Frequent genomic imbalances suggest commonly altered tumour genes in human hepatocarcinogenesis

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Summary Hepatocellular carcinoma (HCC) is one of the most frequent-occurring malignant tumours worldwide, but molecular changes of altered tumour genes in human hepatocarcinogenesis

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representative 2 μm sections obtained from formalin-fixed, paraffin-embedded tissues. Tissues used for DNA extraction and subsequent CGH analyses were especially controlled for containing more than 90% tumour tissue and the lack of significant necroses or preservation artifacts by histological analysis of corresponding frozen sections. Grading of HCCs was performed as recommended by the World Health Organization (WHO) UICC (Ishak et al, 1994).

Cell lines HEP-3B, HEP-G2, and SK-HEP1 were purchased directly from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were stored and cultured as suggested by the supplier.

DNA-extraction and CGH

Genomic DNA was obtained from frozen tissues (tumours), cell lines or peripheral blood cells of normal persons (reference DNA) by phenol/chloroform extraction according to a standard protocol (Sambrook et al, 1989). The quality of genomic DNA was controlled by submitting unrestricted aliquots to 1% agarose gel electrophoresis prior to the labelling reaction.

For chromosomal metaphase preparations, peripheral blood lymphocytes were obtained from normal persons, cultured and stimulated with phytohaemagglutinin (1.5%; Gibco BRL, Eggenstein, Germany) in McCoy’s 5A medium (Gibco BRL). After culturing for 72 h, metaphase arrest was performed with colcemid (0.3 μg/ml; Gibco BRL). All further preparations were performed according to a standard protocol (Vogel and Speit, 1985).

CGH was performed according to Lichter et al (1994) with slight modifications. Slides containing the prepared metaphases were rehydrated in graded ethanol, 0.1 × standard saline citrate (SSC), and 2 × SSC, subsequently heat stabilized in 2 × SSC at 75°C for 30 min, denatured in 0.07 N NaOH and dehydrated in graded ethanol. Test and reference DNAs were labelled by nick translation (CGH-nick translation kit; Vysis, Downers Grove, IL, USA). Hybridization on chromosomal metaphase preparations was carried out at 37°C (low stringency) or 55°C (high stringency) for 3 d. After the hybridization reaction the slides were washed in 2 × SSC and 2 × SSC Tween 20 at room temperature and 1 × SSC at 75°C.

The ratio profiles of each individual chromosome were determined using a cytovision 3.1 software (Applied Imaging, Sunderland, UK). A FITC/TRITC ratio ranging from 0.75–1.25 indicated balanced genomic material (Du Manoir et al, 1995). In most cases the complete hybridization reaction and analyses were performed twice, under low (37°C), and high stringency (55°C) hybridization conditions, and only those imbalances of genomic material reproducible under both conditions were accepted. Chromosomal regions showing significant frequencies of genomic alterations were searched for potential tumour-relevant genes using the OMIM-NCBI database (www.ncbi.nlm.nih.gov/omim/searchmap.html), which is currently estimated to contain more than half of the expected human genes annotated by chromosomal localization (Deloukas et al, 1998). The current analysis focussed on chromosomal regions that were frequently altered in the CGH analysis. These chromosomal regions that were screened for those genes that either had an already established or suspected function in hepatocarcinogenesis or a known oncogenic or tumour-suppressive function, matched to the respective type of genomic imbalance and were likely to act in carcinoma cells (Figure 1).

RESULTS

All HCCs that were analysed met the histological pre-evaluation criteria and resulted in well-interpretable metaphases and CGH ratio profiles. In all HCCs and cell lines with the exception of 3 HCCs, significant genomic imbalances were detected (Table 1). All cell lines showed a rather high number of genomic imbalances. In tumour No. 6, the CGH ratio profiles showed a gain of 1q and an amplification of 8q. In FISH analyses of isolated interphase HCC nuclei with centromeric probes from both chromosomes, significant numerical over-representations of the hybridization signals for each of the test probes were detectable compared to the reference probes (Figure 1A and B, Table 2).

