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Losses of Chromosome Arms 4q, 8p, 13q and Gain of 8q Are Correlated with Increasing Chromosomal Instability in Hepatocellular Carcinoma

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Key Words
Hepatocellular carcinoma · Chromosomal instability · Comparative genomic hybridization · Fluorescence in situ hybridization

Abstract
Objective: Chromosomal instability is a key feature in hepatocellular carcinoma (HCC). Array comparative genomic hybridization (aCGH) revealed recurring structural aberrations, whereas fluorescence in situ hybridization (FISH) indicated an increasing number of numerical aberrations in dedifferentiating HCC. Therefore, we examined whether there was a correlation between structural and numerical aberrations of chromosomal instability in HCC. ***Methods and Results:*** 27 HCC (5 well, 10 moderately, 12 lower differentiated) already cytogenetically characterized by aCGH were analyzed. FISH analysis using probes for chromosomes 1, 3, 7, 8 and 17 revealed 1.46–4.24 signals/nucleus, which correlated with the histological grade (well vs. moderately, $p < 0.0003$; moderately vs. lower, $p < 0.004$). The number of chromosomes to each other was stable with exceptions only seen for chromosome 8. Loss of 4q and 13q, respectively, were correlated with the number of aberrations detected by aCGH ($p < 0.001$, $p < 0.005$; Mann-Whitney test). Loss of 4q and gain of 8q were correlated with an increasing number of numerical aberrations detected by FISH ($p < 0.020$, $p < 0.031$). Loss of 8p was correlated with the number of structural imbalances seen in aCGH ($p < 0.048$), but not with the number of numerical changes seen in FISH. ***Conclusion:*** We found that losses of 4q, 8p and 13q were closely correlated with an increasing number of aberrations detected by aCGH, whereas a loss of 4q and a gain of 8q were also observed in the context of polyploidization, the cytogenetic correlate of morphological dedifferentiation.

Introduction

Despite the high incidence of hepatocellular carcinoma (HCC) with more than 250,000 deaths/year worldwide [1], little is still known about the basic pathomechanisms leading to malignancy in these tumours. In most patients, HCC is found in a setting of chronic inflammation in close association with aetiological factors such as

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hepatitis B and C or ingestion of aflatoxins [2]. Ozturk [3] was the first to describe mutations of p53 as an initiating event in HCC. Functional losses of retinoblastoma-1 as well as overexpression of growth factors such as insulin-like growth factor-II and transforming growth factor-α were also described as molecular changes during the development of HCC [4–6].

According to comparative genomic hybridization (CGH) analysis in more than 800 cases to date (public NCBI database PubMed), HCCs most frequently display chromosomal gains of 1q, 8q, 17q and 20q, and losses of 4q, 6q, 8p, 13q, 16q and 17p. Clinical impact has been reported for losses of 8p and 13q in studies using this technique [7]. An association has been described with aetiological factors such as viral infections [8]. Losses of 4q and 13q have been reported more frequently in low-differentiated HCC (ldHCC) [9]. These data were affirmed by array CGH (aCGH), a high-resolution microarray-based technology that allows detection of DNA copy number changes at the gene level [10, 11]. Moreover, fluorescence in situ hybridization (FISH) has revealed a stepwise increase in aneuploidy in HCC with respect to the morphological dedifferentiation [12].

The occurrence of both structural aberrations and aneuploidy makes HCC an interesting model for the analysis of chromosomal instability. Thus, we evaluated histologically and cytogenetically well-characterized HCC specimens by FISH analysis using centromere and locus-specific probes.

Patients and Methods

Patients and Samples

Analysis was performed on tumour specimens of 27 patients suffering from HCC who were treated at the Hannover Medical School (MHH). Their clinical data are shown in table 1. Tumour material from these patients was obtained from surgical specimens taken for diagnostic purposes. Unfixed tissue samples (0.5 cm in diameter) were snap-frozen within 30 min after resection and stored at –80°C until use for DNA extraction, as permitted by the Ethics Committee of the MHH. At the same time, a sample of each tissue was formalin-fixed and paraffin-embedded. Additional histological examination and FISH analysis were performed on these specimens.

Histological Classification

Histological classification according to the WHO criteria [13] was performed by two experienced pathologists (L.W., P.F.) and revealed 4 well (wdHCC), 10 moderately (mdHCC-G2) and 5 poorly (pdHCC) differentiated HCC. In 8 cases, definitive grading was not possible on the basis of the histological examination: in 1 case between wdHCC and mdHCC, and in 7 cases between mdHCC and pdHCC (table 1). The latter cases were used together with the pdHCC cases as ldHCC.

