Clonal Origins of Postoperative Recurrent Hepatocellular Carcinoma

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In the traditional view, recurrent hepatocellular carcinoma (RHCC) is a sign of tumor metastasis and late stage of development, and it has lost the chance of radical cure [1]. But with the deepening understanding of the theory of clonal origin in HCC, more and more evidence showed that there are two major patterns for clonal origin of RHCC, namely, monoclonal or monocenter origin and polyclonal or multicenter origin [2–4]. However, these two patterns are difficult to determine accurately based on clinical manifestations and histomorphological observations. Therefore, to carry out study of RHCC clonal origin model, looking for molecular markers of tumor clone detection, and establishing the corresponding molecular pathological examination method, is not only an important basic theoretical problem but also a practical guidance for the clinical understanding of clonal origin patterns of RHCC, scientifically formulating individualized strategy to prevent and treat RHCC and improving therapeutic effectiveness and long-term survival rate of RHCC [5].

4.1 Occurrence of Recurrent Hepatocellular Carcinoma

It has been estimated that the global annual number of new cases and deaths of primary hepatocellular carcinoma (primary hepatocellular carcinoma, PHCC) were both more than 600,000 in the world, of which more than 50% occurred in China [6]. As the number of cases of surgical resection of PHCC increases, the incidence of RHCC has risen accordingly. According to different authors' reports, the 5-year cumulative recurrence rates after excision of PHCC can reach 60–100%, with liver recurrent tumors accounted for 80–95% [7]. The number of RHCC surgeries in our department was 830 cases during a period of 26 years from 1985 to 2011 and has been increasing significantly in the last 5 years, including cases with multiple recurrences and surgeries, according to incomplete statistics (Fig. 4.1). Therefore, the study on the histogenesis and pathogenesis of RHCC is of practical significance to formulate clinical individualized therapeutic strategy for RHCC.

4.2 Clonal Origin of Recurrent Hepatocellular Carcinoma

4.2.1 Monoclonal and Polyclonal Origin

The origin of RHCC has long been a major concern and discussion subject since at least 20 years ago [2]. Two major origin patterns of RHCC are concerned. One is intrahepatic metastasis (IM) origin, derived from intrahepatic micrometastases which cannot be recognized with the naked eyes and excised entirely during surgery due to the microvascular invasion (MVI) [8–10], and the residual cancer cells will proliferate post-surgery of PHCC. Obviously, IM pattern has the same clonal origin with the primary tumor and is also termed as monoclonal or single center origin. The other is multicentric occurrence (MO) origin, derived from cancer-adjacent hepatocytes, or de novo tumor clone, which undergo long-term genomic variation leading to carcinogenesis, due to persistent HBV/HCV infection in the patients of chronic hepatitis or liver cirrhosis [11–13].

4.2.1.1 IM-RHCC

Monoclonal origin hypothesis of tumor was proposed in the 1970s that tumors are derived from accumulation of mutation and clonal proliferation of single cells in the tumor cell population. RHCC is traditionally considered as monoclonal in origin, arising from intrahepatic metastasis or recurrence of the residual cancer cells post PHCC surgery, which prompted the initiation of clinical course into the late stage of invasion and metastasis.