Overall gains of genomic material outnumbered losses in frequency (Table 1). When the genomic imbalances were sorted by chromosomal location (Figure 1, Table 1B), gains were most frequently detected in: 1q (57%, core region: 1q21-24), 8q (52%, core region: 8q24.1-24.3), 17q (29%) and 20q (29%). Losses were

Interphase fluorescence in situ hybridization (FISH)

Interphase FISH was performed in selected representative cases (3) with a limited number of frequently occurring genomic imbalances in order to confirm the results of the CGH analysis. Isolated interphase nuclei were obtained according to Hopman et al (1992) except that fixation was performed in methanol/acetic acid (3:1). Hybridization reactions were performed with CEP- and LSI-DNA FISH probes (Vysis). Fluorescence microscopy and photomicroscopy were performed with a Leica DM-RBE microscope (Leica, Bensheim, Germany). Hybridization was carried out simultaneously with a test probe directed against a chromosomal region showing genomic imbalance (1q: 1q12 (sat. II and III); 8: 8p11.1-q11.1 (α-sat. DNA); 13q: 13q14 (RB-1 locus)) and a reference probe derived from a region without significant alteration according to the respective CGH ratio profile (10p11.1-q11.1 (α-sat. DNA); 17p11.1-q11 (α-sat. DNA)). In each analysis more than 100 interphase nuclei were evaluated by fluorescence microscopy. The number of signals per nucleus were counted for each probe. Then the ratio of test probe signal/reference probe signal was determined for each signal interphase nucleus. The nuclei were then grouped according to this signal ratio.

cDNA-arrays

cDNA expression analysis was performed in tumour No. 6 containing the frequently occurring gains of genomic material at 1q and 8q (as well as X) in order to test in an unbiased manner whether the genomic imbalances were associated with alterations in expression of genes located in these chromosomal regions. Atlas Human cDNA Expression Arrays (Clontech, Palo Alto, CA, USA) were performed as recommended by the supplier.

Filter analysis and semiquantitative evaluation was performed with a Storm 840 phosphofluoroimager (Molecular Dynamics, Sunnyvale, CA, USA) using the Image QuaNT software (version 5.0; Molecular Dynamics).
less frequent but significantly altered regions were present in 4q (33%, core region: 4q22-28), 6q (19%), 13q (19%), and 16q (14%). The same alterations were also in part represented in the 3 analysed cell lines. There was no indication for certain genomic
There were no correlations of the most frequent imbalances (gains of 1q and 8q and losses of 4q and 13q) compared to the grade of tumour differentiation, with the exception of 13q losses that were not detected in highly differentiated (G1) HCCs.

Since gains of genomic material may correlate with activated oncogenes and losses with the inactivation of tumour suppressor genes, a database analysis (OMIM-NCBI) was performed in order to identify known genes with potential relevance for the tumorigenic process located in these chromosomal regions. A number of genes of specific interest in the context of HCC that were identified by this search are shown in Figure 1.

To further analyse the potential consequences of genomic imbalances on gene expression, cDNA array analyses (Clontech) were performed with tumourous and non-tumourous liver RNA from tumour No. 6 (with 1q; 8q and X gains). Of the 518 genes evaluated, 126 genes showed significantly altered (> 3-fold) expression compared to the non-tumourous liver tissue; of those, 8 genes located in these chromosomal regions showed significant