The control group for FISH analysis consisted of tissue samples of 5 nonneoplastic and histologically normal donor livers taken as biopsies before transplantation.

Cytogenetic Characterization of Tumours

All tumours included in this study were cytogenetically characterized by either conventional CGH (cCGH; 4 samples, not previously published) or aCGH (23 samples) in a previous study [14].

Using these techniques, chromosomal imbalances were observed in 27/27 cases (karyotypes given in online suppl. table 1, www.karger.com/doi/10.1159/000151712). The number of chromosome arms affected by gains and/or losses varied from 2 to 27 per case. Gains and losses were found in up to 19 and 21 arms, respectively. The mean number of gains and losses in wdHCC was 5.0 (gains 3.6; losses 1.4), in mdHCC 13.5 (gains 7.2; losses 6.3), and in ldHCC 15.6 (gains 10.4; losses 5.2). The most frequently affected chromosomal regions were 1q in 24 cases, 8q in 18 cases, 8p in 14 cases, 4q in 10 cases, and 13q in 8 cases. aCGH detected high-level amplifications of 8q exceeding values of 1.5 in 4 cases. In cases N71, N86 and N87 the amplification affected the whole long arm. In case N37 only amplification of band 8q24 was observed. cCGH revealed a high-level amplification of the whole arm of 8q in case N121.

For 21 samples analyzed by FISH mRNA expression data were available measured by array-based global mRNA analysis as reported recently [15].

FISH

FISH was performed as described previously [16], using a set of probes specific for the centromeres of chromosomes 1 and 8 (CEP1, CEP8; Abbott, Wiesbaden, Germany), for the centromeres of chromosomes 3, 7 and 17 (CEP3, CEP7, CEP17; Abbott) and a combined probe set for chromosome 8 as well as the 8q24 and 8p22 gene loci (CEP8/*CMYC/LPL*; Abbott). For each tumour and each of the control samples 50 nuclei were analyzed for all of these probes. Selection of centromere probes used during FISH analysis was based on the percentage of cases bearing aberrations of these chromosomes previously reported by CGH analysis [9]: chromosomes 1 and 8 were reported to be aberrant in >40% of cases, chromosomes 7 and 17 in >20%, and chromosome 3 in <20%. A probe for 8q24 was selected since a chromosomal gain as well as amplification of the chromosome arm 8q or parts of it were observed by a/cCGH in 5 cases during this study. In all of these samples 8p was lost, too. Additionally, in a previous study it was shown that the ratio of signals for 8p22 and 8q24, respectively, was significantly disturbed in the majority of samples [14]. We therefore completed the analysis of these for an additional seven tumours to complete the full set of cases. Evaluation was done with a probe specific for the *lipoprotein lipase (LPL)* gene localized on 8p22. Since loss of chromosome Y has been reported as a frequently occurring event [17], all tumours occurring in male patients were also analyzed by FISH for chromosomes X and Y (CEPX, CEPY; Abbott).

Evaluation of signals was performed with an epifluorescence microscope (Axioskop 2; Zeiss, Oberkochen, Germany) equipped with a set of fluorescein/rhodamine/aqua blue and DAPI filters and a 100-watt mercury lamp.

Table 1. Clinical, morphological and serological data of the 27 patients analyzed

