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Exome Analysis of the Evolutionary Path of Hepatocellular Adenoma-Carcinoma Transition, Vascular Invasion and Brain Dissemination

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Abstract

Hepatocellular adenoma (HCA) is a rare benign liver tumor, predominantly seen in young women. Its major complications are malignant transformation, spontaneous hemorrhage, and rupture. We describe a case of a young female with no underlying liver disease who presented with acute abdominal pain and was found to have a 17 cm heterogeneous mass in the left lobe of the liver. She underwent left hepatectomy and pathology revealed a 14 cm moderately differentiated hepatocellular carcinoma (HCC) arising in a shell of a HCA. At that time, vascular invasion was already present. She rapidly developed recurrent multifocal hepatic lesions and subsequent spread to the brain, leading to her death 18 months after surgery. To investigate the underlying genetic events occurring during hepatocellular adenoma-carcinoma transition and extra-hepatic dissemination, we performed whole-exome sequencing of DNA isolated from peripheral blood leucocytes, HCA, HCC, tumor thrombus and brain metastasis. Our data show a step-wise addition

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Author names in bold designate shared co-first authorship.

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of somatic mutations and copy number variations with disease progression, suggesting a linear tumor evolution, which is supported by clonality analysis. Specifically, using a model based clustering of somatic mutations, one single founding clone arising in the HCA, which included *CTNNB1* and *IL6ST* driver mutations, was identified and displayed an increasing clonality rate in HCC, tumor thrombus and brain metastasis. Our data highlight the feasibility of performing whole-exome capture, sequencing and analysis using FFPE samples, and we describe the first genomic longitudinal study of hepatocellular adenoma-carcinoma transition, vascular invasion and brain metastasis with detailed clinicopathologic annotation.

Keywords

whole exome sequencing; hepatocellular adenoma; hepatocellular carcinoma; hepatocellular adenoma-carcinoma transition; brain metastasis; FFPE tissue molecular analysis

Introduction

Hepatocellular adenoma (HCA) is a rare benign liver tumor, predominantly seen in young females. HCAs are known to be associated with prolonged exposure to estrogens (i.e. oral contraceptives, OCPs), anabolic androgens, and rare genetic disorders, namely maturity-onset diabetes of the young type 3 (MODY 3, caused by mutation in the hepatocyte nuclear factor 1-alpha gene, *HNF1A*), glycogen storage disease type 1a, and McCune-Albright syndrome. The most severe complications of HCAs are malignant transformation and spontaneous hemorrhage and rupture, both associated more often with large adenomas measuring 5 cm in maximum diameter. Additionally, β -catenin activation is known to be an early event in HCA transformation[1] and exon 3 β -catenin mutated-HCAs have a higher risk of malignant transformation[2]. The application of massively parallel sequencing technologies to study tumors, including HCC, has refocused the field of cancer genetics from hypothesis-driven to hypothesis-generating, overcoming the limitations and biases of candidate gene approaches. Genomic profiling of HCAs has recently identified genetic and epigenetic events associated with adenoma-carcinoma transition, such as recurrent genetic alterations in *CTNNB1*, *IL6ST*, and *TERT* promoter; increased occurrence of copy number variation (CNVs); and hypomethylation of liver-specific genes[1]. Currently, HCAs are classified into five major molecular subgroups; (i) *HNF1A*-mutated HCAs[3]; (ii) inflammatory HCAs characterized by JAK/STAT pathway activation[1,4-6]; (iii) exon 3 *CTNNB1*-mutated HCAs (half of them also have an inflammatory phenotype/genotype)[2]; (iv) exon 7 or 8 *CTNNB1*-mutated HCAs, with a lower risk of malignant transformation as compared to exon 3 *CTNNB1*-mutated tumors[1,7]; and (v) HCAs characterized by sonic hedgehog pathway activation via fusion of the *INHBE* and *GLII* genes[8]. Less than 10% of HCAs remain molecularly unclassified. However, the prognosis for those afflicted with HCA(s) is not predictable, and it remains clinically challenging to manage and counsel these patients. Furthermore, the incidence of HCAs has been increasing in the last several decades, likely due to the introduction and rising use of OCPs, and improvements in diagnostic imaging leading to ever increasing incidental findings in patients undergoing abdominal imaging studies for evaluation of unrelated or nonspecific abdominal symptoms.

