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Characterization of genomic clones using circulating tumor DNA in patients with hepatocarcinoma

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Background: Hepatocarcinoma (HCC) is often diagnosed at an advanced stage with poor prognosis. A non-invasive method concerning circulating tumor DNA (ctDNA) has been recognized as a promising biomarker. ctDNA has been widely studied to monitor tumor dynamics and measure tumor burden. However, the results of previous studies for biomarkers for HCC have generally been inconsistent and limited in clinical application.

Methods: HCC usually represent a mixture of different cancer cell clones differing in mutation content, which can be used to monitor important features such as treatment responses. In this study, we used Bayesian cluster with PyClone to identify clonal population structures based on variations from ctDNA of a 4 HCC patients dataset.

Results: As a result, reductions in cellular prevalences of private mutation clusters were observed between preoperative and postoperative plasma samples, which reflected the treatment responses of surgery. We also identified expansion and sharing sub-clonality of initially minor clones in plasma samples after treatment, and located clusters in patients which might be used as actionable targets.

Conclusions: These results provided a more complete picture of the liver cancer pathogenesis. The comparison of preoperative and postoperative plasma samples showed that ctDNA can be used to real-time sampling of clonal evolution in patients with hepatocarcinoma. In addition to dynamic monitoring of disease progression and response to therapy, characterizing of dynamics of genomic clones can be used to determine the benefit of new therapeutics and guide therapy.

Keywords: Hepatocarcinoma (HCC); circulating tumor DNA (ctDNA); PyClone; clustering

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Introduction

Cell-free tumor DNA (ctDNA), harboring tumor-specific mutations, is released by apoptotic and necrotic tumor cells and found in the cell-free fraction of blood (1). Plasma

derived ctDNA has been recognized as a potential non-invasive surrogate for tumor tissue biopsies, also known as “liquid biopsy” (2). Massively parallel sequencing (MPS) studies of ctDNA have revealed that ctDNA is a potential marker related to various forms of human cancers (3-5). It

can be used as a potential marker to predict disease risk, patient outcomes or response to treatment.

As is known, accurate and timely detection of treatment responses is pivotal because patients' survival rates rapidly decline with late detection and delayed salvage surgery (6,7). The monitoring of treatment response is essential to avoid continuing ineffective therapies, to prevent unnecessary side effects, and to determine the benefit of new therapeutics. Treatment response is generally assessed with the use of serial imaging, but radiographic measurements often fail to detect changes in tumor burden. For example, patients with stage II or III NSCLC (non-small-cell lung cancer) undergoing definitive radiotherapy often have surveillance CT or PET-CT scans that are difficult to interpret owing to radiation-induced inflammatory and fibrotic changes in the lung and surrounding tissues. Plasma protein biomarkers (AFP, CEA, PSA, and CA15-3) are also commonly used in clinical management to reflect treatment responses of patients (8,9). However, the application of plasma protein biomarkers is limited because of low sensitivity and poor clinical correlations. The positive proportion of patients for these biomarkers is usually between 50–70% in cancer patients. Furthermore, it has also been reported that these biomarkers can also be found in lower concentrations in the serum of individuals without cancer (10,11). MPS studies of multiregional and repeated metastatic tumour biopsies analysis have been applied to guide choice and sequence of therapy (12,13). However, this is challenging due to tumor heterogeneity, which limited efficacy and duration of response to treatment. In this case the analysis of ctDNA could provide a whole picture of somatic mutations in a patient, avoiding the need to perform repeated invasive biopsy procedures. Using ctDNA to evaluate tumor heterogeneity as a prognostic factor and monitor therapeutic response in patients could guide choice and therapy. Liver cancer is one of the most common cancers and cause of cancer related death in China (14). However, studies comparing before and after treatment plasma samples of HCC patients to establish the extent of clonal heterogeneity captured in ctDNA is extremely limited.