Patient			pTNM classification	Diameter cm	Grading	Hepatitis serology[a]	ISHAK score
No.	age	gender					
120	59	f	pT3 N0 MX	4.2	wd	neg.	A1B0C0D1F2
59	74	f	pT2 NX MX	3.5	wd-md	B/C	A3B0C1D3F6
25	54	m	pT2 N0 MX	1.5	wd	B	A1B0C1D2F6
77	53	f	pT3 NX MX	18	wd	neg.	no cirrhosis
20	68	f	pT2 NX MX	5	wd	neg.	n.a.
15	68	m	pT3 NX M1	6.5	md	neg.	A2B0C1D2F6
13	65	f	pT4 NX MX	14	md	neg.	no cirrhosis
1	10	m	pT2 N0 MX	2.5	md	neg.	A1B0C0D1F6
9	81	m	pT2 NX MX	9	md	B	no cirrhosis
62	75	m	pT3 N0 MX	2.5	md	neg.	A2B0C1D1F
80	65	m	pT3 NX MX	9	md	B	no cirrhosis
69	73	m	pT2 NX MX	2.5	md	neg.	A2B0C1D2F4
87	47	m	pT2 NX NX	3.3	md	C	A3B0C1D3F6
37	71	m	pT2 NX MX	6	md	neg.	no cirrhosis
74	35	m	pT2 NX MX	6	md	neg.	no cirrhosis
121	64	m	pT3 N0 MX	13	ld?	n.a.	no cirrhosis
45	73	f	pT2 NX MX	10	ld?	C	A2B0C1F2
86	57	m	pT3 NX MX	8	ld?	neg.	no cirrhosis
91	37	m	pT2 NX MX	15	ld?	B	A1B0C1D2F0
71	59	m	pT3 NX MX	17	ld?	B	no cirrhosis
90	9	m	pT3 NX M1	3	ld?	B	no cirrhosis
40	50	m	pT3 N0 MX	11	ld+	B	no cirrhosis
47	65	m	pT3 NX MX	8	ld?	B	A1B0C1D1F2
122	8	m	pT4 N0 MX	15	ld+	neg.	no cirrhosis
82	19	m	pT3 N0 MX	4	ld+	B	A1B0C1D1F6
123	37	f	pT3 N0 MX	4	ld+	C	A2B0C1D2F6
16	59	f	pT4 N1 M1	13	ld+	B/C	no cirrhosis

wd = Well differentiated; md = moderately differentiated; ld? = lower differentiated HCC not unequivocally classifiable between moderately and poorly differentiated HCC; ld+ = clearly poorly differentiated HCC; neg. = no serological evidence of hepatitis B and/or C; B/C = serologically proved hepatitis B/C; n.a. = not available.

[a] Analysis performed for HBsAG, anti-HBs, anti-HBc and anti-HCV.

Immunohistochemistry

Immunostaining for p53 (DO7; Dako, Hamburg, Germany) was done for all cases with the exception of sample N120. Tissue sections of 2–3 μm were dewaxed in xylene, rehydrated and put into Tris-buffered saline. Antigen retrieval was done by continuous heating for 30 min in a microwave oven (900 W) in citrate buffer (pH 6, 0.01 M). Detection was performed as described in detail elsewhere [18].

Cytometry

Cytometric data were obtained after Feulgen staining using a Nikon Eclipse 200 microscope and the Ahrens ICM Cytometry system (Bargteheide, Germany). Intensity of Feulgen stainings was measured using a 40× objective. Calculation of DNA content was performed with the relevant software package strictly following the manual of the supplier.

Results

FISH Analysis of the Control Group

FISH analysis of the 5 control cases displayed mean signal numbers between 1.54 and 1.94 (table 2) for every single centromere probe (specific for chromosomes 1, 3, 7, 8 and 17). Due to cutting artefacts of the histological sections, mean values of 2 were not obtained. According to Ward et al. [19], the threshold for defining an imbalance was set at the mean plus/minus 3× standard deviation (SD) leading to values of 1.23–1.31 for defining losses and 1.96–2.14 for defining gains of a chromosome.

Table 2. Control cases (C1–5) for defining thresholds in FISH

a FISH results for the centromeric probes of chromosomes 1, 3, 7, 8 and 17[a]

	Mean number of signals for chromosomes					Mean for	Ratio of signals for chromosome and the mean number of signals for chromosomes 1–17				
	1	3	7	8	17	1–17	1	3	7	8	17
C1	1.54	1.58	1.52	1.76	1.74	1.63	0.95	0.97	1.08	1.08	1.07
C2	1.82	1.80	1.76	1.80	1.94	1.82	1.00	0.99	0.99	0.99	1.06
C3	1.54	1.58	1.70	1.52	1.70	1.61	0.96	0.98	0.95	0.95	1.06
C4	1.64	1.62	1.74	1.72	1.68	1.68	0.98	0.96	1.02	1.02	1.00
C5	1.62	1.46	1.54	1.58	1.56	1.55	1.04	0.94	1.02	1.02	1.01
Mean	1.63	1.61	1.65	1.68	1.72	1.66	0.98	0.97	1.01	1.01	1.04
Thresholds	1.29[b]/1.96[c]	1.23/1.98	1.31/1.99	1.31/2.04	1.31/2.14		0.87[d]/1.10[e]	0.91/1.02	0.86/1.16	0.86/1.16	0.94/1.14