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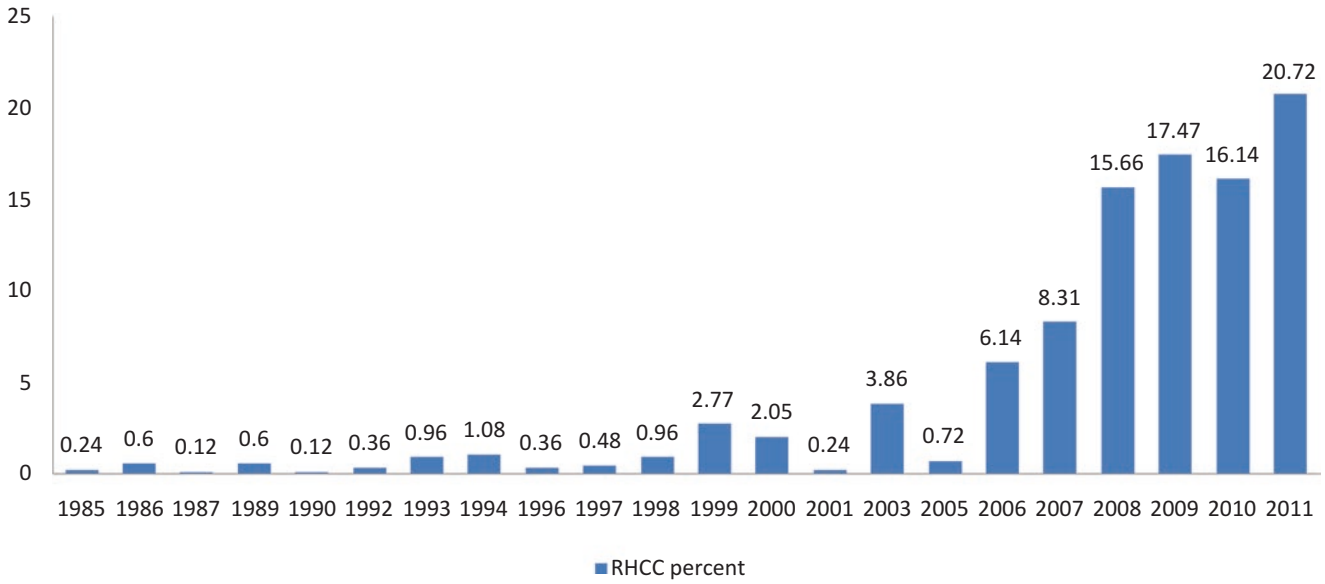

Fig. 4.1 Surgical resection of RHCC in Eastern Hepatobiliary Surgery Hospital

As we know, an adult liver contains about 500 thousand–1 million hepatic lobules, mainly composed of liver cell plates and hepatic sinusoid, and each lobule is surrounded by 3–4 portal areas, containing vasculature of interlobular artery, interlobular vein, and interlobular bile ducts, which facilitates the MVI and intrahepatic metastasis of HCC and is the histological basis for IM-RHCC. The reported incidence of MVI in PHCC is 15% to more than 60% [8–10], and the larger the tumor is, the higher risk of MVI is, which is the reason that more than 80% of the RHCC are mainly within the liver. According to our statistics of a group of HCC cases, the MVI rate in small HCC (≤3 cm in diameter) was 6.9%, among which even a microcarcinoma (0.6 cm in diameter) has also been observed with MVI [14]. Moreover, MVI has been recognized as one of the most important pathological indicators of both postoperative recurrence and metastasis risk and clinical prognosis after surgery [15].

4.2.1.2 MO-RHCC

The monoclonal theory of tumor proposed in 1976, that monoclonal proliferation is one of the main features of the tumor, laid an important theoretical foundation for the differentiation of tumor lesions and proliferative lesions, supported by most researchers considering multiple nodules, recurrent lesions, satellite lesions, and extrahepatic metastasis as monoclonal in origin. Until the late 1980s, with the development of molecular biological techniques which promoted the research of clonal origin of HCC, MO-RHCC was then discovered.

More attention has been paid to the mechanism of polyclonal origin of RHCC since the late 1990s. One of the reasons is the fact that more than 80% of PHCC patients in China have HBV-related chronic hepatitis or liver cirrhosis. HBV-DNA integrate into the genome of the host liver cell randomly, and precancerous lesions such as atypical hyperplasia and dysplastic nodules distribute throughout the liver with heterochronous carcinogenesis and multicenter origins. These contribute to the pathological basis of MO-RHCC. According to current RHCC molecular detection, the proportion of MO-RHCC is about 15–30%.

The interval between PHCC excision and RHCC occurrence is variable from less than 1 year to more than 10 years, making it difficult to preserve paired fresh tissue specimens for a long period to detect molecular clones. Supported by the National Natural Science Foundation of China, the author explored the microsatellite LOH pattern difference detection and determined the six molecular clone types or origin patterns of RHCC, via screening loss of heterozygosity (LOH) of high-frequency microsatellite DNA: type I, single nodular polyclonal RHCC via de novo tumor clone; type II, single nodular monoclonal RHCC via intrahepatic metastasis of PHCC; type III, single nodular monoclonal RHCC with its intrahepatic metastasis nodules; type IV, polyclonal and multinodular MO-RHCC; type V, single nodular polyclonal RHCC with its intrahepatic metastasis nodules; and type VI, combined polyclonal MO-RHCC and metastatic nodules from PHCC [16].