In this study, we performed a Bayesian clustering analysis of preoperative and postoperative plasma samples collected from four HCC patients. We detected changes of genomic clones between preoperative and postoperative plasma samples. We identified reductions in cellular prevalences of private mutation clusters between preoperative and postoperative plasma samples, which correlated with disease status after surgery. In addition, we identified expansion

and sharing sub-clonality of initially minor clones in plasma samples, and located mutation clusters which might be used to guide therapy. These results, from four patients with HCC, suggested that ctDNA could be used to reflect clonal population structures and captures sub-clonal dynamics in real time.

Methods

Data extraction

The study consisted of 4 patients with HCC from the Department of Surgery of the Prince of Wales Hospital, Hong Kong. These patients were initially recruited for the study in reference 1 (15). The maximal dimension of the tumor is 13.0 mm for HCC1, 6.2 mm for HCC2, 4.2 mm for HCC3 and 6.2 mm for HCC4. In this study, we focused on the data from the plasma ctDNA samples of the four patients, which were kindly provide by Y.M.D. Lo. The study and the protocols used were approved by the Institutional Ethics Committee of BGI (NO. BGI-IRB 15136). Written informed consent was obtained from all of the participants when enrolled in the study.

Data processing

Detailed information of the materials and methods was described in reference 1 (15). In short, after genomic DNA extraction of the 4 HCC patients, the DNA library was diluted and hybridized to the paired-end sequencing flow cells. DNA clusters were generated on a cBot cluster generation system (Illumina) with the TruSeq PE Cluster Generation Kit v2 (Illumina), followed by 51×2 or 76×2 cycles of sequencing on a HiSeq 2000 system (Illumina) with the TruSeq SBS Kit v2 (Illumina).

The analysis pipeline included data filtration, alignment, somatic variants detection, annotation and clustering analysis for both preoperative and postoperative plasma ctDNA samples (*Figure 1*). We sorted the bam file, and used bedtools (bedtools v2.25.0) to change the bam file to fq file (filtered some reads with the same read ID) for further analysis. The pipeline started from the clean reads generated in the above mentioned step: first, the clean reads with a length of 50 bps were mapped to the human reference genome (hg19) from the UCSC database using BWA (Burrows Wheeler Aligner). Second, after removing PCR derived duplications using Picard and realigning by GATK (<http://www.broadinstitute.org/>

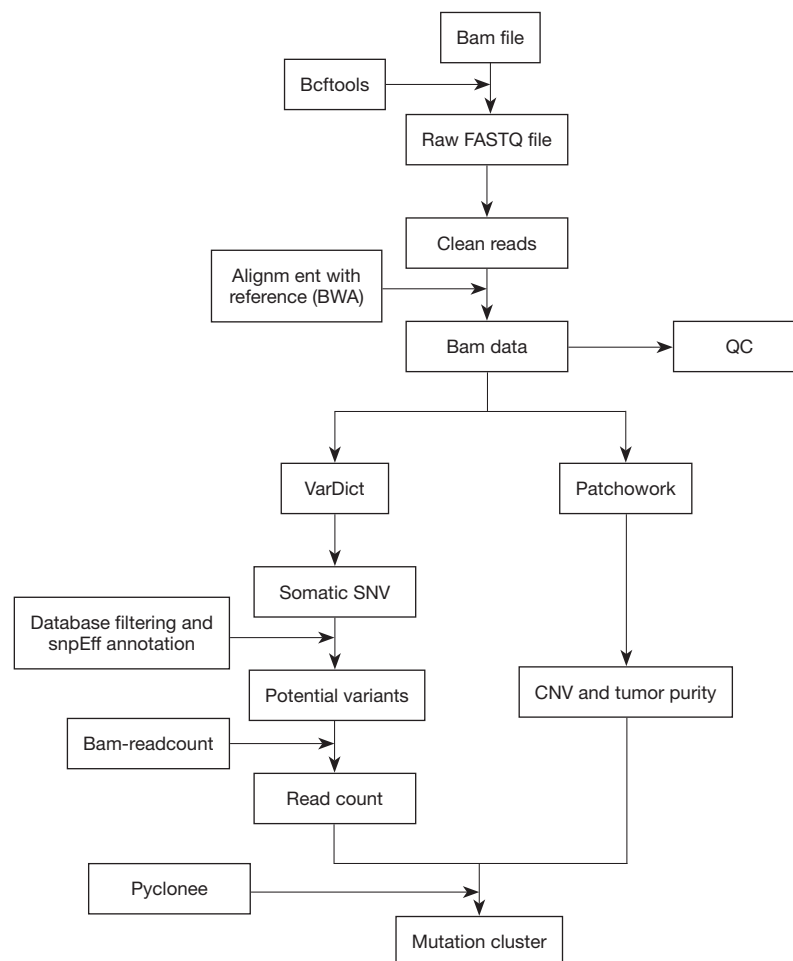


Figure 1 Flow diagram of bioinformatics analysis.