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**Table 1** Genomic imbalances in HCCs and liver tumor cell lines. (A) Listing of cases; (B) Summary

| No. | Sex | G | Etiology | Gains                          | Losses                  |
|-----|-----|---|----------|--------------------------------|-------------------------|
| 1   | m   | G1| HBV      | 1q, 2, 5p, 5q, 6p, 7p21-22, 7q, 8p21.1-21.3, 13q31-34, 17p | 3p, 6q, 12q15-24.3, 21q21-22 |
| 2   | m   | G2| ethanol  | 1q12-25, 1p, 2p, 3q, 6p11-21.2, 9p, 17q, 19q12-13.2, 20q, Xq22-26 | 3p, 4q, 6q31-34, 8p, 9q, 10q, 22-26, 13q, 16q, 20p, Y |
| 3   | m   | G2| HBV      | –                              | –                       |
| 4   | f   | G2-3| hemochrom. | 8q24.2-24.3, 11q12-14          | 4q                     |
| 5   | m   | G1| Ø        | 8q11.1-1.36, 15p, 15q11.1-12, 22p, 22q | –                      |
| 6   | m   | G1| Ø        | 1q, 8q21-1.2-3.3, Xp, Xq21-28  | –                      |
| 7   | m   | G3| HBV      | 1q                              | –                      |
| 8   | m   | G3| ethanol  | 1q, 1p33-11, 4p16-14, 15q13-21, 17q  | 4q24-25, 11q24-25, 13q, 14q33, 21q, Yq |
| 9   | m   | G2| ethanol  | 1q                              | –                      |
| 10  | f   | G1| Ø        | –                              | –                      |
| 11  | m   | G1| Ø        | 14p13-12, 15p13-13              | –                      |
| 12  | m   | G1| HBV      | 7p, Xq                         | 4q, 5p51.5-15.3, 5q11-21-21, 10q, 16q21.2-23 |
| 13  | m   | G1| Ø        | 1q11-31, 17q                    | 16q23-24               |
| 14  | m   | G2| HBV      | 1q, 6, 8q, 11p15, 13q33-34, 14q32, 15q11.2-15, 21 | 8q22-23, Y |
| 15  | m   | G2-3| HBV     | 8q                              | Y                      |
| 16  | m   | G2| ethanol  | –                              | –                      |
| 17  | m   | G3| Ø        | 1p, 1q11-33, 3p11-14, 3q, 4p, 6p12-21.3, 8q12-24.1, 12q, 17q, 20 | 13q, 14q, Y11.2 |
| 18  | f   | G1| Ø        | 1q21-32, 5p, 5q11-1-14, 6p, 7p12-15, 8, 9p11-21, 17q, 18, 20, X | 4q, 11, 12p21-13, 16q21-24 |
| 19  | m   | G2| HBV      | 8q, 17q21-25, 20q13-2.13.3      | Yq                     |
| 20  | m   | G2| HBV      | 1q21-41, 6, 7p, 8q13-24.1, 20 | 4q, 8p, 12p, Y11.2 |
| 21  | f   | G2-3| Ø       | 1q, 2p32-37, 7p14-22, 8q, 12q21-24.1, 13q31-14, 20q | 4p16, 4q22-28, 6q, 10q25-16, 13p, 13q11-14, 16p12-13.3, 21q, 22, X |

**Table 1** (Continued)

| *HEP-3B* | 1q, 3p21.6-29, 4p, 6p, 7p, 11p, 13qter, 19p, 20q |
| *SK-HEP1* | 6p, 17 |
| *HEP-G2* | 2, 6p, 16p, 16q, 17q, 19p, 20 |

**B**

| No. of cases | % | Genomic imbalance |
|--------------|---|-------------------|
| Gains        | 12| 57 1q (core region: 1q21-25) |
|              | 11| 52 8q (core region: 8q21.2-24.3) |
|              | 6 | 29 17q (core region: 17q21.3-25) |
|              | 6 | 29 20q |
|              | 5 | 24 7p |
| Losses       | 7 | 33 4q (core region: 4q22-28) |
|              | 4 | 19 13q (core region: 13q12-14) |
|              | 4 | 19 6q |
|              | 3 | 14 16q |

*: cell line; G: grade; Ø: no defined etiology (HBV and haemochromatosis negative).