Differences of the above six types reflect the different mechanisms and pathways of RHCC providing a reference for clinical treatment of RHCC patients individually based on the clonal characteristics of HCC. As reported in the current literature, MO-RHCC and IM-RHCC constituted 15–30% and 70–85% of total RHCC, respectively, and the patients' average survival times post-surgery were

130 months and 80 months ($P < 0.05$), respectively, suggesting the better curative effect of reoperation for MO-RHCC [17].

4.2.2 Multifocal Growth and Multicentric Origin

Field cancerization theory was proposed by Slaughter in 1953 and remains to constitute the theoretical basis of pathogenesis of epithelial tumor. He hypothesized that one or multiple precancerous epithelial cells underwent sequential tumor genetic or epigenetic transformation to form primary field tumor (PFT) due to the impact of carcinogenic factors, whose persistent existence would facilitate the same genetic mutation and the formation of second field tumor (SFT) derived from the precancerous epithelial cells around the PFT [18, 19]. Theoretically, the molecular range of precancerous lesions is larger than the actual range of solid tumors. And dynamic multistage evolution and clonal selection of precancerous lesions in the region would lead to multifocal tumors with or without heterochrony. Different from tumors with multicentric origins, if PFT, SFT, and local recurrence (LR) of PFT share the same molecular variation or genetic alterations, they are determined as the same or monoclonal origin. However, if a tumor derives from another region, it's determined as a second primary tumor (SPT) and is a multicentric origin tumor. Thus, not all multifocal or recurrent tumors are multicentric origin tumors arising from de novo tumor clone, and it is apparently difficult to differentiate the clonal origins of PET, SFT, LR, and SPT clinically or histologically despite their different pathogenesis.

Slaughter's field cancerization hypothesis has already been confirmed in a variety of common epithelial tumors and is a practical reference for the investigation of clonal origin of RHCC. Studies have shown the significant difference among genetic methylation frequencies of HCC tissues, surgical margin, chronic hepatitis, and liver cirrhosis, suggesting the regional canceration in the liver, which reveals the variation and complexity of pathogenesis and origins of RHCC. Previous discussions of RHCC diagnosis, prevention, and treatment are more related to IM-RHCC, while we now should take the prevention and treatment of MO-RHCC into account, including the determination of molecules of PHCC, the prevention and repairing of genetic mutations and the progression of precancerous lesions in HBV/HCV infection areas, and early identification of precancerous cells with normal morphology and highly malignant tendency in the field. So, local canceration hypothesis is of practical significance to guide surgical resection, prevention, diagnosis, and treatment of PHCC. It can be deduced from the hypothesis that hepatic cells around HCC(T) have already had the genetic mutations to varying degrees and are in different stages of canceration in the patients with HBV/HCV infection, while their morphology remains basically normal. And these precancerous cells will continue the process of carcinogenesis to form LR or SPT after the so-called radical excision. Therefore, only when the PFT and all the cells with cancerous genetic mutations and biochemical alterations are excised will it be possible to completely prevent the recurrence of all forms of monoclonal tumor relapses or new tumor rerecurrences theoretically (Fig. 4.2).

Moreover, the clonal origin of liver tumors may involve malignant transformation and clonal selection within different tissues in terms of hepatic progenitor cells (HPCs). As mentioned above, determination of the clonal nature of tumors should be based on molecular pathology due to the complex and diverse clone types of RHCC. Microsatellite loss of heterozygosity (LOH) was applied for clone identification to diagnose the first case of multi-origin primary malignant tumors of the liver, which received the first HCC resection and later resection of intrahepatic cholangiocarcinoma and fibrosarcoma [20]. To improve molecular pathodiagnosis in the deparment of pathology, great importance should be attached to set up tumor molecular cloning detection methods [21].