gatk/gatkdocs/org_broadinstitute_sting_gatk_walkers_indels_RealignerTargetCreator.html), the bam results were then used to do variants detection. We also included local realignment around indels. Somatic single nucleotide variants (SNVs) were identified by applying VarDict (16) with a P value threshold of 0.01. We only focused on the variants in the exome region in this study. The identified somatic variants were directly annotated by Catalogue of Somatic Mutations in Cancer (COSMIC) database (version 76), dbSNP (human_9606_b150_GRCh37p13), and dbNSFP (version 3.2 Academic, <http://varianttools.sourceforge.net/Annotation/DbNSFP>) using snpEff (snpEff_v4_3p_core) (17) and our own PERL scripts. After annotation, we used bam-readcount (version 0.8.0) to generate metrics at single nucleotide positions of the candidate SNVs, which were used to filter out false positive calls. Detailed filtering parameters followed the best

practice guidelines. The ploidy, tumor content, and absolute copy number were estimated using Patchwork with default parameters (18).

Clustering using PyClone

After candidate non-synonymous SNVs calling and filtering, clonal population structures were identified based on variations from plasma ctDNA using Bayesian cluster with PyClone (version 0.13.0) (19). PyClone_binomial density was used to model read counts. In order to ensure convergence, MCMC sampling with iterations of 1,000,000 was used to approximate the posterior distribution of the model. We discarded clusters with fewer than two mutations assigned to them. This is mainly to exclude clusters with a single mutation, where PyClone gains no power to estimate cellular prevalence.

Table 1 Tumor-associated somatic variants in plasma ctDNA samples

Sample	Time point	No. of SNVs from plasma	No. of SNVs seen in tumor tissue (%)
HCC1	Before treatment	248	19 (7.66)
	After treatment	207	17 (8.21)
HCC2	Before treatment	298	46 (15.44)
	After treatment	304	42 (13.82)
HCC3	Before treatment	410	42 (10.24)
	After treatment	360	27 (7.50)
HCC4	Before treatment	161	13 (8.07)
	After treatment	177	16 (9.04)

HCC, hepatocarcinoma.

Statistical analysis

To assess statistical significance, we analyzed the number of tumor associated SNVs in plasma samples after treatment. In addition, the changes of cellular prevalences in preoperative and postoperative plasma samples were compared, and P values were calculated accordingly.

Results

Identification of somatic variants

We used the above mentioned pipeline to analyze the preoperative and postoperative plasma samples obtained from all 4 HCC patients. We only retained reads with an average mapping quality score of more than 30 (Phred). On average, the sequencing depth after removing of duplicate reads is 16.75-fold of the human genome in the plasma samples for the four HCC patients.

To reduce false positives, we used the following criteria in potential somatic SNV detection: (I) a mutation was kept only when the sequencing depth is more than 10-fold; (II) a mutation was kept when the variant frequency is more than 5%; (III) a mutation was kept only when it is present at less than 1% mutant allelic frequency in the paired normal sample (PBLs); (IV) only bases with Phred quality scores of more than 30 (less than 0.1% probability of a sequencing error) were kept; (V) the mutations were non-synonymous variants; (VI) we carried out analysis only focusing on the mutations which have been previously reported two or more times in COSMIC territory (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>). This threshold was applied in order to lower the false-positive detection rate, and to

only focus on tumor associated mutations. Finally, a total of 2,165 candidate non-synonymous SNVs for the 4 patients (*Table 1*) were selected for analysis of the number of tumor tissue associated SNVs.

