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Original Article

Genetic heterogeneity in hepatocellular carcinoma and paired bone metastasis revealed by next-generation sequencing

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Abstract: Although bone involvement is reported as uncommon in hepatocellular carcinoma (HCC), its incidence has significantly increased in the last decade due to the longer survival of HCC patients related to recent progresses made both in the diagnosis and treatment of the disease. A better understanding of the pathogenic mechanisms underlying the spread of bone metastases in HCC is important. The primary tumor and its corresponding metastases are different at the molecular marker expression or gene status levels and that these differences may affect the clinical outcome of anticancer therapy, particularly in molecularly targeted therapies for the treatment of cancer. No study has investigated the genetic heterogeneity between HCC and paired bone metastasis. In this study, we investigated the genetic heterogeneity in HCC and paired metastasis using a next generation sequencing (NGS) platform to illustrate the molecularly targeted therapy related genes mutations.

Keywords: Hepatocellular carcinoma, bone metastasis, genetic heterogeneity, next generation sequencing

Introduction

Liver cancer in men is the fifth most frequently diagnosed cancer worldwide but the second most frequent cause of cancer death. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death. Half of these cases and deaths were estimated to occur in China [1]. Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, accounting for 70% to 85% of the total liver cancer burden worldwide [2]. The primary risk factor for HCC is liver injury from diverse causes that leads to hepatic cirrhosis in most patients. An estimated 78% of HCC cases and 57% of cases of liver cirrhosis are caused by chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) [2, 3]. HCC is the sixth most prevalent cancer worldwide and the third leading cause of cancer-related death, although its geographical distribution is heterogeneous with the highest incidence in sub-Saharan Africa and Eastern Asia [4]. Bone is an uncommon site of metastasis in HCC, with the incidence ranging from 3% to 20% [5]. Although bone involvement is reported as uncommon in HCC, its incidence has significantly increased in the last decade due to the longer survival of HCC patients related to recent progresses made both in the diagnosis and treatment of the disease [5-7].

A better understanding of the pathogenic mechanisms underlying the spread of bone metastases in HCC is important. Some retrospective studies have described the characteristics of bone metastasis from HCC [5, 8-10]. However, few data are yet available about bone involvement in patients with HCC, and no agreement has yet been reached about the treatment strategy for extrahepatic HCC metastases. The nature and the characteristics of bone metastases in HCC have not been fully explored in literature, presumably because HCC skeletal involvement was rarely diagnosed.

Intratumor heterogeneity is a recognized characteristic of human tumors, and occurs on multiple levels, such as the genetic (detected by

Table 1. Clinical characteristics of two hepatocellular carcinoma patients with metachronous bone metastasis received surgery

	Patient 1	Patient 2
Gender (Male/Female)	Male	Male
Age (Years)	39	58
AFP level (ng/ml)	8.4	27.1
ALT (IU/L)	Not available	Not available
AST (IU/L)	Not available	Not available
HBV	Positive	Positive
HCV	Negative	Negative
BCLC staging (A/B/C)	Not available	A
Child's score (A/B/C)	Not available	A
Hepatocellular carcinoma (number of masses)	2	2
Bone metastases	Metachronous	Metachronous
Overall survival (months)	57.5	42.3
Chemotherapy. (Yes/No)	Yes	Not available
Radiotherapy (Yes/No)	Yes	No
Molecularly targeted therapy (Yes/No)	Yes	No
Primary tumor ID	10-13656-1	10-42240-1
Metastatic tumor ID	12-60901-3	13-39592-3

Abbreviations: HBV, hepatitis B virus infection. HCV, hepatitis C virus infection. AST, aspartate aminotransferase. ALT, aspartate aminotransferase. AFP, alpha-fetoprotein. BCLC staging, Barcelona clinic liver cancer staging.