4.3 Clinical Features of Recurrent Hepatocellular Carcinoma

4.3.1 Pathological Diagnostic Criteria

Shimada et al. described the characteristics of multiple nodular intrahepatic metastasis (IM) of HCC grossly and microscopically including ① obvious derivation from portal vein tumor thrombus, ② multiple satellite nodules around the primary tumor, and ③ histological similarities between solitary tumors adjacent to the primary tumor [22]. As to RHCC, IM can be diagnosed if the recurrent tumor has a moderate to low degree of differentiation and a similar or lower grade to the original tumor. Besides, the Liver Cancer Study Group of Japan put forward the histological diagnostic criteria of MO including [23] ① moderately to poorly differentiated primary tumor and highly differentiated recurrent tumor, ② highly differentiated PHCC and RHCC, ③ RHCC with precancerous lesions or highly differentiated HCC surrounding poorly differentiated HCC or nodule-in-nodule appearance, and ④ RHCC with higher differentiation than that of PHCC. And the diagnostic criterion for IM is RHCC with poorer differentiation than that of PHCC. However, these histological criteria can't be applied to diagnose most RHCC, due to the extremely small proportion of highly differentiated HCC and high-grade dysplastic nodules (HGDN) in clinical practice. Moreover, the determination of differentiation degree and precancerous lesions is not completely object, easily affected

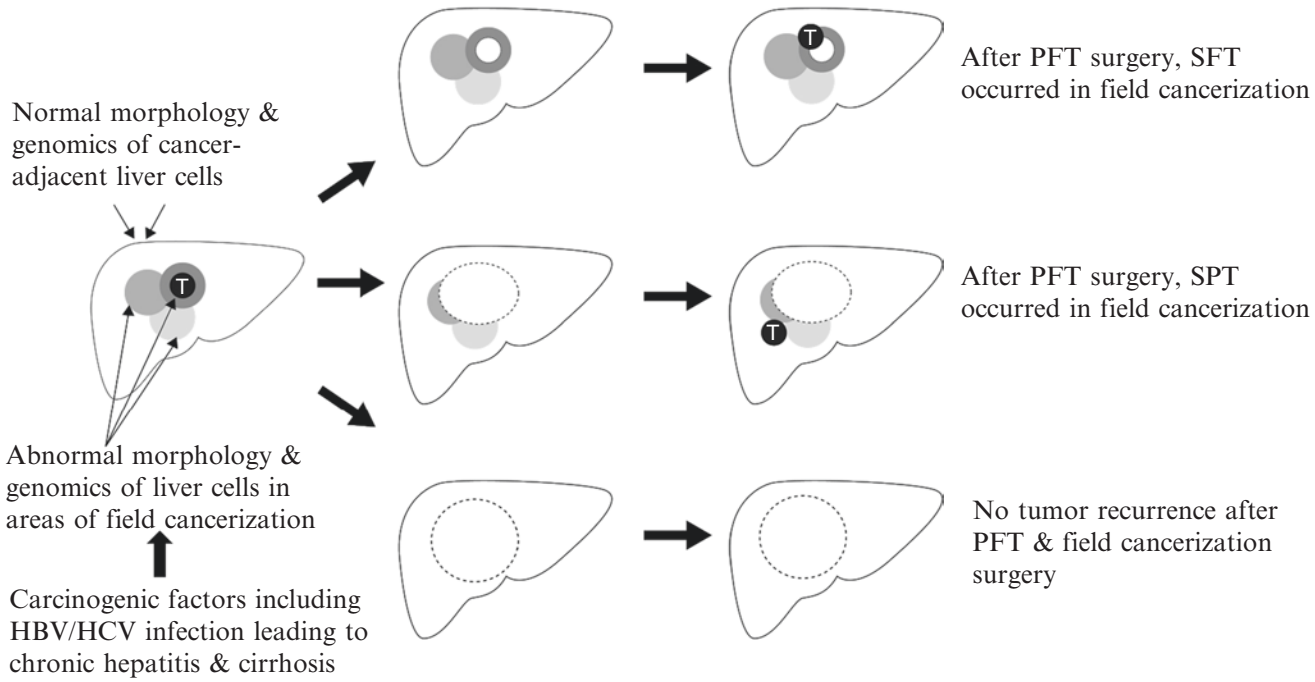

Fig. 4.2 Schematic diagram of the relationship between HCC and regional carcinogenesis and treatment mode

by subjective factors such as the working experience of the pathologists. Thus it is inaccurate to determine the clonal origin of RHCC simply based on the morphohistology of the tumors.

