

RESEARCH COMMUNICATION

Sex-related Differences in DNA Copy Number Alterations in Hepatitis B Virus-Associated Hepatocellular Carcinoma**Zhong-Zheng Zhu^{1&*}, Dong Wang^{2,3&}, Wen-Ming Cong⁴, Hongmei Jiang⁵, Yue Yu², Bing-Ji Wen¹, Hui Dong⁴, Xiao Zhang², Shu-Fang Liu⁶, Ai-Zhong Wang⁷, Guanshan Zhu⁸, Lifang Hou^{2,9}****Abstract**

Background: Males have a higher prevalence of hepatocellular carcinoma (HCC) than females in general, but the reasons for the sex disparity are still obscure. DNA copy number alteration (CNA) is a major feature of solid tumors including HCC, but whether CNA plays a role in sex-related differences in HCC development has never been evaluated. **Methods:** High-resolution array comparative genomic hybridization (CGH) was used to examine 17 female and 46 male HCC patients with chronic hepatitis B virus (HBV) infection in Shanghai, China. Two-tailed Fisher's exact or χ^2 tests was used to compare CNAs between females and males. **Results:** The overall frequencies and patterns of CNAs in female and male cases were similar. However, female HCC tumors presented more copy number gains compared to those in males on 1q21.3-q22 (76.5% vs. 37.0%, $P = 0.009$), 11q11 (35.3% vs. 0.0%, $P = 0.0002$) and 19q13.31-q13.32 (23.5% vs. 0.0%, $P = 0.004$), and loss on 16p11.2 (35.3% vs. 6.5%, $P = 0.009$). Relative to females, male cases had greater copy number loss on 11q11 (63.0% vs. 17.6%, $P = 0.002$). Further analyses showed that 11q11 gain correlated with 19q13.31-q13.32 gain ($P = 0.042$), 11q11 loss ($P = 0.011$) and 16p11.2 loss ($P = 0.033$), while 1q21.3-q22 gain correlated with 19q13.31-q13.32 gain ($P = 0.046$). **Conclusions:** These findings suggest that CNAs may play a role in sex-related differences in HBV-associated HCC development.

Keywords: Array comparative genomic hybridization - copy number alteration - hepatocellular carcinoma - sex

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide, with 55% of cases occurring in China where hepatitis B viral (HBV) infection is highly prevalent (Parkin et al. 2005). The incidence of HCC is higher in males than in females in general, with male to female ratio ranging between of 2 to 1 and 4 to 1 (El-Serag and Rudolph 2007). Epidemiologic studies have suggested that such a difference might be partly due to higher exposure to HCC risk factors in males than in females such as HBV or hepatitis C viral (HCV) infection, alcohol consumption, and cigarette smoking (El-Serag and Rudolph, 2007). Accumulating evidence indicates that genetic and hormonal factors may also be important in the higher HCC incidence in males (Yu and Chen, 1993; Rudolph et al., 2000; Takemoto et al., 2005; Naugler et al. 2007; Yeh and Chen 2010). Nevertheless,

the exact reasons for the sex disparity in HCC incidence remain largely unknown (Wands, 2007).

DNA copy number alteration (CNA), i.e., gain and loss of either specific genomic regions or even entire chromosomes, is a major feature of solid tumors including HCC (Midorikawa et al., 2007; Minguetz et al., 2009). It has been shown that CNAs play a significant role in the tumorigenesis by copy number-induced alterations in gene expression level and other molecular mechanisms (Kuiper et al. 2010; Thompson et al. 2010). As a powerful technique, comparative genomic hybridization (CGH) allows for the complete copy number analysis of a tumor genome in a single experiment and has been widely used in cancer studies (Pinkel and Albertson, 2005). CGH analyses of HCCs have identified a number of recurrent CNAs and some of these have been associated with specific clinicopathological characteristics (Rashid et al., 1999; Wong et al., 1999; Zondervan et al., 2000;

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Guan et al., 2001; Laurent-Puig et al., 2001). However, whether CNAs play a role in sex-related difference in HCC development has never been evaluated. In the present study, we examined 17 female and 46 male HCC patients in China using high-resolution array CGH to examine sex-related difference in CNAs in HBV-associated HCCs.

Materials and Methods

Subjects

The tumor samples were collected from 63 HBV-associated HCC patients who underwent surgery at Eastern Hepatobiliary Surgery Hospital, Shanghai, China between 2007 and 2008. Patients consisted of 17 females and 46 males with an average age of 50 years (range, 32–72 years). All patients were ethnic Han Chinese, and none had received radiation therapy or chemotherapy before surgery. Final diagnosis of HCC was pathologically confirmed and only patients with samples containing >80% of tumor cells were enrolled. Tissues were frozen in liquid nitrogen within 0.5 h after surgery resection and kept at -80°C until DNA extraction. The study protocol was approved by the Institutional Review Board of the participant hospital, and written informed consent for this study was obtained from all patients.