Table 2. Fifty known cancer genes

Gene name				
ABL1	EGFR	GNAQ	KRAS	PTPN11
AKT1	ERBB2	GNAS	MET	RB1
ALK	ERBB4	HNF1A	MLH1	RET
APC	EZH2	HRAS	MPL	SMAD4
ATM	FBXW7	IDH1	NOTCH1	SMARCB1
BRAF	FGFR1	IDH2	NPM1	SMO
CDH1	FGFR2	JAK2	NRAS	SRC
CDKN2A	FGFR3	JAK3	PDGFRA	STK11
CSF1R	FLT3	KDR	PIK3CA	TP53
CTNNB1	GNA11	KIT	PTEN	VHL

the method of mutation analysis), protein (detected by the method of immunohistochemical analysis) and macroscopic level in a wide range of tumors, such as breast, colorectal (CRC), non-small cell lung (NSCLC), prostate, ovarian, pancreatic, gastric, and brain cancer and renal clear cell carcinoma. In recent years, many studies have focused on the heterogeneity found in primary tumors and related metastases with the consideration that evaluation of metastatic rather than primary sites could be of clinical relevance. Numerous reports have evaluated the genetic heterogeneity in primary tumors and corresponding metastases in a range of solid tumors such as breast cancer [11], CRC [12] and NSCLC [13]. As discussed earlier, the primary tumor and its corresponding metastases are different at the molecular marker expression or gene status levels and that these differences may affect the clinical outcome of anticancer therapy, particularly in molecularly targeted therapies for the treatment of cancer [14, 15]. Monaco et al. suggested that the EGFR and KRAS status of primary lung carcinomas might not predict the status in the corresponding metastases. Their observation may have important implications for molecular testing for EGFR-targeted therapies [16]. A retrospective study investigated the role of PTEN loss, AKT phosphorylation and KRAS mutations in primary colorectal tumors and their corresponding metastases on the activity of cetuximab plus irinotecan [17]. This study gave us direct evidence to reveal that the genetic heterogeneity in primary colorectal tumors and their corresponding metastases have different responses to molecularly targeted therapy.

However, no study has investigated the genetic heterogeneity between HCC and paired bone metastasis. Here, we hypothesize that there is genetic heterogeneity between HCC and paired bone metastasis, which might result in the therapeutic failure of molecularly targeted therapy for treatment of HCC bone metastasis. In this study, we investigated the genetic heterogeneity in HCC and paired metastasis using a next generation sequencing (NGS) platform to illustrate the molecularly targeted therapy related genes mutations.

Patients and methods

Patients and tumor tissue samples

The institutional ethical committee approved the current retrospective study. A written in-

Table 3. Hepatocellular carcinoma primary tumor (sample number: 10-13656-1) detected gene mutations

Gene symbol	Mutation site	Amino acid change	Sequencing Depth	Mutation frequency
STK11	c.465-17G>A		1430	7.55%
FGFR3	c.1974G>A	p.Leu658Leu	1210	7.36%
FGFR3	c.1977C>T	p.Pro659Pro	1258	7.79%
FGFR3	c.1974G>A	p.Leu658Leu	1210	7.36%
FGFR3	c.1977C>T	p.Pro659Pro	1258	7.79%
GNA11	c.736_737insA		1071	6.72%
GNA11	c.735+7_735+8insA		1071	6.72%
TP53	c.1018A>G	p.Met340Val	2807	13.36%
VHL	c.464-25G>A		3425	8.76%
JAK3	c.420+51_420+50insGG		1414	5.37%
JAK3	c.420+42_420+41insA		1390	5.32%
JAK3	c.415G>A	p.Ala139Thr	2656	5.42%
KRAS	c.116C>T	p.Ser39Phe	3491	8.42%
ALK	c.3628G>A	p.Glu1210Lys	2187	22.04%
SRC	c.*92G>A		1995	16.74%
ERBB2	c.2501G>A	p.Ser834Asn	2652	5.51%
ERBB2	c.2506C>T	p.Leu836Leu	2579	5.62%
SMAD4	c.455G>A		9800	22.69%
SMAD4	c.454+5G>A		9800	22.69%
SMAD4	c.955+19T>C		5736	6.17%
SMAD4	c.1551C>T	p.Ser517Ser	7742	15.37%
KIT	c.232_233delAA	p.Lys78fs	1171	6.23%
KIT	c.1681G>A	p.Glu561Lys	3557	5.17%
KDR	c.795G>A	p.Ser265Ser	2876	8.48%
GNAS	c.2532T>C	p.Arg844Arg	8144	7.81%
ATM	c.1803C>T		5740	13.33%
ATM	c.1803-8C>T		5740	13.33%
ATM	c.7328_7329insT	p.Arg2443_Glu2444fs	7768	5.69%
ATM	c.7332_7333delGC	p.Glu2444fs	7720	5.53%