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DISCUSSION

The present CGH study of human HCCs has identified a number of prominent genomic imbalances (Table 1 and Figure 1) that were selectively confirmed by FISH analyses of the respective interphase HCC nuclei. Our data show in part a correlation with one previous analysis (Marchio et al, 1997), although significant differences to this study exist. The overall percentage of genomic imbalances in our material is lower, which may be due to differences in case selection, not limited to HBV-induced HCCs in our study, but more likely to the stringent criteria applied to the acceptance of genomic imbalances in the present study. Some of the previously reported alterations (1p del, 4p del, 17p del, 19q amp, and X del) were not detected or did not reach significance in our analysis. In contrast there is a good match in the type of genomic imbalances of our study to a second, recent one (Wong et al, 1999) although the percentage of imbalances in our study is slightly but consistently lower for each single type of imbalance. It can therefore be speculated that the extent and type of genomic imbalances may be influenced by the underlying etiology of HCC.

Significant gains of genomic material have been found at 1q, 8q, 17q and 20q. These alterations appear to be selected and maintained during hepatocarcinogenesis and thus represent candidate regions for relevant proto-oncogenes or growth-promoting genes. Since 1q and 8q gains and 4q losses are detectable in highly and poorly differentiated tumours at an almost equal frequency, there is no evidence for a correlation with tumour cell differentiation. In the core region of 1q, the retinoic acid receptor gamma gene is located (1q22-23) that was originally cloned from an HCC and which is frequently overexpressed in HCCs (de The et al, 1987; Benbrook et al, 1988). Furthermore the c-myc gene (as well as the myc-activator) is located in 8q24, and is known to be activated in HCC cells (Huber and Thorgeirsson, 1987). The functional relevance of its overexpression due to genomic amplification is well documented (Koskinen and Alitalo, 1993). Correlative cDNA microarray expression screening in tumour No. 6 that showed gain

Table 2 Interphase FISH analysis

| Tumour No. | Probes test/reference | Q ≤ 0.5 (%) | 1 > Q > 0.5 (%) | Q = 1 (%) | Q > 1 (%) |
|------------|-----------------------|-------------|-----------------|-----------|-----------|
| 8          | 13/10  
(13q14(RB-1) / 10p11.1-q11.1 (α-sat. DNA)) | 83.3  | 10.2  | 5.6  | 0.9 |
| 1/17  
(1q12 (sat. II and III) / chr.17p11.1-q11.1 (α-sat. DNA)) | 0  | 1.9  | 30.7  | 67.3 |
| 6          | 8/10  
(8p11.1-q11.1 (α-sat. DNA) / 10p11.1-q11.1 (α-sat. DNA)) | 1.8  | 10.7  | 40.2  | 47.3 |

Q: ratio of the numbers of signals per nucleus using the 2 indicated hybridization probes.

overexpression in the tumour RNA (Table 3), among them the protooncogene c-ski.