Information on age, sex, cigarette smoking, alcohol drinking, HCC family history in first-degree relatives, and chronic infection history of HBV/HCV were obtained through in-person interviews at the first hospital admission by trained personnel. Clinicopathological characteristics including serum HBsAg, anti-HBs, anti-HBc of the IgG type, anti-HCV, alpha-fetoprotein (AFP), liver cirrhosis, tumor location, number of tumors, tumor size, Edmondson-Steiner grade, and tumor stage were collected from pathological and medical reports. All patients were positive for chronic HBV infection and negative for chronic HCV infection according to diagnostic criteria of chronic HBV/HCV infection.

Measurement of CNAs using array CGH

Genomic DNA was extracted from the frozen tissue specimens using the Genomic DNA purification Kit (Qiagen, Valencia, CA, USA). Array CGH analysis was performed using the Agilent Human Genome Microarray Kit 244K (Hu-244A, Agilent, Shanghai, China) according to the manufacturer's instructions. The array-CGH platform is a high resolution 60-mer oligonucleotide-based microarray containing 236,381 probes spanning coding and non-coding genomic sequences with median spacing of 7.4 and 16.5 kb, respectively. The probe sequences and gene annotations are based on NCBI Build 35 of the human genome and UCSC version hg17 released in May 2004. The hybridization imaging was scanned on the Agilent G2565BA DNA microarray scanner, and image analysis was performed using the Feature Extraction software version 9.5 (Agilent, Shanghai, China).

Circular binary segmentation (Olshen et al., 2004; Venkatraman and Olshen, 2007) was applied to the log₂ ratios to divide the genome into regions with the same copy number level while adjacent regions have different copy numbers. Segmentation was performed for each

Table 1. Demographic and Clinicopathological Characteristics According to Sex

Variables	Females	Males	P value
Age (years), mean (SD)	48.2(10.0)	50.6(10.4)	0.403
Cigarette smoking			
No	17(100.0)	25(54.3)	0.001
Yes	0(0.0)	21(45.7)	
Alcohol drinking			
No	16(94.1)	30(65.2)	0.026
Yes	1(5.9)	16(34.8)	
HCC family history in first-degree relatives			
No	15(88.2)	42(91.3)	0.657
Yes	2(11.8)	4(8.7)	
AFP ($\mu\text{g/L}$)			
≤ 20	3(17.6)	15(32.6)	0.350
> 20	14(82.4)	31(67.4)	
Cirrhosis			
No	9(52.9)	16(34.8)	0.249
Yes	8(47.1)	30(65.2)	
Tumor location			
Left	2(11.8)	13(28.2)	0.430
Right	13(76.4)	28(60.9)	
Left and right	2(11.8)	5(10.9)	
Number of tumors			
Single	14(82.4)	33(71.7)	0.522
Multiple	3(17.6)	13(28.3)	
Tumor diameter (cm), mean (SD)	8.3(4.2)	6.7(4.6)	0.221
Tumor grade [†]			
II	3(17.6)	10(21.7)	0.722
III	14(82.4)	36(78.3)	
Tumor stage [‡]			
T1	5(29.4)	21(45.6)	0.412
T2	5(29.4)	8(17.4)	
T3	6(35.3)	16(34.8)	
T4	1 (5.9)	1 (2.2)	

[†]According to the Edmondson-Steiner grading system; [‡]Tumor stage corresponds to the T (tumor) description according to the TNM (tumor-nodes-metastasis) classification (the Six Edition)

of the multiple samples independently and separately. For each region, the segmented log₂-ratio was assigned to be the average log₂ ratios of the contained probes in that region. The R package DNACopy was used for the segmentation. Data from probes mapped to sex chromosomes X and Y were eliminated. A total of 3,500 regions were generated from this analysis. An absolute segmented log₂ ratio of >0.5 was used as the threshold for the gain or loss in DNA copy numbers for each region.