4.3.2 Clinical Diagnostic Criteria

Differentiation between recurrence of a residual lesion and a de novo tumor post-surgery is a key to develop therapeutic strategy and predict clinical prognosis scientifically. However, due to the inaccuracy of determining the clonal origin of a RHCC according to clinical manifestations, doctor's experience plays a major role. Currently, single center recurrence (IM-RHCC) refers to recurrence within 2 years post-surgery (short-term recurrence), and multicentric recurrence (MO-RHCC) refers to recurrence more than 2 years after tumor excision (long-term recurrence). Li et al. [24] detected the pattern of P53 mutation via PCR-SSCP and divided 12 cases into two groups, single center recurrence (6.5 ± 3.25 months) and multicenter recurrence (33.8 ± 17.8 months). They concluded that recurrence within 2 years post-surgery derived from single center or multicenters, while recurrence more than 2 years after surgery mainly derived from multicenters and thus was secondly primary carcinoma. However, our previous researches on molecular clone detection showed there was overlap of recurrence interval between IM-RHCC and MO-RHCC. For instance, in a molecular clone detection, monoclonal origin or recurrence of residual lesions was found in a RHCC 8 years post-surgery [4]. The author also reported a patient who occurred hepatic and colon metastasis from breast cancer 13 years after the surgery of breast cancer, and the patient received a second surgery for the metastatic tumors [25], indicating that residual cancer cells could stay silent or in a tumor-dormant state in vivo for a long time and proliferate again due to certain microenvironment changes even to metastasize.

The difference of serum AFP levels between PHCC and paired RHCC patients was also found to be associated with the difference of clonal origins. The recurrence intervals in group A (significantly different serum AFP levels between PHCC and RHCC patients) and group B (similar serum AFP levels between PHCC and RHCC patients) were 34.1 ± 3.8 months and 24.6 ± 2.7 months, respectively ($P < 0.05$), and recurrence intervals in group A type II and group B type II (recurrent tumors of different liver lobes) were 39.4 ± 5.9 months and 21.3 ± 4.1 months ($P < 0.05$), suggesting RHCC in group A has the features of MO-RHCC, such as the relatively longer growth periods of neoplasms in multistage growth pattern, and the difference of serum AFP levels may also reflect differences in tumor cell clone characteristics. Huang et al. [26] divided 82 RHCC into IM type and MO type post-surgery according to histological criteria; the recurrence intervals were 10.78 ± 7.9 months and 47 ± 31.69 months ($P < 0.001$), suggesting a possible relationship between recurrence intervals and tumor clonal origins. In principle, IM-RHCC-derived residual tumor post the

first HCC excision is usually complicated with MVI or satellite foci formation, and this should primarily consider multimodality therapy including interventional therapy (such as radiofrequency ablation, hepatic artery embolization chemotherapy, and biological therapy), while MO-RHCC is a de novo tumor in nature and is more suitable to be excised or treated by liver transplantation, the same effect of which can be acquired with the first surgery of PHCC. On this basis, the author proposed the relevance between clonal origin types and individualized treatment modes of RHCC (Fig. 4.3).

Yasui et al. [11] demonstrated a comparative study between 18 cases of MO-HCC and 64 cases of multinodular IM-HCC post surgery, and the results showed that 3-year survival rates and 3-year disease-free survival rates in MO-HCC group were 39% and 70%, respectively, which were significantly higher than those in IM group. However, total post-surgery survival rates in the two groups showed no significant difference despite patients of MO group were in the later AJCC stages at the time of surgery. Arii et al. [27] reported the survival rates of reoperation at 1 year and 3 years in HCC patients with intrahepatic recurrence were 100% and 80% in MO group and 91.7% and 38.1% in IM group, respectively, suggesting a better prognosis of surgical treatment in patients with MO-RHCC. Poon et al. [12] reported 246 cases of HCC post radical resection including 80 cases of early recurrence (≤1 year) and 46 cases of late recurrence (>1 year), among which 9 cases were diagnosed as IM receiving re-excision in early recurrence group and 6 cases were diagnosed as MO receiving re-excision in late recurrence group. The median survival time in the late recurrence group was significantly longer than that in early recurrence group (29.6 months vs. 15.8 months, $P = 0.005$). Huang et al. [26] divided the 82 postoperative cases of RHCC into IM group (54.9%) and MO group (45.1%) histologically, and the results showed significantly lower postoperative recurrence rate, higher postoperative overall survival, and recurrence-free survival rate in MO-RHCC group compared with IM-RHCC ($P < 0.001$). Matsuda et al. [13] reported 29 cases of RHCC with 31 excisions, divided into MO group (18 cases), IM group (4 cases), and undefined group (4 cases) histologically, and the 1-, 3-, and 5-year survival rates of patients were 100%, 69.7%, and 58.1% in MO group and 57.1%, 14.3%, and 14.3% in IM group ($P = 0.0016$).