Identification of reliable predictive biomarkers of malignant transformation is necessary to determine optimal therapy (medical versus surgical).

Here, we describe a rare case of a 33-year-old female with no significant past medical history who presented with acute abdominal pain and was found to have a 17 cm left hepatic lesion for which she underwent left hepatectomy. Pathology revealed a 14 cm moderately differentiated HCC in a background of HCA. Vascular invasion was present. The patient rapidly developed recurrent multifocal hepatic lesions and subsequent spread to the brain, culminating with her death 18 months after surgery. We hypothesized that a comprehensive longitudinal genomic and pathological analysis of the patient's HCA, HCC, tumor thrombus and brain metastasis would inform the evolutionary path of this atypical HCC presentation.

Materials and Methods

Blood and tumor samples

The study was performed in accordance with the Yale Institutional Review Board standards. DNA was extracted from a sample of the patient's peripheral leucocytes, as well as from formalin-fixed paraffin-embedded (FFPE) samples of her typical HCA, atypical steatotic HCA, HCC, tumor thrombus and brain metastasis.

Whole Exome Capture and Sequencing

We performed whole-exome sequencing (WES) of DNA isolated from typical HCA, atypical steatotic HCA, HCC, tumor thrombus, brain metastases, and matching peripheral blood leucocytes with mean target coverage of 205×, 235×, 223×, 194×, 163× and 78×, respectively. The quality control on raw reads, alignment, marking PCR duplicates, local realignment and base quality score was performed as detailed previously[9].

Exome Sequencing Analysis

a. Somatic Variant Calling—We used the Haplotype caller implemented in GATK (version 2.5)[10] for all five tumor stages and matching blood. We identified the somatic variants as previously described[11].

b. Somatic Copy Number Variation (CNV) analysis—CNV segments were calculated using the segments called by the ExomeCNV R package, and depth of coverage log ratio (between tumor sample and blood) was calculated using the GATK-Depth of Coverage tool. The called segments were corrected by calculating minor allele frequencies (BAF) in each CNV segment. The admixture rate, i.e., the rate of normal tissue contamination in the tumor samples, was calculated using the BAF shift of germ line heterozygous mutations in the regions of copy number loss using qpure R package[12]. The calculated admixture rates for the vascular invasion and the brain metastasis were 0.5 and 0.2, respectively. However, for the samples with no loss of heterozygosity (LOH), i.e. HCA, atypical HCA and HCC, the admixture rate could not be calculated, and it was estimated to be closer to the brain metastasis (a solid tumor), and a default admixture rate of 0.3 was used.

c. Clonality Analysis—We performed clonality analysis by integrating the CNV information, admixture rate and non-reference variant allele frequency in the somatic variant data as previously described[5]. Clonality rate corresponded to the percentage of tumor cells harboring the identified somatic mutation. Mclust package in R was used to cluster the unique 37 protein altering somatic mutations in four tumors based on their clonality rate distributions. Bayesian Information Criteria (BIC) was used to find the model with the optimal number of clusters (<http://www.stat.washington.edu/mclust/>).

d. Mutational Signature Analysis—Mutational signature analysis comprised all somatic single nucleotide polymorphisms, including protein altering, synonymous and (captured) non-coding variants.

Sanger Sequencing of genomic DNA

Sanger sequencing of genomic DNA isolated from HCA and HCC FFPE samples for recurrent *TERT* promoter mutations in two “hot-spots” (chr5:1,295,228, G>A/T, and chr5:1,295,250, G>A) was performed by PCR amplification, using forward primer: 5′-CAGCCCTGGGGCCCCAGGCGCCGCACG-3′, and reverse primer: 5′-CTGGGAGCCCCGGCCTGGCCCCGACAG-3′ for mutation at chr5:1,295,228; and forward primer: 5′-CTGGGGCCCCAGGCGCCGCACGAACG-3′, and reverse primer: 5′-GCTGCTCCGGGCGGACCCGGGGGTC-3′ for mutation at chr5:1,295,250.

Droplet Digital PCR (ddPCR)

Bio-Rad IL6ST deletion ddPCR assay (forward primer: 5′-ATTCTCTGCTTCTACCCAG-3′; reverse primer: 5′-CCTCATGCACTGTTGATTAT-3′; deletion probe: cagacttcaatGacagtagaa; WT probe: caatGTTGACAAAATACacagt) was performed using genomic DNA isolated from peripheral leucocytes, HCA, HCC and brain metastasis.

