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Original Paper

Loss of Heterozygosity at Chromosome 13q in Hepatocellular Carcinoma: Identification of Three Independent Regions

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Loss of heterozygosity (LOH) on chromosome 13q is one of the most common genetic alterations in hepatocellular carcinoma (HCC) and might be involved in liver cancer development through inactivation of tumour suppressor genes. In order to narrow down the region of 13q loss, we examined the pattern of loss of heterozygosity (LOH) in tumours from 88 HCC patients, using 18 microsatellite markers on 13q. Thirty-eight of the 88 tumours (43%) showed LOH for at least one marker. Of these, two tumours (5%) showed 13q whole arm allelic loss, while the remaining 36 tumours (95%) had partial allelic loss. The LOH pattern defined by the 36 tumours suggested the existence of at least three different smallest common deleted regions which might be involved in the carcinogenesis of HCC. The first, the most centromeric in the 13q12.3 is, close to the *BRCA2* gene, defined by *D13S171*; the second, the most telomeric region in the 13q31-32 band, is defined by *D13S154* and *D13S157*; the third, the intermediate region at 13q14.3, which is near the *RB* gene, is defined by loci *D13S268*. The rate of LOH at 13q31-32 was significantly higher in Hepatitis B-surface antigen (HBsAg)-positive patients than HBsAg-negative HCC patients, pointing to a candidate gene related to the development of HBsAg-positive HCCs.

Key words: loss of heterozygosity, hepatocellular carcinoma, chromosome 13q
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INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the leading malignancies in the world [1]. In Taiwan, it ranks highest in terms of cancer mortality [2, 3]. Different aetiological factors such as hepatitis viral infection, dietary aflatoxins or chemical carcinogens are associated with the development of liver cancer [2–5]. Nevertheless, the molecular mechanism involved remains to be clarified.

Recent studies have indicated that in HCC, frequent aberrations are present in several genomic regions [6–23]. It has been suggested that accumulation of these genetic changes, which affect the expression of oncogenes and tumour suppressor genes, occur in a stepwise manner during the development and progression of HCC. There are two candidate genes at 13q that might have a role in the development of HCC [15, 16, 22, 23]. One of the candidate genes is the *RB* gene, which is located at 13q14. Inactivation of this gene has been shown in the carcinogenesis of many human cancers [15, 24–26]. However, Kim and colleagues found that although loss of heterozygosity (LOH) on 13q was found in approximately 50% of high grade primary ovarian carcinomas, most of the tumours showed normal RB nuclear protein staining patterns [23]. They therefore suggested that the *RB* gene is probably not the target of the frequent allelic losses on chromosome 13q in this cancer. Zhang and colleagues also reported infrequent somatic mutations of the *RB* gene in HCCs although nearly half of the tumour cells lacked pRb [15]. Recently, a second gene, *BRCA2*, responsible for familial breast cancer, was localised to 13q12-13 after linkage analysis of families carrying hereditary breast cancer [27, 28]. These results indicated the presence of a putative tumour suppressor gene, other than *RB*, on chromosome 13q.

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In our previous report, we detected (by comparative genomic hybridisation) frequent DNA loss (20%) in the chromosomal region 13q [29]. To identify the putative tumour suppressor genes on 13q, we performed deletion mapping in 88 HCCs using highly polymorphic microsatellite markers. Here, we present evidence that LOH occurs in three regions of this chromosomal arm.

PATIENTS AND METHODS

Patients

Primary HCC tissues and their corresponding non-neoplastic liver tissues were obtained from 88 HCC patients receiving surgical resection at the National Taiwan University. The diagnosis of HCC was confirmed by histology. Both tumour and non-tumour parts were frozen immediately after surgery and stored at -135°C until use. A total of 88 tumours were studied. The tumour size was <3 cm in 81 tumours (92%) and between 3 and 5 cm in seven tumours (8%). 71 patients were males (81%) and 17 were females (19%). 47 (53%) were positive for hepatitis B surface antigen (HBsAg), and 41 (47%) were negative. Hepatitis C virus antibody (anti-HCV) was positive in 4 of the 47 patients (9%) who were positive for HBsAg. However, in the 41 patients negative for HBsAg, 32 patients (78%) were positive for anti-HCV and 9 (22%) were negative for anti-HCV.

