In this issue of Clinical Cancer Research, Friemel and colleagues (1) assessed the morphologic and genetic heterogeneity of 23 hepatocellular carcinomas (HCCs). To this purpose, they performed geographic sampling of 120 different regions in each of these tumors. They collected different pathologic features and performed immunostaining to assess the activation of the WNT/β-catenin pathway (β-catenin, glutamine synthase), the presence of stem-cell markers (CK7, EPCAM, and CD44), as well as CD34 and AFP expression. They also performed sequencing of TP53 and CTNNB1 (coding for β-catenin), two of the most frequently mutated genes in HCC. Morphologic heterogeneity was observed in 87% of the cases, and heterogeneity at the immunohistochemical (IHC) and morphologic levels in 61% of the cases. Strikingly, genetic intratumor heterogeneity was noted in 22% of the cases and was systematically associated with heterogeneity at both the pathologic and IHC levels. This suggested that, at least in these cases, genotype and phenotype were strongly correlated. The authors also showed how different CTNNB1 mutations could be observed in different regions of the same tumor. As predicted, intratumor heterogeneity was more frequent in larger tumors, suggesting that heterogeneity increases as tumors progress. The authors postulate that genetic and phenotypic intratumor heterogeneity is frequent in HCC and questioned the use of single biopsy to identify prognostic and predictive biomarkers.

Phenotypic heterogeneity has been widely described by pathologists. However, our understanding of genetic intratumor heterogeneity has been limited until recently, due to our incomplete structural knowledge of the cancer genome and due to the lack of technology sensitive enough to easily detect tumor subclones. Luckily, the introduction of next-generation sequencing enabled scientists to draw the genetic landscape of the most frequent tumors. Moreover, it has allowed analytic refinements to identify low-frequency mutations and consequently to assess subclonality. As other cancers, HCC is a disease of the genome, where somatic mutations affecting driver genes lead to a selective proliferative advantage. Mutations can also affect stochastically passenger genes without clear functional consequences in a cell’s malignant phenotype. In addition, these genetic defects tend to accumulate in transformed hepatocytes. This phenomenon, also observed in almost all malignancies, is not linear what confers remarkable plasticity to tumor evolution (2, 3). In primary renal carcinoma, the pivotal study of Gerlinger and colleagues (4) has shown that a minority of mutations were ubiquitous and shared between the different regions of the primitive tumor and the different metastatic sites. In contrast, some genetic alterations were exclusively shared by all the metastasis, suggesting that within tumors are epigenetic changes (e.g., DNA methylation) and genetic intrinsic heterogeneity persists. This study confirmed similar observations described concomitantly in other solid tumors and hematologic malignancies (3).

A Darwinian theory has been proposed to describe the evolution of tumor clones that arose and diverge from the tumor cell considered as the most recent "common ancestor." The selective pressure due to microenvironment, carcinogenic exposure, and the acquisition of driver mutations led to the emergence of clones with growth advantage or metastatic ability (3). Selective pressure could also be exacerbated by treatment, and emergence of clones harboring new mutations has been implicated in secondary resistance to targeted therapies (5). Consequently, tumor plasticity would become a major issue when designing second-line/third-line therapies in advanced tumor stages. Besides genetic heterogeneity, other potential sources of phenotypic diversity within tumors are epigenetic changes (e.g., DNA methylation) and microenvironment (hypoxia gradients, local oxidative stress; ref. 2). Considering that most HCCs arise in the background of chronic liver disease with different levels of fibrosis and persistent inflammation, the impact of tumor microenvironment in intratumor heterogeneity surely deserves further exploration (Fig. 1). Genetic intratumor heterogeneity has been barely evaluated in HCC, and despite the relevant information provided by Friemel and colleagues, the study leaves some
unanswered questions. For example, the study only focused on CTNNB1 and TP53 mutations, but there are other somatic mutations that are identified using deep sequencing targeting ARID1A, ARID2, NFE2L2, KEAP1, RPS6KA3, MLL2, MLL3 or MLL4, for which there are no data regarding intratumor distribution (6). Moreover, we recently identified TERT promoter mutations as the most frequent somatic genetic defect in HCC, with an overall frequency of 60% (7). TERT promoter is also recurrently mutated in precancerous nodules. It is one of the earliest genetic alterations involved in malignant transformation in HCC and could be considered as a tumor "gatekeeper." We could speculate that TERT promoter mutations are present in the common ancestor cell, being transmitted to its progeny and hence present in most tumor cells. As noted by the authors, further studies are still needed to decipher HCC intratumor genetic heterogeneity using unbiased approaches such as whole-exome or whole-genome sequencing, preferable by ultra-deep sequencing (2, 3). Another issue raised by Friemel and colleagues is the reliability of molecular classification and genetic analysis using one single sample of resected tumors or using a single tumor biopsy. They suggest that tumor heterogeneity could challenge the use of molecular classification and development of biomarker-driven targeted therapies in the field of HCC. However, such assumption needs to be balanced considering the available data. Several molecular prognostic signatures derived from the tumor have been validated in large and independent studies despite tumor heterogeneity (8, 9). Also, all biomarker-based molecular therapies approved in solid malignancies are based on the traditional genetic analysis of the tumor, that did not account for genetic heterogeneity. Some examples are vemurafenib for BRAF V600E–mutated melanoma, cetuximab for wild-type RAS colorectal cancer, or crizotinib for ALK translocated non–small cell lung cancer.

The fact that independent tumor clones ("multicentric carcinogenesis") can arise on cirrhotic livers adds an additional layer of complexity (Fig. 1). New data are required to delineate intertumor molecular heterogeneity and accurately distinguish between intrahepatic metastasis and multicentric carcinogenesis (i.e., de novo tumor) on a cirrhotic background. Until we understand the nature, mechanisms, and consequences of this molecular diversity, it will be difficult to evaluate its impact in clinical practice. Accurate assessment of intra- and intertumor heterogeneity will also be pivotal to understand primary or secondary resistance to targeted therapies. Coupled with the analysis of the tumor genome, circulating tumor DNA will be an interesting tool to monitor tumor heterogeneity longitudinally and easily in clinical practice (10).
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Writing, review, and/or revision of the manuscript: J.-C. Nault, A. Villanueva

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References

# Intratumor Molecular and Phenotypic Diversity in Hepatocellular Carcinoma

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