formed consent was obtained for both two patients. We reviewed the electronic medical records of consecutive patients in whom HCC and metachronous bone metastasis was newly diagnosed from January 2009 to October 2014 at the Department of Orthopedics, the First Affiliated Hospital, Zhejiang University School of Medicine (**Table 1**). The diagnosis of HCC was mainly based on recommendations of the American Association for the Study of Liver Diseases [18]. Both patients underwent blood investigations, which included complete blood count, liver function tests, and tests for viral markers of hepatitis B and C infection. Serum alpha-fetoprotein (AFP) was estimated using a particle enzyme immunoassay (Axsym System; Abbott Laboratories, Abbot Park, Illinois, USA; normal value <20 ng/ml). Upper gastrointestinal endoscopy was done in each case to detect the presence of esophageal varices. Patients with underlying cirrhosis were classified into Child's A, B or C based on the Child-Pugh classification [19]. Staging of HCC was done based on the Barcelona Clinic Liver Cancer (BCLC) staging protocol [20]. Both patients were evaluated pre-operatively using abdominal computed tomographic (CT) scan, magnetic resonance imaging (MRI), or fluorine-13 fluorodeoxyglucose positron emission tomography/computed tomographic scan (^{18}F-FDG PET/CT).

DNA extraction

Formalin-fixed paraffin-embedded (FFPE) tissues were sectioned, and hematoxylin and eosin (H&E) stained slides were reviewed by

Table 4. Hepatocellular carcinoma bone metastasis (sample number: 12-60901-3) detected gene mutations

Gene symbol	Mutation site	Amino acid change	Sequencing Depth	Mutation frequency
HRAS	c.-15G>A		5301	37.94%
HRAS	c.-21G>A		5329	37.27%
STK11	c.816C>T	p.Tyr272Tyr	8705	40.69%
FGFR3	c.1190T>C	p.Leu397Pro	1541	12.07%
GNA11	c.889+49G>T		2138	72.83%
GNA11	c.889+61_889+62insCA		2183	9.89%
TP53	c.1101G>A		4077	14.62%
TP53	c.1100+7G>A		4077	14.62%
VHL	c.346C>T	p.Leu116Phe	6318	6.38%
VHL	c.426T>C	p.Val142Val	6382	6.71%
JAK3	c.415G>A	p.Ala139Thr	2656	5.42%
FLT3	c.2053+87delC		1674	6.57%
ERBB2	c.2308G>A		9077	12.49%
ERBB2	c.2307+1G>A		9077	12.49%
SMAD4	c.1591C>T	p.Arg531Trp	13368	5.22%
KDR	c.3984G>A	p.Lys1328Lys	8372	5.22%
CDH1	c.1021T>C	p.Tyr341His	8831	6.87%
CDH1	c.1047C>T	p.Asp349Asp	8666	7.74%
GNAQ	c.953C>T	p.Pro318Leu	11157	6.63%
AKT1	c.75C>T	p.Arg25Arg	3671	15.94%
APC	c.2684C>T	p.Ser895Leu	14562	5.60%
APC	c.4073C>T	p.Ala1358Val	16662	6.74%
APC	c.4081C>T	p.Pro1361Ser	16706	6.60%
HNF1A	c.737T>C	p.Val246Ala	5033	5.03%
SMO	c.566G>A	p.Ser189Asn	3754	10.07%
NOTCH1	c.5120C>T	p.Ala1707Val	7070	21.19%
NOTCH1	c.4715G>A	p.Gly1572Asp	4258	13.69%

one surgical pathologist (FL) to confirm the tumor content in each section. Ten serial sections (4 μm) were cut from selected tissue areas with tumor tissue were microdissected from those slides using the H&E slides as templates. The pathologic diagnosis of each case was confirmed on routine H&E slides. All samples sent for DNA extraction contained a minimum of 20% DNA derived from tumor cells. The tissues were deparaffinized with 1 mL xylene at 56°C for 10 min, washed with 1 mL 100% ethanol for 5 min at RT, and then dried at 37°C for 10 min. QIAamp DSP DNA FFPE tissue kit (Qiagen, Valencia, CA, USA) was used to extract the genomic DNA from FFPE samples. DNA concentration was determined by Qubit dsDNA HS assay kit on the Qubit Fluorometer according to the manufacturing protocol (Life Technologies, Carlsbad, CA, USA). DNA quality (A260/280 and A260/230) was measured by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Library construction