b Results obtained for chromosomes X and Y

	Mean number of signals for chromosomes			X mean for 1–17	Y mean for 1–17
	X	Y	Y/X		
C1	0.83	0.78	0.94	0.51	0.51
C2	0.91	0.83	0.91	0.50	0.48
C3	0.85	0.87	1.02	0.53	0.53
C4	0.79	0.85	1.08	0.47	0.46
C5	0.80	0.78	0.98	0.52	0.53
Mean	0.84	0.82	0.99	0.50	0.50
Thresholds	0.69[b]/0.98[c]	0.70/0.94	0.79[d]/1.18[e]	0.44[d]/0.57[e]	0.44/0.59

c Since there was a major difference in the length of probes used for 8q24 and 8p22, with 750 vs. 170 kb, respectively, FISH was performed in the control cases to avoid systematic errors due to variations in the hybridization efficiency

	Mean number of signals for			Ratios of q24/p22
	cen8	8q24	8p22	
C1	1.88	1.82	1.84	0.99
C2	1.88	1.90	1.82	1.04
C3	1.40	1.36	1.42	0.96
C4	1.74	1.72	1.78	0.97
C5	1.70	1.76	1.72	1.02
Mean	1.72	1.71	1.72	1.00
Threshold				0.89[f]/1.11[g]

For all control samples, as also done for tumour samples, 50 nuclei were counted.

[a] The mean number of signals for these probes is clearly below 2 due to cutting artifacts typically occurring in tissue sections.

[b] Thresholds defining loss of a chromosome.

[c] Thresholds defining gain of one or more chromosomes.

[d] Thresholds indicating a disturbed ratio of chromosomes by a relative loss of a chromosome.

[e] Thresholds indicating a disturbed ratio of chromosomes by a relative gain of a chromosome.

[f] Threshold indicating a relative loss of 8q24 in comparison to 8p22.

[g] Threshold indicating a relative gain of 8q24 in comparison to 8p22.

Additional statistical analysis was performed to determine whether changes in the number of signals for the centromeric regions reflected aneuploidy of a single chromosome or polyploidization in the tumour nuclei. For this purpose, the mean number of signals for each individual chromosome in each tumour sample was compared to the overall mean number of signals from the five chromosomes collectively. For example, trisomy 8 in an otherwise diploid karyotype would give a value of 1.5, whereas monosomy 17 would give a value of 0.5. In the control group consisting of five cases, 25 ratios were calculated revealing values of ratios between 0.93 and 1.08 with an SD of 0.04.

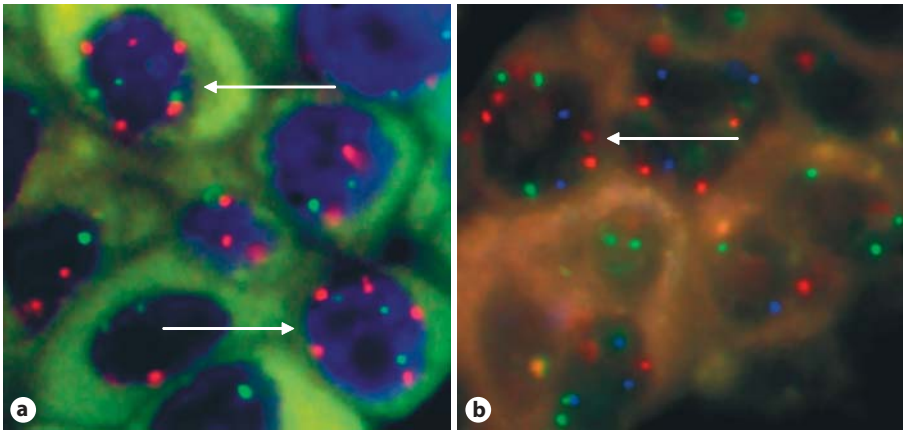

Fig. 1. FISH analysis of specimen N121 for chromosomes 1 (red) and 8 (green) (**a**) and chromosomes 3 (red signals), 7 (green signals), and 17 (blue signals) (**b**). The mean number of signals is clearly increased (4.08 for chromosome 1, 3.04 for chromosome 3, 3.84 for chromosome 7, 7.64 for chromosome 8, 2.60 for chromosome 17). Thus, this tumour was graded as HCC-G2–3. Interestingly, signals are arranged in a circle at the periphery of the nuclei. This is similar to the spheric order described by Nagele et al. [38] as 'rosettes'. This may also be representative of a maintained basic structure found even in lower differentiated HCC.