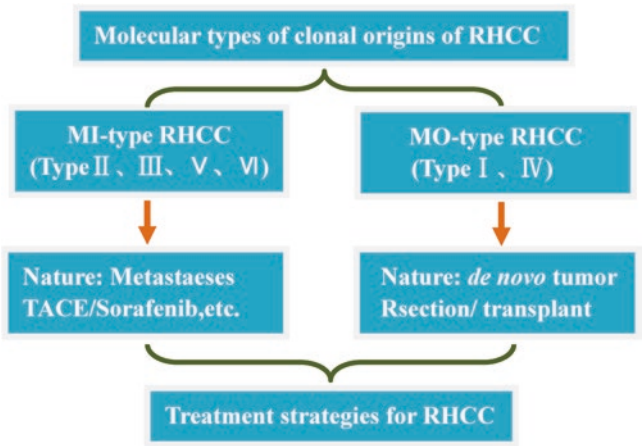

Fig. 4.3 Schematic diagram of clone classifications and individual diagnosis and treatment pattern of RHCC

As to liver transplantation for HCC patients, the core indicators in widely adopted criteria including Milan criteria, Pittsburgh criteria, and UCSF criteria are the diameter of the tumor, tumor thrombus in main vessels, and number of tumor nodules. It has begun to attract attention on the impact of clonal origin of multinodular HCC on long-term survival in liver transplantation patients. Finkelstein et al. [29] reported better postoperative survival in liver transplantation patients of multinodular MO-HCC compared with that of IM-HCC group, indicating the objective reference of molecular diagnosis in TNM staging, liver transplantation recipient screening, and prognosis assessment. Gehrau et al. [29] present a HCC diagnosis flow chart, and they proposed that patients with multiple HCC (>2 nodules) combined with molecular detection as MO type can be delivered in the assessment for liver transplantation, and for patients of IM type, TACE and targeted drug therapy with sorafenib would be the better choice.

4.4 Molecular Pathodiagnosis of HCC Clonal Origin

The existence of MO-RHCC and IM-RHCC has been confirmed by using molecular biological techniques, including DNA ploidy analysis, p53 gene mutation analysis, HBV-DNA integration analysis, X-chromosome inactivation pattern (XCIP) detection, comparative genomic hybridization (CGH) analysis, DNA methylation profile analysis, microsatellite LOH, chromosomal LOH, microRNA (miRNA) profile analysis, etc. [30–32]. Cheung et al. [33] studied 22 HCC nodules in six patients using cDNA microarrays containing 23,000 genes and found clonal relevance among HCC nodules via integrated analysis of 90 metastasis-related genes' differential expression, p53 gene mutation pattern and protein expression, HBV integration pattern, and comparative genomic hybridization of genes, but which is limited in clinical application because of its complicated process, heavy workload and high cost, and so forth. Microsatellite DNA is a good marker for overall stability of cellular genome and can facilitate PCR analysis with denser loci and more accurate location. Hence, analysis of chromosome with multiple high-frequency LOHs and a panel of microsatellite loci is beneficial to improve the accuracy in diagnosing the clonal origin of RHCC. Ng et al. [34]

compared microsatellite LOH, p53 mutation type, and HBV-DNA integration mode in terms of their properties in differentiating the clonal origin of multinodular HCC and concluded that microsatellite LOH is most widely used, suitable for samples with small DNA content, such as samples obtained via fine needle aspiration biopsy or liver biopsy and formalin-fixed paraffin-embedded (FFPE) tissue.