Statistical analysis

Two-tailed Fisher's exact test or χ^2 test, as appropriate, was used to compare the prevalence for each CNA of the 3,500 regions between females and males. Gains were tested against no gain and losses were tested against no loss. All of the tests were two-tailed and a P value of <0.01 was considered statistically significant to reduce the effect of multiple testing in this large dataset. Differences between sex groups in age, cigarette smoking, alcohol drinking, HCC family history, serum AFP, cirrhosis, tumor location, number of tumors, tumor size, Edmondson-Steiner grade, and tumor stage were evaluated using Fisher's exact test, χ^2 test or Student's t-test. A P value of <0.05 was considered statistically significant. Statistical

Table 2. Chromosomal Regions of Gains and Losses Present Differently in Female and Male HCC Tumors

Cytoband†	Map position (start – end) †	Size (bp)	No. (%) in females (n = 17)	No. (%) in males (n = 46)	P value
Gain					
1q21.3-q22	148 146 197 – 152 934 820	4 788 624	13 (76.5)	17 (37.0)	0.009
11q11	55 124 730 – 55 195,049	70 320	6 (35.3)	0 (0.0)	0.0002
19q13.31-q13.32	49 842 066 – 50 295 822	453 757	4 (23.5)	0 (0.0)	0.004
Loss					
11q11	55 124 730 – 55 195 049	70 320	3 (17.6)	29 (63.0)	0.002
16p11.2	34 369 010 – 34 505 579	136 570	6 (35.3)	3 (6.5)	0.009

†Cytoband and map position are based on the public UCSC database [Human Genome Browser, May 2004 Assembly (hg 17)]

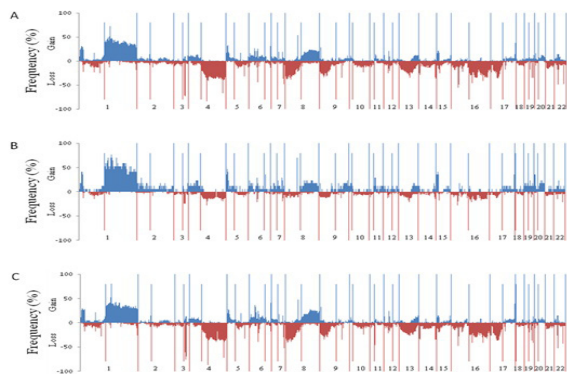


Figure 1. Genome-wide Frequencies of Copy Number Alterations in all 63 Cases of HCC (A), in 17 Female Cases (B), and in 46 Male Cases (C). The genome-wide frequencies of all gains (blue, top plot) and losses (dark red, bottom plot) for each test region are shown. A total of 3500 test regions were ordered (x-axis) according to the map positions and the chromosome order from 1pter to 22qter. The boundaries of individual chromosome and location of centromere are indicated by long and short vertical bars, respectively

analyses were conducted using the Stata 10.1 (Stata Corporation, College Station, TX).

Results

Table 1 shows the demographic and clinicopathological characteristics of the 63 HCC cases according to sex. No significant differences between female and male cases were found in the distributions of age, HCC family history, serum AFP, cirrhosis, tumor location, number of tumors, tumor size, Edmondson-Steiner grade, or tumor stage. Compared with females, more males were cigarette smoker ($P = 0.001$) and alcohol drinker ($P = 0.026$).

The overall pattern of CNAs in the 63 HBV-HCCs examined is shown in Figure 1A. The observed frequent CNAs, such as gain on 1q and 8q and loss on 1p, 4q, 8p, 9p, 10q, 13q, 16q, and 17p, were consistent with previous reports using CGH in HCC (Marchio et al., 1997; Terracciano and Tornillo, 2003; Moinzadeh et al., 2005; Baudis, 2007). Similar overall patterns of CNAs between female and male HCCs were observed (Figure 1B and 1C, respectively). By comparing the prevalence of the 3,500 test regions between females and males, however, sex-related difference of CNAs in tumor tissue DNA was revealed (Table 2). Compared with the male, female HCC patients had more frequent copy number gains on 1q21.3-q22 (76.5% vs. 37.0%, $P = 0.009$), 11q11 (35.3%

vs. 0.0%, $P = 0.0002$) and 19q13.31-q13.3 (23.5% vs. 0.0%, $P = 0.004$), and loss on 16p11.2 (35.3% vs. 6.5%, $P = 0.009$). However, the DNA copy number loss on 11q11 was more frequent in male than in female (63.0% vs. 17.6%, $P = 0.002$).

The potential associations between the sex-related CNAs, including gain on 1q21.3-q22, 11q11 and 19q13.31-q13.32, and loss on 11q11 and 16p11.2, were evaluated to determine if these CNAs presented concordantly in the set of HCC cases. The results showed that 11q11 gain correlated with 19q13.31-q13.32 gain ($P = 0.042$), 11q11 loss ($P = 0.011$) and 16p11.2 loss ($P = 0.033$). Another significant correlation was observed between 1q21.3-q22 gain and 19q13.31-q13.32 gain ($P = 0.046$).