Sequencing library was prepared by Illumina TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturing protocol. In brief, genomic DNA sample was fragmented into 350 or 550 bp in AFA fiber snapcap microTUBE using Covaris M220 (Covaris, Woburn, MA, USA). End repair and size selection were performed according to the fragment size, followed by 30 end adenylation. Finally, multiple indexing adapters were ligated to the ends of the DNA fragments. Library concentration was determined using Qubit according to the manufacturing protocol. For low DNA input samples, PCR-free library was further amplified with Illumina p5 (AATGATACGGCGACCACCGA) and p7 (CAAGCAGAAGACGGCATACGA) primers in NEB Next High-Fidelity 2XPCR Master Mix (NEB, Ipswich, MA, USA).

Hybrid capture and ultra-deep next generation sequencing

The 5'-biotinylated probe solution is provided as capture probes, the baits target 416 cancer-related genes. 1 μg of each DNA-fragment sequencing library is mixed with 5 μg of human Cot-1 DNA, 5 μg of salmon sperm DNA, and 1 unit adaptor-specific blocker DNA in hybridization buffer, heated for 10 minutes at 95°C, and held for 5 minutes at 65°C in the thermocycler. Within 5 minutes, the capture probes are added to the mixture, and the solution hybridization is performed for 16-18 hours at 65°C. After hybridization is complete, the captured targets are selected by pulling down the biotinylated probe/target hybrids using streptavidin-coated magnetic beads, and off-target library is removed by washing with wash buffer. The PCR master mix is added to directly amplify (6-8

Table 5. Genes mutations detected in hepatocellular carcinoma primary tumor (sample number: 10-13656-1) and its paired bone metastasis (sample number: 12-60901-3)

Number	Mutant genes in HCC primary tumor and bone metastasis	Mutant genes in HCC primary tumor	Mutant genes in HCC bone metastasis
1	ABL1	ALK	AKT1
2	ATM		APC
3	CTNNB1	FGFR3	CDH1
4	EGFR	GNAS	HNF1A
5	ERBB2	KDR	NOTCH1
6	ERBB4	KIT	SMAD4*
7	FLT3	KRAS	VHL*
8	GNA11	SMAD4	
9	GNAQ	SRC	
10	HRAS	STK11	
11	JAK3	VHL	
12	NPM1		
13	NRAS		
14	SMO		
15	TP53		

*, The same mutant genes with different mutation sites in hepatocellular carcinoma primary tumor and bone metastasis.

cycles) the captured library from the washed beads. After amplification, the samples are purified by AMPure XP beads, quantified by qPCR (KAPA Biosystems, Boston, MA, USA) and sized on bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Libraries are normalized to 2.5 nM and pooled. Deep Sequencing is performed on Illumina HiSeq 4000 using PE75 V1 Kit. Cluster generation and sequencing is performed according to manufacturer's protocol.

Sequence alignment and processing

Quality control (QC) was applied with Trimmomatic 1. High quality reads were mapped to the human genome (hg19, GRCh37 Genome Reference Consortium Human Reference 37) using modified BWA aligner 0.7.12 2 with BWA-MEM algorithm and default parameters to create SAM files. Picard 1.119 (http://picard.sourceforge.net/) was used to convert SAM files to compressed BAM files which were then sorted according to chromosome coordinates. The Genome Analysis Toolkit 3 (GATK, version 3.4-0) was modified and used to locally realign the BAMs files at intervals with indel mismatches and recalibrate base quality scores of reads in BAM files.

Validation of SNPs/Indels and CNVs

Single nucleotide variants (SNVs) and short insertions/deletions (indels) were identified using VarScan 2 2.3.9 4 with minimum variant allele frequency threshold set at 0.01 and *p*-value threshold for calling variants set at 0.05 to generate Variant Call Format (VCF) files. All SNVs/indels were annotated with ANNOVAR, and each SNV/indel was manually checked with the Integrative Genomics Viewer (IGV) 5. Copy number variations (CNVs) were identified using ADTEx 1.0.4 6.

Data collection and follow-up

The clinical, laboratory, and radiologic records of the two patients were retrospectively reviewed (**Table 1**). Liver function tests were checked in the patients every three months in order to evaluate hepatic functional reserve. The results of two HCC patients with metachronous bone metastasis were analyzed. Follow-up cross-sectional imaging (contrast-enhanced CT or MRI) was performed one month after treatment. Further treatments were based on clinical evaluation, laboratory values and imaging response. Patients were followed-up every 3 months. The patients were followed up until death or until the date of last follow-up. Follow-up was finished on February 28, 2015.