RNA isolation and Quantitative RT-PCR

RNA was isolated from background normal liver parenchyma, HCA and HCC FFPE samples and cDNA was synthesized. Expression was assessed by quantitative RT-PCR using Taqman (Applied Biosystems) gene expression assay (*TERT*, Hs00972656 m1). Taqman gene expression assay for RNA ribosomal 18S (Hs99999901 s1) was used as internal control. HCT116 colon cancer cell line was used as a positive control for *TERT* gene expression assay.

Liver Immunohistochemistry

Immunohistochemistry was performed in FFPE liver tissues with the following antibodies: anti-CRP (monoclonal rabbit, 1/400 dilution; Abcam), anti-LFABP (polyclonal rabbit, 1/100 dilution; Abcam), anti-β-catenin (monoclonal mouse, 1/200 dilution; BD Biosciences); anti-GS (monoclonal mouse, 1/400 dilution; BD Biosciences), and anti-SAA (monoclonal mouse, 1/100 dilution; Dako).

Case Report

A 33-year-old female with no underlying liver disease or other significant co-morbidities presented with acute abdominal pain and was found to have a 17 cm left hepatic mass. She underwent urgent left hepatectomy in the setting of presumed intra-tumoral hemorrhage. Macroscopically, the liver tumor was multinodular with areas of extensive hemorrhage and necrosis (Figure 1A). The scant non-neoplastic liver had intact architecture with no fibrosis or other significant abnormality (Figure 1B). Pathological analysis of the tumor showed some residual HCA (Figure 1C-D) surrounding a 14 cm moderately differentiated HCC (Figure 1E). The grossly identifiable residual HCA was 3 cm in greatest dimension, though areas of HCA were histologically evident within the larger tumor. Some foci of HCA had nuclear atypia and increased hyaline globules, features that were more prominent in steatotic areas (left aspect of Figure 1C and 1D). Immunohistochemical characterization revealed heterogeneous serum amyloid A (SAA), glutamine synthetase (GS) and c-reactive protein (CRP) stains with areas of atypical steatotic hepatocytes expressing SAA, variably (weak to moderate) expressing GS, and having weak positivity for CRP, designated as atypical steatotic HCA (Figure 1C and D). The adenoma demonstrated weak to moderate expression of liver fatty acid binding protein (LFABP), without the characteristic loss described in *HNF1A*-mutated adenomas (data not shown). Reticulin stain was intact in typical and atypical HCA (data not shown). HCC was diffusely positive for GS (Figure 1E) and showed heterogeneous CRP and negative p-catenin stain (data not shown). Detailed results of immunohistochemical staining of the different regions are shown in supplementary Table 1. Though resection margins were negative, there was vascular invasion (Figure 1F) as well as invasion into but not through the liver capsule. Tumor pathologic stage was T3aNxMx, stage IIIA. Staging chest CT and bone scan ruled out pulmonary and osseous metastases. As expected, based on tumor vascular invasion, she developed multifocal liver recurrence shortly after surgery, for which she was started on systemic therapy with sorafenib 400mg twice daily. She tolerated it reasonably well, reporting mild-to-moderate hair loss and mild hand-foot-skin reaction. She also underwent bi-lobar selective internal radiation therapy with Yttrium-90 as advised by consensus of the institutional Multidisciplinary Liver Tumor Board. However, three-month surveillance MRI showed persistent liver progression of multifocal HCC. She underwent a second Y90 treatment of the right hepatic lobe approximately 10 months after her initial diagnosis. Her course was complicated by biliary obstruction secondary to progression of infiltrative HCC. Sixteen-months after surgical resection, she developed persistent severe headache and nausea and she was found to have a 5 cm right frontal lobe mass with surrounding vasogenic edema and midline shift. She underwent palliative right frontal craniotomy for resection of the brain tumor, confirmed by pathology to be metastatic HCC (Figure 1G), which showed rare positive β -catenin nuclear staining (Figure 1H). Ultimately, she developed liver failure with worsening mental status culminating in death 18 months after her initial presentation and HCC diagnosis.

In light of such an atypical presentation, we performed genomic analysis to infer the evolutionary path of hepatocellular adenoma-carcinoma transition and uncommon metastatic dissemination to the brain.