Figure 2 Representative interphase FISH analyses in tumour Nos 6 and 8. (A) Interphase FISH with a satellite-DNA centromeric probe specific for chromosome 1 (red) and a satellite-DNA centromeric probe for chromosome 17 (green) (tumour No. 6). (B) Interphase FISH with a satellite-DNA-probe specific for chromosome 6 (green) and a satellite-DNA-probe specific for chromosome 10 (red) (tumour No. 6). (C) Interphase FISH with a LSI-probe for 13q14 covering the RB-1 gene (green) and a satellite-DNA-probe for chromosome 10 (red) (tumour No. 6).
Chromosomal macrodeletions were most frequently found in 4q. A tumour-suppressor gene relevant for HCC development has already been suspected at this location from several LOH studies (13q12.3) gene. LOHs in 13q are relatively frequent in HCCs (up to 40%) and additionally sequence abnormalities in the RB-1 gene have so far not been identified. In contrast other LOHs frequently observed at 17p (up to 64%), 1p (30–83%) and 11p (up to 44%) did not correlate with losses of genomic material in our CGH analysis and are thus unlikely to result from macrodeletions. Thus macrodeletions were not found to encompass the p53 locus at 17p13.1, in consistence with the fact that frequently the loss of p53 function in HCCs is due to point mutations at codon 249 (Fujimoto et al, 1994; Bressac et al, 1991), and not to deletions as described for glioblastomas (Albertoni et al, 1998). Furthermore no significant gains of genomic material have been found at chromosomal locations of growth factors that are frequently overexpressed in HCCs, such as IGF-II (11p15.5) and TGFβ (4q31.2), suggesting that at least for some genes gains of genomic material may support overexpression. SKI appears particularly to be activated by overexpression and to represent a potential target gene since it has been recently shown to suppress transcriptional repression exerted by the Smad-mediated TGF-β activated pathway (Sun et al, 1999). This growth-suppressive pathway is deactivated at least in a wide variety of malignant epithelial tumours (Hata et al, 1998).

In 4 cases, deletions encompass 13q, a chromosomal region that contains not only the RB-1 (13q14.1-14.2), but also the BRCA-2 (13q12.3) gene. LOHs in 13q are relatively frequent in HCCs (up to 49%) and additionally sequence abnormalities in the RB-1 gene have been detected in up to 25% of HCCs (Murakami et al, 1991). Loss of function of the BRCA-2 gene has so far not been described in HCCs, but beside its well-documented role in breast cancer (Wooster et al, 1994, 1995), it also occurs in sporadic cancer of the exocrine pancreas (Schutte et al, 1995; Teng et al, 1996), an organ with its cellular origin embryologically closely related to hepatic tissue.

There are 3 cases that show deletions of 16q, which contain the cadherin gene cluster including E-cadherin at 16q22.1. Loss of E-cadherin expression has been linked to the gain of invasive potential (Fri xen et al, 1991; Vleminkx et al, 1991; Berx et al, 1995) and has recently been identified as the cause of hereditary gastric cancer (Guilford et al, 1998). LOHs at 16q have on one hand been observed in 40–70% of the cases in HCC studies (Nishida et al, 1992; Yeh et al, 1996) but have conversely been correlated with advanced stages (Nishida et al, 1992) and differentiated morphology of HCC (Shimoyama and Hirohashi, 1991). Functional inactivation of E-cadherin in carcinomas may additionally arise epigenetically through hypermethylation (Graff et al, 1995). Interestingly the Fanconi anemia gene is also located at 16q24.3 (Gischwend et al, 1996), and it may be hypothesized that in addition to loss of E-cadherin expression, somatic activation of a chromosomal breakage phenotype may support HCC progression. Another chromosomal breakage syndrome, ataxia telangietasia, may carry a slightly increased risk of hepatocellular carcinoma (Weinstein et al, 1985).

Chromosomal macrodeletions were most frequently found in 4q. A tumour-suppressor gene relevant for HCC development has already been suspected at this location from several LOH studies (frequency: 17–77%) (Bu et al, 1989; Zhang et al, 1990; Konishi et al, 1993; Yeh et al, 1996; Chou et al, 1998). A common chromosomal breakpoint mapped to 4q has also been found in 4 out of 50 HCCs (Pasquinelli et al, 1988) as well as an HBV integration site in an HCC at 4q32.1. Furthermore caspase genes (caspase-6 (4q25), caspase-3 (4q35)) and the smad1 gene (4q28) are located at 4q. Nevertheless a definite target tumour-suppressor gene at 4q has so far not been identified.

In conclusion, our CGH analysis of HCCs induc es several hypotheses regarding activated or suppressed genes relevant to hepatocarcinogenesis. Some of them (RB-1 and E-cadherin) are reflected by preliminary expression data but many more including the ones mentioned events (Figure 1) will have to be tested by appropriate expression analyses.

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