The number of tumor-associated SNVs ranged from 161 to 410 in the plasma samples of the 4 HCC patients (*Table 1*). In plasma samples, 7.66–15.44% of the tumor associated SNVs were detected in tumor tissue before treatment. After treatment, 7.50–13.82% of the tumor associated SNVs in the plasma samples were detected in tumor tissue. We did not detect significant reducing of the number of tumor associated SNVs in plasma samples after treatment (Paired samples *t*-tests, P value =0.3736). This suggested that total number of mutations cannot reflect the treatment response and monitor tumor dynamics in the 4 HCC patients. There might be new mutations detected in patients after treatment.

Mutation clusters in preoperative and postoperative plasma samples

We used PyClone (19), a Bayesian clustering method to group sets of somatic mutations into putative clonal clusters. We carried out PyClone clustering analysis focusing on all the mutations (both reported/not reported in COSMIC). All the mutations were listed in the Table (online: <http://tcr.amegroups.com/public/system/tcr/supp-tcr.2018.03.17.pdf>). An average of five major clusters were identified in the plasma samples of the 4 HCC patients (*Figure 2*). In the preoperative and postoperative plasma samples of each patient, we identified mutation clusters detected in all plasma samples, and private mutation

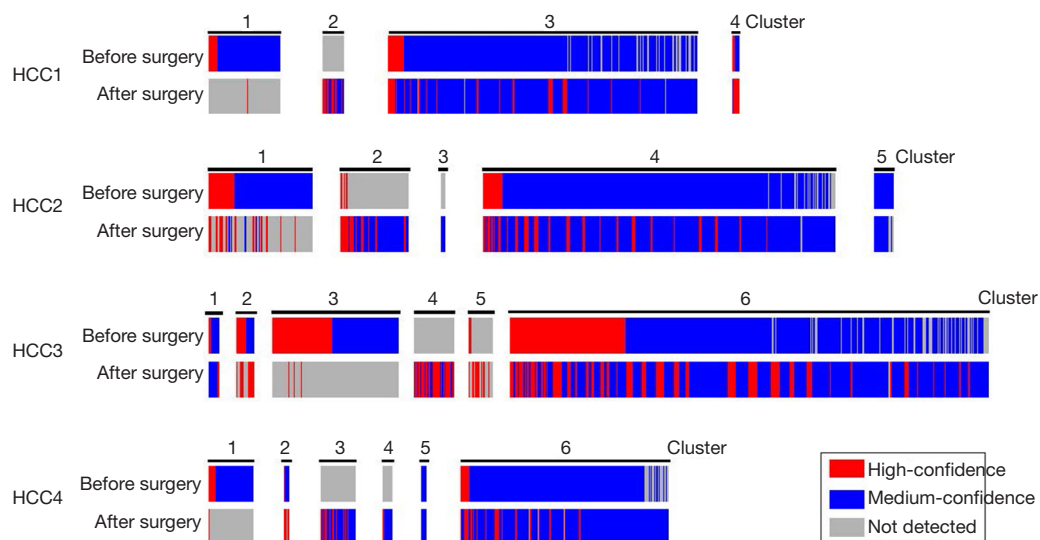


Figure 2 Mutation clusters in plasma ctDNA samples. Red rectangles indicated high-confidence mutations with sequencing depth of more than 30 and allele fraction >5%; blue rectangles indicated mutations with sequencing depth of less than 30 but with AF >5%; gray rectangles indicated mutations not detected.

clusters detected only in one of the plasma samples. Each patient has a large mutation cluster detected in all plasma samples (cluster 3 in HCC1, cluster 4 in HCC2, cluster 6 in HCC3, and cluster 6 in HCC4), which contain most of the mutations. We also detected 10 private mutations clusters: cluster 1, 2 in HCC1, cluster 1, 3, 4 in HCC4 and so on (*Figure 2*). Private mutation clusters suggested the changes in plasma samples between preoperative and postoperative plasma samples. For example, mutations with high confidence (mutations with sequencing depth of more than 30 and allele fraction >5%) in cluster 3 of HCC3 were detected mainly in the plasma sample before treatment, while most of these mutations were not detected in the plasma sample after treatment.