Consistent with previous work done by us[13] and others[14], this study provides further evidence that WES using DNA extracted from formalin-fixed paraffin embedded (FFPE) samples is feasible and reproducible. We detected 3, 6, 17, 29, and 32 somatic mutations in HCA, atypical steatotic HCA, HCC, vascular invasion and brain metastasis, respectively (Figure 2A).

Mutational signature analysis revealed that atypical steatotic HCA, HCC, vascular invasion and brain metastasis had an increased rate of C>T transitions and C>A transversions, which are signatures frequently seen across various cancer types (Figure 2B)[15].

Step-wise addition of somatic mutations through the progress of disease indicates a linear evolution, which is also supported by the clonality analysis (Figure 2C-D). Interestingly, *CTNNB1* (p.S45P) and *IL6ST* (c.567-578del12) driver mutations were detected in HCA and progressively increased in clonality during tumor progression and dissemination. The primary HCC mass had a total of 17 protein-altering somatic mutations. The intravascular HCC sample had 29 somatic mutations, 16 of which were shared with the primary HCC. The brain metastasis further gained 6 private mutations and shared 26 somatic mutations with the intravascular tumor sample (Figure 2C). Except for mutations in *CTNNB1* and *IL6ST* genes, there were no other known cancer driver genes mutated in these samples. Specifically, *TERT* promoter hot-spot mutations (chr5:1,295,228, G>A/T, and chr5:1,295,250, G>A) were not detected in HCA and HCC samples by Sanger sequencing (supplementary Figure 1); and *TERT* mRNA expression was also not detected in HCA and HCC samples (data not shown). A splice site variant in the *HAS2* gene, which encodes hyaluronan synthase 2, was identified across all samples. Overexpression of hyaluronan synthase 2 correlates with tumorigenesis and breast cancer metastasis. A highly conserved mutation in *CFP* encoding complement factor properdin was also noted in the pre-neoplastic lesion and showed increased clonality with tumor progression and dissemination to extra-hepatic sites, but its biological significance is currently unknown. Mutations in *YY1AP1* (p.P337R) and *PLXNB2* (p.Q1379H), which encode for YY1-associated protein 1 and plexin-B2, respectively, were detected initially in vascular invasion and persisted in the brain metastasis sample. YY1AP1 has been proposed as an oncogenic driver in a subgroup of HCC associated with poor prognosis, based on expression data[16]. Moreover, overexpression of plexin-B2, an axon guidance receptor, correlates with glioma invasion, vascularization and poor prognosis[17]. We also calculated the clonality of the somatic mutations to assess the evolution of the malignancy: HCC “diagnosis-to-death” progression (Figure 2C-D). Model based clustering on the clonality of somatic mutations across 5 tumors revealed 5 clones, which presented a single founding clone (clone 1) from the adenoma sample that was present in all tumors (Figure 2D). Clone 1, with *CTNNB1* somatic mutation displayed an increasing clonality rate with malignant evolution, indicating monoclonal seeding. Emergence of other clones in atypical steatotic HCA and HCC, and the preservation of some of these clones throughout vascular invasion and brain metastasis also present a linear evolution pattern (Figure 2D).

CNV analysis revealed that the HCA and HCC did not have any large-scale CNV events, whereas the intravascular tumor and the brain metastasis samples had deletion events that affected 1.16% and 5.94% of their genomes, respectively (Figure 2E). Large-scale deletions

on chromosome 8p, chromosome 13p and whole chromosome deletion on chromosome 18 were shared between the intravascular tumor and the brain metastasis (Figure 2F).

Since this “N of 1” case describes an aggressive lethal HCC in a young patient with no identifiable risk factors or underlying findings of advanced liver disease, we investigated the presence of germ line damaging (truncating, frame-shift and splice-site mutations) and missense conserved (no more than one species different of amino acid conservation) mutations in genes previously associated with an increased risk of inherited (familial) gastrointestinal cancer(s). We found that the patient carried a novel heterozygous splice-site *Ataxia Telangiectasia Mutated (ATM)* mutation (Supplementary Table 2). ATM has an important role in DNA damage response, regulating DNA repair, cell-cycle check-points and cell death upon the presence of DNA strand breaks.