Results

HCC primary tumor (sample number: 10-13656-1) detected gene mutations

We analyzed fifty molecularly targeted therapy related genes (**Table 2**) to illustrate the genetic heterogeneity in HCC and paired metastasis using a next generation sequencing (NGS) platform as described in Materials and Methods. HCC primary tumor (sample number: 10-13656-1) involves 15 mutant genes, 30 independent mutation sites, and an average mutation frequency of 11.1% (**Table 3**).

HCC bone metastasis (sample number: 12-60901-3) detected gene mutations

HCC bone metastasis (sample number: 1260-901-3) involves 18 mutant genes, 27 independent mutation sites, and an average mutation frequency of 12.34% (**Table 4**).

Table 6. Hepatocellular carcinoma primary tumor (sample number: 10-42240-1) detected gene mutations

Gene symbol	Mutation site	Amino acid change	Sequencing Depth	Mutation frequency
TP53	c.504C>A	p.His168Gln	2566	5.85
TP53	c.501_502insG	p.Gln167_His168fs		8.38
VHL	c.484T>C	p.Cys162Arg	3256	5.04
ERBB2	c.2310A>G		2302	8.95
ERBB2	c.2310A>G	p.Glu770Glu	2302	8.95
ERBB2	c.2498T>C	p.Met833Thr	2937	11.68
FGFR1	c.839-15T>C		1877	9.59
PDGFRA	c.2003G>A	p.Gly668Asp	2752	6.29
PDGFRA	c.2003G>A		2752	6.29
EGFR	c.286G>A	p.Val96Met	4311	11.53
EGFR	c.2184+63delC		1260	14.29
KIT	c.2597T>C		2263	9.68
KIT	c.2596+2T>C		2263	9.68
KDR	c.3496G>A	p.Ala1166Thr	2746	10.31
KDR	c.2962G>A	p.Glu988Lys	2143	27.2
GNAQ	c.650T>C	p.Ile217Thr	10582	7.4
GNAQ	c.496C>T	p.Arg166Cys	2698	13.86
ATM	c.5918+37T>C		2371	18.52
ATM	c.7328G>A	p.Arg2443Gln	7559	14.14
APC	c.2674G>A	p.Glu892Lys	8094	10.49
PTPN11	c.1448C>T		4161	12.62
PTPN11	c.1448-8C>T		4161	12.62
MET	c.2986C>T	p.Pro996Ser	28255	17.48
FGFR2	c.880G>A	p.Val294Met	4164	17.65
SMO	c.654G>A	p.Gln218Gln	4469	6.58
SMO	c.1264+41A>G		2321	69.19
SMO	c.1599C>T	p.Ser533Ser	4612	22.61
ABL1	c.1010T>C	p.Met337Thr	5156	6.92
ABL1	c.1143-44A>T		1067	6.28
PIK3CA	c.2719G>A	p.Ala907Thr	5771	8.14
ERBB4	c.884-23A>T		1163	5.93
ERBB4	c.764G>A	p.Ser255Asn	4417	28.59

Genes mutations detected in HCC primary tumor (sample number: 10-13656-1) and its paired bone metastasis (sample number: 12-60901-3)

HCC primary tumor (sample number: 10-13656-1) and paired bone metastasis (sample number: 12-60901-3) identified 15 common mutant genes. Ten unique mutations in the primary cancer were identified. We found 7 unique mutations in metastatic cancer, including 2 genes, which are different from mutations in the primary mutation. Five new mutations were only found in metastatic site (**Table 5**).

HCC primary tumor (sample number: 10-42240-1) detected gene mutations

HCC primary tumor (sample number: 10-42240-1) involves 19 mutant genes, 32 independent mutation sites, and an average mutation frequency of 13.81% (**Table 6**).

HCC bone metastasis (sample number: 13-39592-3) detected gene mutations

HCC bone metastasis (sample number: 1339592-3) involves 10 mutant genes, 32 independent mutation sites, and an average mutation frequency of 9.19% (**Table 7**).

Genes mutations detected in HCC primary tumor (sample number: 10-42240-1) and its paired bone metastasis (sample number: 13-39592-3)

HCC primary tumor (sample number: 10-42240-1) and paired bone metastasis (sample number: 13-39592-3) identified 21 common mutant genes. Twelve unique mutations in the primary cancer were identified. We found 6 unique mutations in metastatic cancer, including 4 genes, which are different from mutations in the primary mutation. Two new mutations were only found in metastatic site (**Table 8**).