DNA isolation

To avoid contamination, cryosections of 5–7 μm were prepared from the tumour and non-tumour liver tissues, and setions containing predominantly neoplastic cells (>90%) were used for the extraction of genomic DNAs by conventional procedures [5].

Table 1. *Loss of heterozygosity at* 18 *microsatellite loci on chromosome* 13*q in HCCs*

	Locus symbol	Chromosomal location	Frequency of LOH (%) (LOH/informative cases)
centromere ↑			
	D13S260	13q12.3	11/73 (15)
	D13S171	13q12.3	11/59 (19)
BRCA2 gene →			
	D13S267	13q12.3	10/56 (18)
	D13S220	13q12.3	6/29 (21)
	D13S218	13q13.1-14.1	9/56 (16)
	D13S328	13q14.2	7/62 (11)
	D13S168	13q14.3	14/68 (21)
	D13S268	13q14.3	15/66 (23)
	D13S155	13q14.3-21.1	11/60 (18)
RB1 gene →			
	D13S153	13q14.1-14.3	10/84 (12)
	D13S176	13q14.3	7/43 (16)
	D13S276	13q21.2	5/50 (10)
	D13S279	13q21.3	6/41 (15)
	D13S156	13q21.3	10/75 (13)
	D13S269	13q21.3	5/47 (11)
	D13S157	13q31	14/58 (24)
	D13S154	13q31-32	12/78 (15)
	D13S174	13q32.3	9/70 (13)
telomere ↓			

BRCA2 gene is located between *D13S171* and *D13S267*. Intron 2 of *RB1* gene is contained on *D13S153*.

Microsatellite polymorphism analysis

In this study, we used 18 microsatellite markers mapped on the long arm of chromosome 13 for the mapping of putative suppressor genes on 13q. These markers are shown in Table 1. These primers covered two candidate loci for human tumour suppressor genes, *RB1* and *BRCA2*, on the long arm of chromosome 13. The *BRCA 2* gene is located between *D13S260* and *D13S267*. Intron 2 of the *RB1* gene is contained on *D13S153*. All primers were obtained from Research Genetics (Huntsville, Alabama, U.S.A.). Short tandem repeats were amplified by polymerase chain reaction (PCR). All these samples including tumour and non-tumour part were systematically studied by using the microsatellite markers (Table 1) [30]. A total of 18 microsatellite markers were used. Each marker was amplified by PCR in 25 μl volumes of a mixture containing 25 ng of genomic DNA template, 5 pmol of each primer, 75 μM of each deoxyguanosine triphosphate, deoxythymidine triphosphate, and deoxycytidine triphosphate, 7.5 μM deoxyadenosine triphosphate, 1.5 mM magnesium, 1X PCR buffer, 2.5 μCi of [^{33}P]dATP 5U/μl and 0.5U Taq polymerase. The PCR was performed in a thermocycler which can fit a 96-well microtitre plate (PTC-100-96V, MJ Research Inc., Watertown, Massachussetts, U.S.A.). Reactions were performed for 27 cycles under the following conditions: 30 sec at 94°C for denaturation, 75 sec at 55°C for primer annealing and 30 sec at 72°C for primers extension. Finally, PCR product was further incubated in 72°C for a further 6 min. The pipetting and dispensing of the PCR reagents was performed by a Robotic workstation (Biomek 2000, Beckman, Palo Alto, U.S.A.). After completion of PCR, the PCR products were run on a 6% polyacrylamide gel electrophoresis (PAGE) gel, followed by autoradiography. The signal intensity of each allele amplified from tumour DNA was compared with that from the corresponding normal counterpart DNA. Representative examples of autoradiograms showing LOH are illustrated in Figure 1. Absence of or decreased intensity of one allele in the tumour sample was defined as LOH. The assessment of LOH was carried out as previously described [6, 7, 22].