Cellular prevalence of mutation clusters

Previous studies used PyClone to infer cellular prevalence in tumour biopsies and to follow clonal dynamics in serially transplanted tumour xenografts (12,20). In this study, we used PyClone to estimate cellular prevalences in preoperative and postoperative plasma samples of the four HCC patients (*Figure 3*). Cellular prevalence is defined as the proportion of cancer cells with the mutation. We used PyClone to investigate the changes of estimated cellular prevalence in the preoperative and postoperative plasma samples.

The cellular prevalence of the plasma samples were grouped into putative mutation clusters. For each of the 4 cases, we observed reductions in cellular prevalence of mutation clusters between preoperative and postoperative plasma samples (P value <0.001), which reflected the treatment responses after surgery (for example, cluster 1, 4 in HCC1, cluster 1, 5 in HCC2, cluster 2, 3 in HCC3 and cluster 1, 2, 5 in HCC4 respectively).

Notably, several cases contained mutation clusters with low (mean of 0.0005–43.27%) prevalences in the preoperative plasma samples but high (mean of 20.25–63.23%) prevalences in the postoperative plasma samples (P value <0.001) (*Figure 3*), implying expansion of initially minor clones. Those clones could be recognized as the emergence of treatment-resistant clones, which can be used as potential targets of therapy. We detected a mutation *ERBB2*-c.G2524C, which only existed in mutation cluster 2 of the postoperative plasma sample in HCC1. *ERBB2*, commonly referred to as HER2, is amplified and/or overexpressed in 20–30% of invasive breast carcinomas. *ERBB2* (c.G2524A) was one of the first *ERBB2* variants to be functionally classified (21). This mutation was shown to be an activating mutation in an *in vitro* assay: along with other *ERBB2* activating mutations, mutation *ERBB2* (c.G2524A) in MCF10A breast cancer cell lines have been shown to be sensitive to the kinase inhibitor neratinib. More recent evidence showed that *HER2* activating mutations