Genes mutations detected in HCC primary tumor (sample number: 10-13656-1; 10-42240-1) and its paired bone metastasis (sample number: 12-60901-3; 13-39592-3)

Of the two HCC primary tumor (sample number 1013656-1; 10-42240-1) identified 18 non common genes; the sample 10-13656-1 has 6 non common genes, and the sample 10-42240-1 number has 12 non common genes (**Table 9**). After analyzing two pairs of HCC primary tumor

Table 7. Hepatocellular carcinoma bone metastasis (sample number: 13-39592-3) detected gene mutations

Gene symbol	Mutation site	Amino acid change	Sequencing Depth	Mutation frequency
PDGFRA	c.1958C>T	p.Pro653Leu	1415	9.47
PDGFRA	c.1960_1961insT	p.His654_Leu655fs	10.22	
PDGFRA	c.1962T>C	p.His654His	1432	10.27
PDGFRA	c.1966A>C	p.Asn656His	1433	9.84
EGFR	c.391G>A	p.Gly131Arg	3743	7.59
EGFR	c.2539A>G	p.Thr847Ala	2582	6.51
KIT	c.222_223insA	p.Thr74_Asn75fs	7.08	
KIT	c.223A>T	p.Asn75Tyr	1080	9.35
KIT	c.226_227insA	p.Glu76_Asn77fs	8.87	
KIT	c.229A>C	p.Asn77His	1108	8.66
KIT	c.230A>G	p.Asn77Ser	1103	9.16
KIT	c.1740C>T	p.His580His	7062	9.8
KDR	c.3405G>A		2008	17.53
KDR	c.3405-1G>A		17.53	
KDR	c.795G>A	p.Ser265Ser	1445	8.37
GNAQ	c.477-30G>A		8.26	
GNAQ	c.477-42G>A		8.58	
PTEN	c.926_927insCC	p.Ala309_Asp310fs	7.36	
PTEN	c.928G>C	p.Asp310His	8209	7.53
PTEN	c.931A>C	p.Asn311His	12188	5.03
APC	c.2697C>T	p.Thr899Thr	6481	7.36
MET	c.3757T>C	p.Tyr1253His	15747	10.48
MET	c.3790C>T	p.Pro1264Ser	15146	7.16
MET	c.3793G>A	p.Val1265Met	15509	12.41
FBXW7	c.1443C>T	p.Ala481Ala	3967	5.14
FBXW7	c.843_844insA	p.Ile281_Ser282fs	8.29	
FBXW7	c.828_832delTCAAC	p.Phe276fs	1710	6.08
FBXW7	c.826T>G	p.Phe276Val	1718	8.56
ERBB4	c.2825C>T	p.Pro942Leu	1901	6
ERBB4	c.1091G>A	p.Gly364Glu	6467	21.97
ERBB4	c.884-20T>C		5.51	
ERBB4	c.831C>T	p.His277His	7092	12.11
			163787	9.19

(sample number 10-13656-1; 1042240-1) and bone metastases (sample number: 12-60901-3; 13-39592-3), we found 13 unique mutations in bone metastases, including 7 genes, which were only different from the mutation sites of primary mutations. Five new mutations were only found in metastatic sites (**Table 9**). This suggests that the importance of genetic mutations after metastasis is declining. If additional sample analysis is added, it will increase the persuasiveness.

Discussion

HCC is the fifth most common cancer in men worldwide [4]. The bone is well known to be the third most frequent site of metastases by all tumors, after the lungs and lymph nodes, and HCC bone colonization has been reported in approximately 20% of patients affected by this tumor [5, 21, 22]. Recently, the progress in both diagnostic modalities and therapeutic procedures, such as surgical resection, radiofrequency ablation, and transcatheter arterial chemoembolization in association with treatments using small molecules as multikinase inhibitors, has prolonged the survival in HCC patients which led to a concurrent worsening of the tumor progression within the skeleton and the formation of bone metastases [5-7, 21]. To date, few data are yet available about bone involvement in patients with HCC, and the nature and the characteristics of bone metastases in HCC have not been fully explored. In recent years, numerous reports have evaluated the genetic heterogeneity in primary tumors and corresponding metastases in a range of solid tumors such as breast cancer, CRC and NSCLC. However, no study has investigated the genetic heterogeneity between HCC and paired bone metastasis. We hypothesize that there would be genetic heterogeneity between HCC and paired bone metastasis.