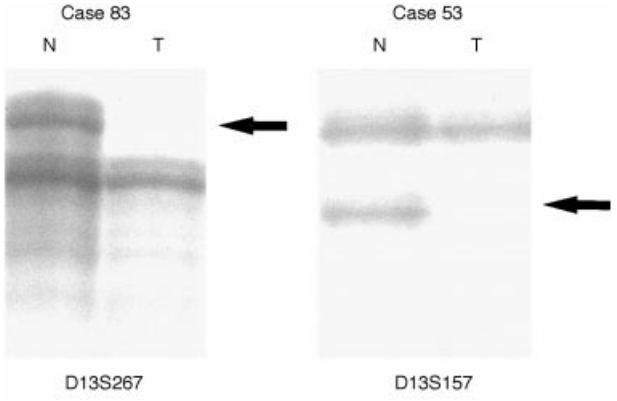

Figure 1. Representative results of loss of heterozygosity observed with 13q microsatellite markers in hepatocellular carcinoma. N and T, matched DNA samples isolated from non-tumour and tumour tissues, respectively. Two tumours with distinct patterns of 13q LOH are depicted: arrows, lost alleles. Case 83 showed LOH at markers *D13S267* and case 53 had evidence of LOH at *D13S157*.

Table 2. Loss of heterozygosity on chromosome 13q31-32 and status of HBsAg in HCC patients

	Status of HBsAg	
	Group A HBsAg(−)	Group B HBsAg(+)
LOH at 13q31-32	4	16*
No LOH at 13q31-32	37	31

*$P = 0.02$.

Statistical analysis

Analysis was carried out to try to correlate the LOH frequency with several clinical aspects, including sex, age, tumour size and status of hepatitis B or C virus infection. The statistical analysis was performed by the computer program STATISTICA (StatSoft, Tulsa, Oklahoma, U.S.A.). *P* values were obtained using Fisher's exact test.

RESULTS

Tumour DNA and corresponding non-tumour DNA from 88 patients with HCC was analysed, using 18 polymorphic markers for the long arm of chromosome 13. All patients were informative for seven or more loci on 13q. The results showed that a high frequency of LOH was observed at *D13S157* (24%), *D13S268* (23%), *D13S220* (21%), and *D13S168* (21%) (Table 1). Examples of LOH at two loci are shown in Figure 1. LOH on 13q for at least one locus was found in 38 of the 88 tumour DNAs (43%). Of these 38 tumours which had alterations on 13q, 2 (5%) showed LOH at almost all of the informative loci tested on the long arm of chromosome 13, whereas the other 36 (95%) showed partial (interstitial and/or telomeric) alterations on 13q (Table 2). The LOH pattern defined by the 36 tumours with partial deletions did not clearly point to a single common region, but suggested the existence of at least three different smallest common deleted regions (Figure 2).

Mapping of the non-contiguous regions of LOH

The deletion mapping of the chromosome 13q of 36 HCCs with LOH was shown in Figure 2. The first, the most centromeric region was defined by samples T24 and T25 which showed loss of a common region at 13q12.3 between *D13S260* and *D13S267*. Two tumours (T5 and T84) lost a single locus at *D13S171*, but showed allelic retention at *D13S260* and *D13S267* (Figure 2). This region is within the region defined by T24 and T25, meaning that these results refine this LOH region and suggest that the first smallest common deleted region (SCDR1) is between *D13S267* and *D13S260*. Within this region lies the locus *D13S171*, which had disappeared from 11 (44%) of the 25 informative tumours with partial 13q alteration. The high frequency of LOH involving this locus and the retention of adjacent loci suggest *D13S171* may be a central part of a deletion region.

The second, most distal region of LOH was defined by a region between loci *D13S157* (13q31) and *D13S154* (13q32) by samples T22, T30, and T41 (Figure 2). Nineteen (53%) of the 36 informative tumours with partially 13q alteration showed LOH between *D13S157* and *D13S154*. Because of the high frequency of LOH involving these loci, we mapped the SCDR2 between *D13S157* (13q31) and *D13S154* (13q32).