Discussion

HCAAs that harbor “hot-spot” somatic mutations in *CTNNB1* and *IL6ST* are known to be associated with a significant risk of malignant transformation[2]. However, this case report describes a rare presentation of hepatocellular adenoma-carcinoma transition complicated by vascular invasion at the time of diagnosis. Despite systemic therapy with sorafenib and Y-90 locoregional therapy, this tumor progressed rapidly with local hepatic infiltration and extra-hepatic metastasis, culminating with the patient's death approximately 18 months after diagnosis. HCC commonly metastasizes to the lungs, regional lymph nodes, peritoneum and adrenal glands and only rarely to the brain (~1.3-2.9%). Even more uncommon is to have access to brain metastatic tissue for genomic analysis.

HCA and HCC samples harbored a known activating *CTNNB1* mutation, p.S45P, which has been reported to activate β -catenin weakly as compared to large in-frame exon 3 deletions and point mutations occurring at the D32-S37 residues [7]. Thus, heterogenous GS staining and negative β -catenin stain seen in the HCA is consistent with recently described weak β -catenin mutant activity. In this case, *IL6ST* deletion was likely the second activating genetic event, since no other known driver mutations were detected at the exome level or in the two hot spots of the *TERT* promoter.

In accordance with previous studies[18-19], our findings show that the vascular and brain metastasis and primary hepatocellular samples share a common ancestor and follow a linear evolution pattern. Moreover, the hepatocellular adenoma area described as having atypical features harbors *CTNNB1* mutation, a higher frequency of *IL6ST* deletion than the typical HCA tissue, and two additional mutations that are currently of unclear significance in *CFP* and *ZPF90* that persisted with increased clonality in vascular invasion and brain metastasis samples.

HCC has significant genetic heterogeneity, a limiting factor in advancing the molecular understanding of this malignancy. However, genomics is a powerful tool to follow tumor progression with great precision at the individual level. We believe that this case report illustrates the importance of investigating atypical “N of 1” cases to help identify common patterns and enable more robust conclusions about HCC origin and progression. In an

editorial from Brannon and Sawyers[20], the authors discuss a new initiative of “diagnosis-to-death” project with longitudinal genomic analysis of tumor progression. As discussed by the authors, this approach aims to provide new insights into disease pathophysiology, through the collection and cross comparison of hundreds of “*N* of 1” cases.

We expect that the collection of multiple single cases undergoing integrative genomic analysis and their cross comparison among HCC cases will yield new insights in HCC biology and advances in Precision Medicine in this field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Pathological features of background liver parenchyma, hepatocellular adenoma (HCA), atypical steatotic HCA, hepatocellular carcinoma (HCC), vascular invasion and brain metastasis. (A) Gross image of left hepatectomy specimen, scale bar, 1cm; (B) Non-neoplastic liver parenchyma with no significant fibrosis or other abnormality (Trichrome stain), scale bar, 50μm. (C) C-reactive protein stain in hepatocellular adenoma with atypical (left aspect of the image) and typical features (right aspect of the figure), scale bar, 50μm; (D) Atypical HCA (left aspect of the image) shows weak to moderate expression of glutamine synthetase (GS), and typical HCA (right aspect of the image) shows heterogeneous GS staining, scale bar, 50μm; (E) Diffusely positive GS stain in HCC, scale bar, 50μm; (F) Representative image of intravascular HCC (tumor thrombus); (G) Brain metastasis of HCC (H&E), scale bar, 50μm; (H) Rare positive nuclear β-catenin stain in brain metastasis of HCC, scale bar, 50μm.

Figure 2.

Genomic analysis of longitudinal hepatocellular adenoma (HCA), atypical HCA (aHCA), hepatocellular carcinoma (HCC), vascular invasion and brain metastasis samples. (A) Number of coding region somatic mutations in the five tumor stages (HCA, aHCA, HCC, vascular invasion and brain metastasis). (B) Distribution of all somatic mutations. (C) Clonality analysis of somatic mutations and (D) schematic representation of the “trunk” mutations and the “private” mutations across the five tumor stages. (E) The percentage of genome altered by CNV events in five tumor stages, which are all deletion events. (F) Circos plot depicting copy number variation analysis with deletions marked by blue segments. (Asterix*) Indicates that IL6ST 12 bp deletion is detected in HCA (44 mutated reads out of 529 reference reads (~8%), and was also validated by ddPCR).