Recent advances in genomics technologies are now providing new opportunities for the analy-

Table 8. Genes mutations detected in hepatocellular carcinoma primary tumor (sample number: 10-42240-1) and its paired bone metastasis (sample number: 13-39592-3)

Number	Mutant genes in HCC primary tumor and bone metastasis	Mutant genes in HCC primary tumor	Mutant genes in HCC bone metastasis
1	HRAS	GNA11	EGFR*
2	SMARCB1	VHL	KIT*
3	KRAS	ERBB2	KDR*
4	FLT3	FGFR1	MET*
5	CTNNB1	RET	FBXW7
6	GNAQ	EGFR	DEAR
7	PTEN	KIT	
8	ATM	KDR	
9	APC	PTPN11	
10	NRAS	MET	
11	SMO	FGFR2	
12	NPM1	ABL1	
13	PIK3CA		
14	ERBB4		
15	TP53		
16	JAK3		
17	FLT3		
18	PDGFRA		
19	ATM		
20	APC		
21	NOTCH1		

*, The same mutant genes with different mutation sites in hepatocellular carcinoma primary tumor and bone metastasis.

sis of tumor DNA and circulating tumor DNA (ctDNA). The technique of next-generation sequencing (NGS) has been directly applied to tumor DNA and ctDNA analysis, to provide an unprecedented, genome-wide view of somatic chromosomal alterations and copy number aberrations [23, 24]. NGS of ctDNA has been demonstrated to be an effective non-invasive tool for monitoring tumor burden, evolution, therapeutic responses and resistance to therapy [25-30].

This is the first study to investigate the genetic heterogeneity between HCC and paired bone metastasis. In this study, we investigated the genetic heterogeneity in HCC and paired metastasis using a next generation sequencing (NGS) platform to illustrate the molecularly targeted therapy related genes mutations. In this study, after analyzing two pairs of HCC and paired bone metastases, we found a total of 18 mutants involved, mainly due to frameshift and missense mutations. These include important driver mutations in cancer cells, known as cancer driven genes such as EGFR, ALK, KRAS, and PI3KCA. If further samples are added, there will be more evidence for the differential analysis of gene mutation status in hepatocellular carcinoma primary tumor and bone metastases. The different mutations of the functional genes in HCC and paired bone metastasis might result in the therapeutic failure of molecularly targeted therapy for treatment of HCC bone metastasis, which should be demonstrated in our following work.

Conclusion

Our study revealed the different gene mutation status in HCC and paired bone metastases. However, whether the different mutations of the functional genes in HCC and paired metastasis would result in the therapeutic failure of molecularly targeted therapy for treatment of HCC bone metastasis should be demonstrated further.

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Disclosure of conflict of interest

None.

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Table 9. Genes mutations detected in hepatocellular carcinoma primary tumor (sample number: 10-13656-1; 10-42240-1) and its paired bone metastasis (sample number: 12-60901-3; 13-39592-3)

Number	Mutant genes in HCC primary tumor and bone metastasis	Mutant genes in HCC primary tumor	Mutant genes in HCC bone metastasis
1	ABL1: del	ALK	DEAR
2	ATM: del or ins	APC	EGFR*
3	CTNNB1: mis	ATM	FBXW7
4	EGFR: mis or del	FGFR1	KDR*
5	ERBB2: mis	FGFR2	KIT*
6	ERBB4: del or ins	FGFR3	MET*
7	FLT3: del	GNAS	AKT1
8	GNA11: sillent	MET	APC
9	GNAQ: del or ins	NOTCH1	CDH1
10	HRAS: mis	PDGFRA	HNF1A
11	JAK3: mis	PIK3CA	NOTCH1*
12	KDR: ins or mis	PTEN	SMAD4*
13	KIT: del	PTPN11	VHL*
14	KRAS: mis or ins or del	RET	
15	NPM1: del or ins	SMAD4	
16	NRAS: sillent	SMARCB1	
17	SMO: mis	SRC	
18	TP53: mis or ins	STK11	

*, The same mutant genes with different mutation sites in hepatocellular carcinoma (HCC) primary tumor and bone metastasis.

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