The third, intermediate region of loss was defined by tumour T37 and T45 between *D13S268* (13q14.3) and *D13S168* (13q14.3) (Figure 2). Nine tumours (T9, T13, T26, T44, T49, T63, T68, T69, and T84 (33%)) showed LOH at a single locus, *D13S268*, but showed allelic retention at *D13S168*. Fourteen (52%) of the 27 informative tumours

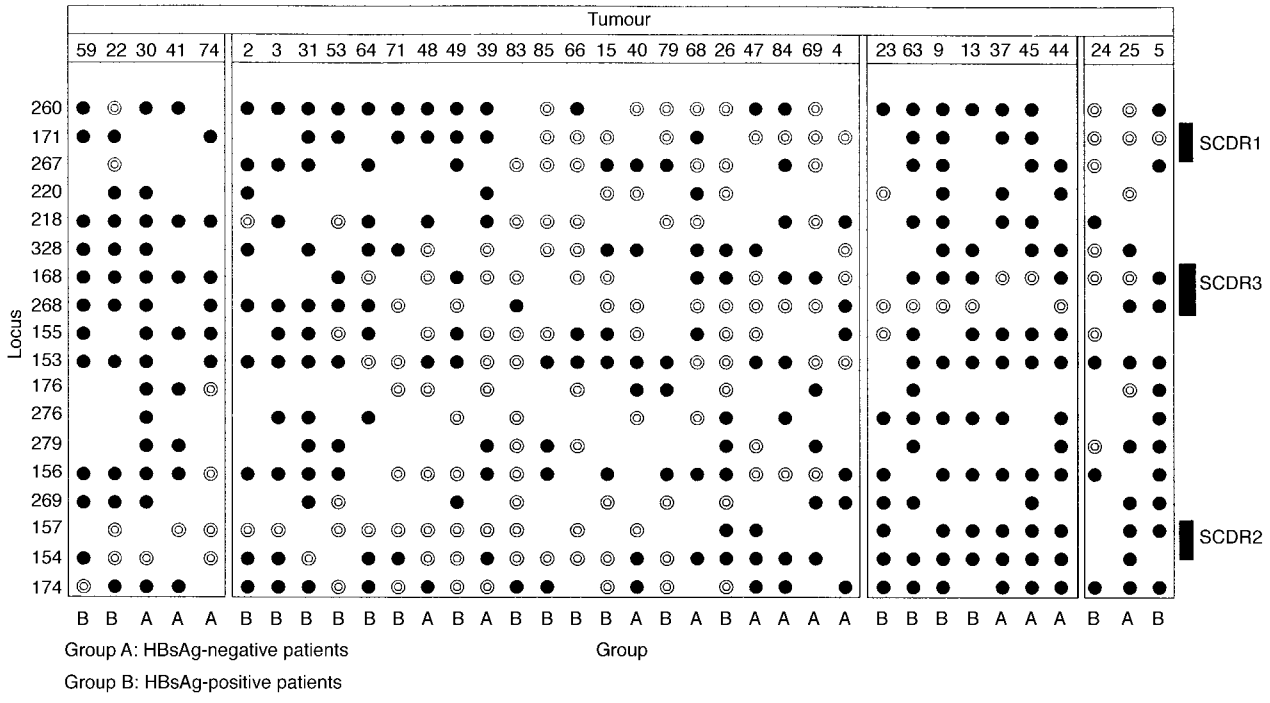

Figure 2. A detailed deletion mapping of chromosome 13q of 36 cases of HCC with LOH at one or more loci. The case number is shown above each column and 18 microsatellite markers are indicated on the left. SCDRs (smallest common deleted regions) are shown on the right. ●, heterozygous (informative loci), ⊙, LOH; blank, homozygous (uninformative loci).

with partially 13q alteration showed LOH at locus *D13S268*. These data demonstrated that the third SCDR3 was mapped to *D13S268* (13q14.3).

On the basis of published sex-averaged framework maps [30], the estimated size of the three distinct commonly deleted regions are 4 cM (SCDR1, 13q12.3), 10 cM (SCDR2, 13q31-32), and 1 cM (SCDR3, 13q14.3).

In this study, 6 cases showed three deletion areas including SCDR1, SCDR2 and SCDR3; 8 cases showed two deletion regions including SCDR1 and SCDR3; 5 cases showed deletion at SCDR2 and SCDR3; 8 cases showed deletion at SCDR2; 7 cases showed deletion at SCDR3; and only one case showed deletion at SCDR1 (Figure 2).

Correlation of LOH on 13q with clinical data

LOH at one or more loci on chromosome arm 13q was analysed according to clinical aspects, including sex, age, tumour size, liver state, serum α-fetoprotein (AFP) and status of hepatitis B or C virus infection. 23 (49%) of the 47 informative patients with HBsAg positivity showed LOH of one or several loci on 13q, whereas LOH was observed in 13 (32%) of the 41 informative patients with HBsAg negativity. Although a trend was found toward a higher frequency of LOH in HBsAg-positive patients than HBsAg-negative patients, these results were not statistically significant.