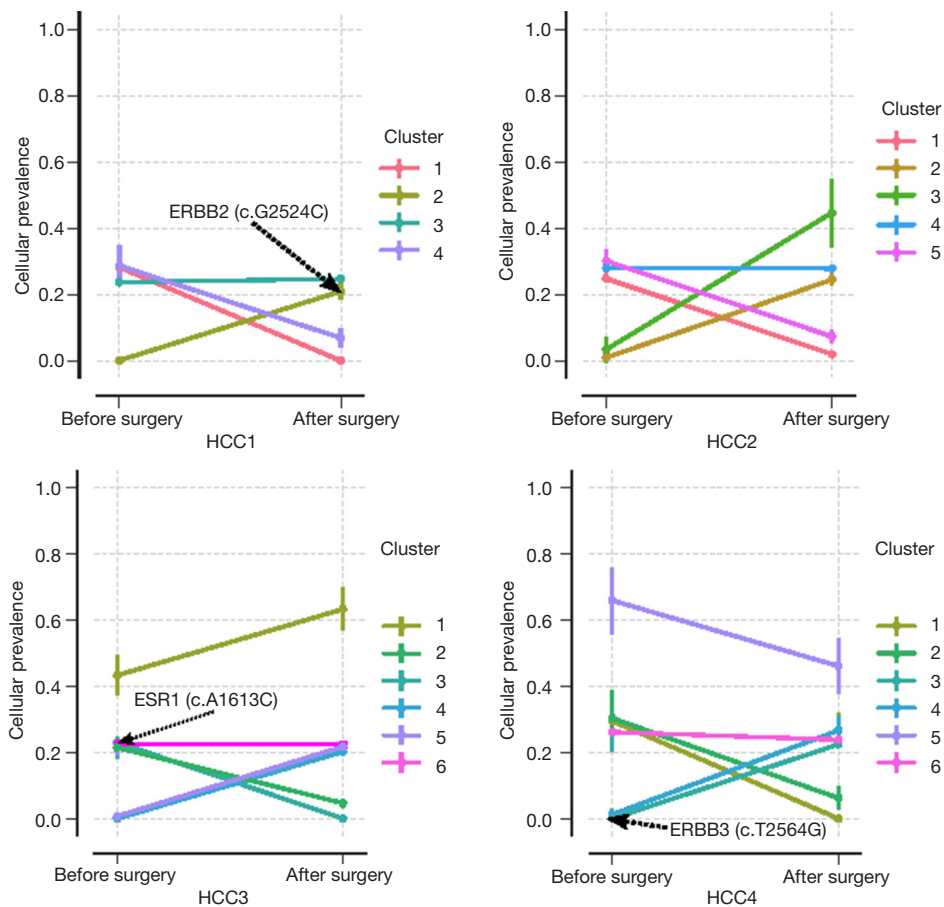


Figure 3 Cellular prevalence of mutation clusters. Inferred cellular frequency of mutation clusters using PyClone in plasma ctDNA samples.

confer sensitivity to a host of tyrosine kinase inhibitors like Neratinib, Lapatinib, Trastuzumab, which means the *ERBB2* (c.G2524C) mutation found in cluster 2 of the post plasma sample could be used as a target for therapy. In patient HCC4, we identified a mutation (*ERBB3*, c.T2564G) in cluster 3 of the post treatment plasma sample (Figure 3). A recent study reported a novel c.T2564C mutation located in exon 21 of the *HER3* tyrosine kinase domain in an adolescent patient with a chemotherapy-resistant advanced NSCLC (22). Protein alignments indicated the c.T2564C mutation was analogous to *EGFR*-L858R and *BRAF*-L597V. Co-expression of wild type *HER2* and *HER3*-c.T2564C in Ba/F3 cells led to sensitivity to afatinib and pertuzumab. This observation suggests that mutation cluster 3 in HCC4 might be used as a potential actionable target. However, the mechanism of mutation *ERBB3*-c.T2564G being an actionable mutation still needs further investigation.

Interestingly, we also identified large major clusters

(containing an average of 333 mutations in each sample) sharing sub-clonality between the preoperative and postoperative plasma samples, which suggests the detection of direct evidence of residual disease in the form of ctDNA. This implicated that after treatment cells containing these mutations still exist. The cellular prevalences of these mutation clusters ranges between 22.55% and 27.90% (Figure 3). We identified a mutation in gene *ESR1* (c.A1613C) in cluster 6 of preoperative plasma sample of HCC3. *ESR1* mutations have been identified in breast cancer, endometrial, ovarian and other cancer types. Mutations in the *ESR1* ligand binding domain have been shown to confer resistance to hormone therapy (23,24). This evidence has led to an increased use of targeted sequencing of the estrogen receptor in breast and ovarian cancer. Mutation c.A1613C is one of these ligand binding domain mutations, and is commonly implicated in this hormone resistance. In this study, c.A1613C mutation

was detected in cluster 6 of preoperative plasma sample of HCC3. The sharing sub-clonality between the preoperative and postoperative plasma samples also suggests resistant in patient HCC3 when applying hormone therapy.

Discussion

In this study, we performed PyClone to investigate putative clonal clusters and infer cellular prevalence in plasma sample of 4 HCC patients. In total, we identified 21 clusters in the plasma ctDNA samples. We also detected serial changes in cellular prevalences of mutation clusters in preoperative and postoperative plasma samples. We found actionable targets in mutation clusters which could be used to guide therapy. The results suggest that ctDNA analysis of plasma samples can be used to investigate clonal population structures and captures sub-clonal dynamics in real time.