However, different detection methods and diagnostic criteria will cause significant difference in the proportions of molecular clonal patterns of RHCC. Ng et al. [34] reported 11 cases of nodular HCC with 25 nodules, among which MO-HCC and IM-HCC accounted for 36% and 64%, respectively. Morimoto et al. [35] reported 19 cases of RHCC with 52.6% MO-RHCC, 26.3% IM-RHCC, and 21.1% undefined cases. In another report conducted by Huang et al. [26], 54.9% IM-RHCC and 45.1% MO-RHCC were defined in 82 postoperative cases according to histological criteria.

It is worth noting that whole-genome microarray has been used to predict the prognosis and risks of intrahepatic recurrence and metastasis via screening of differentially expressed gene profile in RHCC after liver transplantation [28, 29]. And it can also be used to determine the clonal pattern, as shown in the report of miRNA expression profiles of ten cases of RHCC that expressions of miR-602, miR-451, miR-144, and miR-486-5p were significantly upregulated (>2.0 times) and expressions of miR-55 lb., mir-96, and miR-502-3p were significantly downregulated (<0.5 times) in early RHCC within 1 year after surgery, probably related to early recurrence of RHCC [36].

Tao et al. [37] analyzed the genomic variations of tumor tissues in six different regions (T1–T6) of the primary tumor (R0) excised in a RHCC and two recurrent tumors (R1, R2) using the methods of next-generation sequencing (NGS) of exon capture and whole-genome sequencing and detected 214 point mutations, including 205 point mutations (95%) detected in all three tumors, 24 mutations associated with amino acid changes, and 22 major domain insertions and deletions/copy number variations (>1 MB). They demonstrated the R0 tumor cell populations with these somatic mutations consist of four lineages (π0–π3) and are highly clonal, among which π0 cells contain all background mutations but without obvious proliferation. In addition, three protein-encoding mutations (CCNG1, P62, and an insertion and deletion/fusion gene, such as APC) were found to be the promoter mutation, and each lineage may hold only one lineage-specific protein-encoding mutation that initiates the proliferation and metastasis of R2 and R3 tumor cell populations. Furthermore, Alsinet et al. [38] suggested that a small amount of highly invasive and proliferative cells with the new mutation in the primary tumor cell population could form a new tumor nodule and continue to form new tumor nodules through similar clonal selection and proliferation. These results and analysis enrich our understanding in clonal heterogeneity of HCC, clonal selection mechanism during HCC development, and diversity and complexity of RHCC. Therefore, it is necessary to apply new technologies and new theories to the systemic investigation of the pathogenesis and diagnosis and treatment strategies of RHCC.

4.5 Prospection

The application of molecular cloning technique provides guidance for individualized clinical diagnosis and treatment of multiple and recurrent tumors. For instance, traditional classification according to the diameter and recurrent interval divides recurrent head and neck squamous cell carcinomas into LR and SPT, while they are classified into three types, LR, SFT, and SPT, using molecular biological detection methods. As to therapeutic strategy, radiotherapy or re-excision applies to postoperative minimal residual cancer which is highly risky to develop LR, regular diagnostic biopsy or chemotherapy applies to precancerous cells intra- or peri-surgical region which indicate high risk of developing SFT, and routine follow-ups apply to those without precancerous cells and the risk of developing SFT is low [37]. It is worthy of reference to integrate molecular diagnosis and therapeutic strategy in the study of RHCC.

At present, only a few researches on molecular cloning diagnosis of HCC have been reported abroad which focus more on the clonal analysis of multinodular HCC [14], and the results are similar with ours that interventional therapy is a preference for multinodular IM-HCC, while reoperation is suitable for multinodular MO-HCC. However, there are at least six subclonal patterns in multinodular HCC and therefore should be treated with comprehensible strategy according to the clinical pathological features of individuals.

In summary, with the rapid development of molecular tumor surgery, investigation on integrated treatment mode based on molecular clonal diagnosis and clinical individualized treatment of RHCC and multinodular HCC is promising in surgical diagnosis and treatment strategy of HCC. Further researches on molecular hepatopathology should be focused on the establishment of accurate identification and detection methods for tumor clonal origin and molecular boundary, predicting the risk of tumor recurrence, formulating the classifications and treatment pathways of RHCC according to molecular clonal evidence [17].

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