Discussion

In the present study, we demonstrated for the first time that, although the overall CNAs profile were similar between female and male HBV-HCC patients, there exist a significantly different CNAs pattern between these two groups of HCC tumors. In particular, we observed that gains on 1q21.3-q22, 11q11 and 19q13.31-q13.32 and loss on 16p11.2 were more frequently presented in female HCC tumors, while loss on 11q11 was more frequently presented in male HCC tumors.

Chromosome 1q gain is one of the most common CNAs in HCC. It has been reported that 1q gain can be identified in dysplastic nodules (DNs), the premalignant precursor of HCC (Terris et al., 1997; Tornillo et al., 2002; Raidl et al., 2004). Moreover, in an explorative CGH meta-analysis of HCC by Moinzadeh et al. (2005), 1q gain has been demonstrated to be positively correlated with almost all other high-frequency CNAs. These findings suggest that 1q gain may predispose to chromosomal alterations and thus is one of the early genomic events associated with development of HCC. Several studies have further refined 1q gain in HCCs to the 1q21–q22 region (Wong et al., 2003; Midorikawa et al., 2004). In the present study, we observed that gain on 1q21.3-q22 was more frequently presented in female than in male HCC patients. In the 4.79 Mb region within 1q21.3-q22, many cancer-related genes are contained. Four growth-related genes, JTB, HAX-1, SHC1, and CKS1B were shown to be highly expressed in HCC compared to the non-cancerous tissues (Midorikawa et al. 2004; Wong et al. 2003). By correlating expression arrays and array CGH data, a panel of up-regulated genes were located in the 1q21.3-q22

gain region, including HAX1, SNX27, ILF2, UBAP2L, UBE2Q1, PYGO2, FLAD1, FDPS, and DAP3 (Lee et al., 2008; Woo et al., 2009). Additionally, MUC1 oncoprotein has been reported to stabilize and activate estrogen receptor alpha and to contribute to 17beta-estradiol-mediated growth and survival of breast cancer cells (Wei et al., 2006). Further studies are warranted to clarify whether these cancer-related genes play a role in the development of HCC, and, if so, how they might differently affect sex-related HCC development. The chromosomal gain of the 454 Kb window within 19q13.31-q13.32 was also more frequently presented in female HCC cases than in male HCC patients in the present study. Cancer-related genes, such as BCL3, BCAM, and RELB (Drewniok et al., 2004; Maldonado et al., 2010; Slovak et al., 2011) are located in this region.

One of the interesting findings in the present study is on 11q11. We observed that 11q11 loss was more frequently presented in HCC tumors from male cases, while 11q11 gain was limited to female patients. It has been reported that both gain and loss on 11q occurred frequently in DNAs, indicating 11q copy number change is a early genetic event for hepatocarcinogenesis (Raidl et al. 2004). However, our cytoband and map position analysis showed that only four olfactory receptor genes, namely OR4C11, OR4P4, OR4S2, and OR4C6, locate in this 70 Kb window within 11q11. Thus, the sex-related difference in 11q11 copy number changes in HCC tumor may be due to the coexistence with other CNAs which virtually play a role in sex-related HCC development. In line with this hypothesis, our data demonstrated that 11q11 loss significantly correlated with 19q13.31-q13.32 gain. The chromosomal loss of the 137 Kb window within 16p11.2, which was more frequently presented in female HCC tumors, contains no known gene. Thus, coexistence of the loss of 16p11.2 with other identified and unidentified CNAs may explain its sex-related differential presentation in HCC.

The array CGH detection in the present study was based on the high resolution Agilent 244K microarray system, which is capable of producing reliable detection of unbalanced DNA abnormalities and precise identification of small CNAs in a genome (Barrett et al., 2004). Association analyses were performed on the test regions defined on segmentation patterns across the samples instead of individual probes, which reduced the number of association tests and improved statistical power. Several limitations of our study should be noted. Firstly, the number of samples (n = 63) is relatively small and thus significant findings by chance cannot be excluded. Further studies using larger sets of samples are needed to verify our findings. Secondly, our study included only HBV-associated HCC cases, which might make the results less applicable to HCC in general since CNA patterns have been shown to be distinct in HCCs of differing etiologies (Wong et al., 2000; Moinzadeh et al., 2005). Thirdly, all study subjects enrolled were Chinese, therefore our results may not apply to other ethnicities. Finally, given that all cigarette smokers were male in our dataset and that smoking-related CNAs patterns have been reported in tumor DNA of lung tumors (Sy et al., 2003; Massion et

al., 2008), it cannot be excluded that the sex-related CNAs that we observed may partly or merely reflect the smoking-related genomic signatures in this study population.

In summary, our results suggest that CNAs may play a role in sex-related difference in HBV-HCC development. The sex-related CNAs identified in the present study may help map the key genes that contribute differently to HCC development in females and males.

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