The LOH frequency of each locus and SCDR was also examined with respect to clinical parameters. 16 of the 47 (34%) HBsAg-positive HCC patients had LOH at SCDR2 (13q31-32), while only 4 of the 41 (9.8%) HBsAg-negative HCC patients did. A significant relationship ($P = 0.02$) was found between HBsAg-positivity and LOH within the SCDR2 (13q31-32) (Table 2). However, no significant difference between HBsAg positivity and LOH frequency of SCDR1 and SCDR3 was noted. No significant correlation was found between these three SCDRs and other clinical parameters.

DISCUSSION

Although LOH involving 13q are known to occur in HCC [16, 17, 22], precise deleted regions have not been clearly defined. In this study, LOH on 13q was found in 38 of the 88 tumour DNAs (43%). Of the 38 tumours which had alteration on 13q, two tumours (5%) showed LOH of the almost entire long arm of chromosome 13. We also found that high percent (36 of 38: 95%) of tumours had partial deletion on 13q. Furthermore, six of the 36 tumours (18%) had allelic losses in all three LOH regions and 13 tumours (38%) had allelic losses in two of these three LOH regions. These data further support the presence of tumour suppressor genes on the three common regions which might be important in hepatocarcinogenesis.

By using microsatellite polymorphism analysis, Nagai and colleagues identified three regions of common deletion at 13q12-q13, 13q14, and 13q21-32 on chromosome arm 13q [22]. Our data are consistent with their report but provide more accurate and more detailed LOH regions [22]. Nevertheless, our data showed a lower frequency of LOH than previous report by Nagai and colleagues [22], the discrepancy is probably a reflection of differences is tumour size or geographical differences in the two studies.

Our results showed that the SCDR1 region was at 13q12.3 (*D13S171*). The existence of this LOH region on 13q, near 13q12-13, has been reported in HCC and other tissues [16, 20, 31]. Current genetic mapping information suggests that the *BRCA2* gene responsible for familial breast cancer, is mapped to 13q12-13 (between *D13S171* and *D13S267*) [16, 32]. It is possible that *BRCA2* is the candidate tumour suppressor gene for HCC in this area and the role of *BRCA2* in HCC, therefore, needs further investigation.

A second defined LOH region was mapped to 13q31-32, a region of deletion between *D13S154* and *D13S157*. However, to the best of our knowledge, no candidate tumour suppressor genes have been published in this area. The significance of LOH in this area in HCC, therefore, remains to be clarified.

The third smallest deleted region of deletion lies at 13q14.3 between *D13S268* and *D13S168*. A candidate gene in this region (13q14) is *RB*, which is implicated in the development of retinoblastoma and is a well documented tumour suppressor gene [15, 24—26]. Intron 2 of *RB* is contained on the *D13S153* microsatellite [16, 31]. However, the low frequency (12%, 10/84) of LOH at *D13S153* in this study suggests the *RB* gene might not be the candidate tumour suppressor gene in this region in HCC. However we did not analyse whether the *RB* gene is altered or not in HCC in Taiwan. Therefore, we could not rule out the possibility that *RB* is involved in HCC. Nevertheless, according to our data, it is likely that other tumour suppressor genes on 13q might be present in this region.

LOHs on 13q are not restricted to HCCs but have also been observed in a variety of human cancers including breast cancer, ovarian cancer and oral cancer [23, 31, 32]. Wingren and colleagues reported the presence of three similar LOH regions in breast cancers, suggesting that the putative tumour suppressor genes at these locations might be involved in different human cancers [31].

A significant relationship ($P = 0.02$) was found between HBsAg positivity and LOH within the SCDR2 (13q31-32). These results imply that HBV infection might have caused some genetic changes within chromosome 13q31-32, which could be related to the development of HBsAg-positive HCCs. However, the precise role of this putative tumour suppressor gene, as well as those in the other SCDRs in HCC development remain to be determined. Studies will be focused on the refinement of SCDRs and identification of the putative tumour suppressor genes, together with their role in hepatocarcinogenesis.

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