We used VarDict (16) to call somatic variants in this study. As is known, there are several widely used variants callers which were developed to find somatic mutations in whole genome sequencing. For example, MuTect (25) applies a probabilistic framework (Bayesian statistics) to detect somatic mutations and assess confidence in them. FreeBayes (26) is haplotype-based software, it calls variants based on the literal sequences of reads aligned to a particular target, not their precise alignment. VarScan2 (27) employs a robust heuristic/statistic approach to call somatic mutations that meet desired thresholds for read depth, base quality, variant allele frequency, and statistical significance (some statistical test like Fisher's Exact). However, we focused on preoperative and postoperative plasma samples in this study. We compared the results of several callers, Mutect generated too much potential variants in the plasma samples compared to variants obtained from tumor tissue. The results performed by VarScan2 filtered some true variants in the plasma samples when we checking reads covering the position (data not shown). It has been reported that variant allele fraction in ctDNA can be as low as 0.01% (28). The extremely low ctDNA levels may make the above mentioned somatic mutation callers less sensitive when analyzing plasma samples. In addition, these above mentioned tools are not designed to work with sequencing data at multiple time points of the same patient. In this study, we performed VarDict to detect somatic mutations in preoperative and postoperative plasma samples, which could identify low allele frequency mutations with high sensitivity.

The average depth of the plasma samples of the four HCC patients is considered low coverage. For the purpose

of PyClone analysis, less than 1,000 depth is considered low coverage. At low depth PyClone is likely to over cluster mutations as there is insufficient precision to separate clones. Single tumor sample analysis will probably not work well though. This is not PyClone specific, but an intrinsic issue of clone analysis software. So, in this study we focused on multiple samples per patient, and used binomial method, which works better and faster for low coverage samples. We discarded mutation clusters with fewer than two mutations assigned to them. Clusters with fewer than two mutations have more uncertainty about the cellular prevalence estimates than those with multiple mutations. This happens because without clustering PyClone has no idea which possible genotype the mutation has. However, in clusters with several mutations, the model can share information and infer what the common cellular prevalence is and by extension associated genotypes for mutations in the clusters are. In this study, we did not identify mutation cluster appearing as clonally dominant (prevalence ~100%). This could be because the founding point mutation was not identified due to low coverage or if the founding mutation is a larger genomic aberration. Further investigation still need to be performed here.

A recent research reported that metastatic breast cancer (mBC) patients with high heterogeneity (clonal population ≥ 3) in plasma ctDNA had a significantly worse PFS (HR, 2.79; 95% CI, 1.23 to 6.34; $P=0.014$) (29). In this study, we used PyClone to infer putative clonal clusters and cellular prevalence in plasma sample of 4 HCC patients. The more the clusters, the higher heterogeneity in plasma ctDNA. We identified 4 clusters in HCC1, 5 clusters in HCC2, and 6 clusters in HCC3 and HCC4. An average of five major clusters were identified in the plasma samples of the four HCC patients, which is more than metastatic breast cancer reported in another research (29). The size of the tumor does not appear to be correlated with the number of clusters in plasma before surgical resection. We identified only four clusters in HCC1 who had the largest tumor (13 cm) of the four HCC cases. The results could also be used as a prognostic factor to assess tumor heterogeneity in plasma ctDNA in the future, which could provide a more sensitive way to predict and monitor therapeutic response in patients with HCC. Unfortunately, we did not get the prognosis of the 4 HCC patients. As for the treatment responses of surgery, we found that reductions in cellular prevalence of mutation clusters between preoperative and postoperative plasma samples (P value <0.001), which reflected the treatment responses after surgery (for example,

cluster 1, 4 in HCC1, cluster 1, 5 in HCC2, cluster 2, 3 in HCC3 and cluster 1, 2, 5 in HCC4 respectively).

Along with the development of next-generation sequencing, the research of ctDNA has become a hot spot in this field. Our analysis highlights the potential utility of clone analysis for tracking therapy effectiveness in real time. The clone analysis of circulating tumor DNA (ctDNA) could help to establish a more precise target, which could make ctDNA an informative, inherently specific, and highly sensitive biomarker for HCC.

Conclusions

Our findings provided a more complete picture of the liver cancer pathogenesis. The comparison of preoperative and postoperative plasma samples showed that ctDNA can be used to real-time sampling of clonal evolution in patients with hepatocarcinoma. In addition to dynamic monitoring of disease progression and response to therapy, characterizing of dynamics of genomic clones can be used to determine the benefit of new therapeutics and guide therapy.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study and the protocols used were approved by the Institutional Ethics Committee of BGI (NO. BGI-IRB 15136). Written informed consent was obtained from all of the participants when enrolled in the study.

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