to prevent IM recurrences after radical treatments have also been tried in HCC. However, the STORM study showed a negative result of adjuvant sorafenib for patients with HCC after locoregional therapy (71). No significant difference in the median RFS between the sorafenib group and the placebo group reflected that the antiangiogenic activity of sorafenib was insufficient to prevent relapse. In fact, the benefit of sorafenib may be offset by a more invasive phenotype elicited by an adaptive-evasive response (72–74). On the other hand, lack of IM/MO identification for recurrent HCC is a confounding factor for the negative result because MO recurrence should not be attributed to ineffective adjuvant therapy. Efforts to precisely select patients based on factors mostly associated with IM may allow adjuvant therapies more effective. As shown in a recent study, postoperative adjuvant transcatheter arterial chemoembolization (TACE) in HBV-related HCC with an intermediate or high risk of recurrences turned out to serve a favorable effect on RFS and overall survival (75). Lipiodol embolization of preoperative invisible micrometastases with rich microcirculation probably can explain the benefit. Another study recommended the traditional Chinese medicine Huaier granule as postoperative adjuvant therapy after curative resection of HCC in Barcelona Clinic Liver Cancer A or B stages because of significant RFS prolongation and reduced extrahepatic recurrence in Huaier group (76). Although the exact mechanism is unclear, improved innate immunity and activation of tumor cell apoptosis induced by Huaier granule may function to prevent both IM and MO recurrences (77).

For patients with synchronous multifocal HCCs, treatment algorithm has been made based on tumor number, size, distribution, vascular invasion, and hepatic functional reserve (9,78–83). Currently, none of the guidelines include IM/MO discrimination in the treatment algorithms of multifocal HCC. Because patients with MO have greater postoperative survival than patients with IM (13), future efforts to integrate IM/MO discrimination with postoperative surveillance and adjuvant therapies are needed to improve survival in patients with multifocal HCCs. Currently, the studies of multifocal HCC have been focused on resected or transplanted samples with relatively early tumor stages. For advanced multifocal HCCs developed either synchronously or metachronously, clonal evolution should be noted when systemic therapy is chosen. During the past decade, 5 molecular targeted drugs including first-line brivanib, sunitinib, erlotinib, and linifanib and second-line brivanib and everolimus have shown negative results in phase III clinical trials (84). Among other potential reasons, tumor heterogeneity including almost complete distinct genetic alterations among MO-HCC and subclonal mutational divergence among IM-HCC has been proposed to be a major obstacle for effective drug development in

HCC (85). One platform using patient-derived cell line–based model integrated with genetic and pharmacologic data from multiregional cancer samples has proved to render more precise selections of targeted therapy under the condition of intratumor heterogeneity (86). Identification of IM/MO HCC models in the design of clinical trials may help select more effective medicine and target population. For patients with IM-HCC, targeted drug proved to be effective based on the genetic profiling of one biopsy lesion tends to render similar effect to other IM lesions because of the same truncating genetic alterations. On the other hand, patients with MO may benefit from genetic analysis of multiple lesions and combined targeted treatment. Checkpoint inhibitor functioning through block of immune invasion rather than direct suppression of carcinogenetic signaling within the tumor has become a promising therapy for heterogeneous HCC and may turn out to be an optimal choice for both MO- and IM-HCC (87). For patients with multifocal HCC, personalized therapy based on individual genetic, molecular, and immune profiling remains to be achieved in the future.

CONCLUSIONS

For multifocal HCCs, identification of clonal origin and genetic diversity could greatly facilitate tumor staging, prognostic prediction, and treatment allocation. Histopathological assessments used to be the mainstay of making differentiation between IM- and MO-HCC. Various molecular and genetic approaches have been integrated to make more accurate differentiation during the past two decades. Recent NGS-based genomic comparisons not only offer a sensitive and precise way to determine the clonality but also enable detection of new biomarkers with biological, prognostic, and therapeutic significances. Despite potentially promising therapeutic effect of checkpoint inhibitors in multifocal HCC, future efforts to classify targetable factors most associated with IM and to integrate IM/MO discrimination with detailed treatment options are needed to improve survival in patients with multifocal HCCs.

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