For chromosome 8, a set of probes specific for 8q24/cen8/8p22 was used. Since the probe used for 8q24 was 600 kb larger than the probe for 8p22, we examined differences in the hybridization efficiency by comparing the number of signals obtained for 8q24 and 8p22 to avoid a systematic error. In the control cases, this ratio varied between 0.95 and 1.04 with an SD of 0.04. The threshold for defining an imbalance of this ratio was set at 0.88 for a relative loss and at 1.10 for a relative gain of 8q24 in relation to 8p22.

FISH Analysis of HCC

In HCC, the mean number of signals for centromeres of chromosome 1 ranged from 1.46 to 4.12, for chromosome 3 from 1.40 to 4.00, for chromosome 7 from 1.36 to 4.48, for chromosome 8 from 1.48 to 7.64, and for chromosome 17 from 1.46 to 3.87 (table 3, fig. 1). The ratios of the number of signals obtained for the centromeres of chromosomes 1, 3, 7, 8 and 17 to each other ranged between 0.68 and 1.80. In 58/135 analyses, ratios were lower or higher than the thresholds of 0.87 or 1.13 that define losses or gains of chromosomes, respectively. However, ratios exceeding values of 1.5 were only detected in 4/135 analyses. In 3/4 of these cases, this was due to a high number of signals on chromosome 8.

FISH analysis with the combined probe set for cen8/8q24/8p22 (see Patients and Methods for a detailed description of the probe set) revealed values for cen8 ranging from 1.68 to 8.12, for 8q24 ranging from 1.88 to 9.44, and for 8p ranging from 1.18 to 2.52 (table 4, fig. 2). 8q24/8p22 ratios in 17/27 samples were greater than 1.5, suggesting that 8q24 was amplified compared to 8p22. Interestingly, those samples with a high-level 8q amplification detected by c/aCGH (N37, N71, N86, N87, N121) displayed high 8q24 signal numbers (mean range 4.26–9.44) and high 8q24/8p22 ratios (3.44–6.05) during FISH analysis as well. Ratios for the number of signals for 8q24 and the corresponding centromere probe in all tumour samples varied between 0.72 and 1.68 with a mean of 1.12 in wdHCC, 1.18 in mdHCC and 1.27 in ldHCC. These ratios were greater than 1.5 in only four samples. Therefore gain of 8q is accompanied by a corresponding number of centromers of chromosome 8 indicating additional isochromosomes i(8q).

For gonosomes, the mean number of signals for chromosome X ranged from 0.88 to 3.06, and for chromosome Y from 0.0 to 1.67. The ratios of chromosomes X or Y and the number of autosomes ranged between 0.32 and 1.18 for chromosome X and between 0 and 0.73 for chromosome Y. The ratios of the gonosomes to each other ranged from 0 to 1.63. In 10/17 samples, disrupted signal ratios were observed, although ratios greater than 1.5 or less than 0.5 were observed in only 6 cases.

Correlation between FISH/CGH and Histological Grading

The number of aberrations detected by c/aCGH and the changes detected by FISH (online suppl. table 1, www.karger.com/doi/10.1159/000151712) correlated with the histological grading as follows.

The mean number of signals detected by FISH for the five autosomes analyzed here correlated significantly with the histological grading (wdHCC vs. mdHCC, $p < 0.0003$; mdHCC vs. ldHCC, $p < 0.004$; Kruskal-Wallis test). However, the correlation between the c/aCGH data and histological grading displayed a somewhat different picture: a significant difference in the number of structural aberrations seen by c/aCGH ($p < 0.28$) was only observed for wdHCC vs. mdHCC, but not for mdHCC vs. ldHCC.

Table 3. FISH results obtained for the centromeric probes in HCC samples

Patient No.	Histological grade	Mean number of signals for chromosomes							Mean for	Ratio of signals for chromosome and the mean number of signals for chromosomes 1–17								p53 staining % of positive nuclei	DNA cytometry mean content of DNA
		1	3	7	8	17	X	Y	1/3/7/8/17	1	3	7	8	17	X	Y	Y/X		
N120	wd	1.46	1.40	1.36	1.62	1.46			1.46	1.00	0.96	0.93	1.11	1.00				not available	
N59	wd	1.82	1.78	1.80	1.48	1.92			1.76	1.03	1.01	1.02	0.84	1.09				2	
N25	wd	1.92	1.88	1.86	1.84	1.80	0.88	0.92	1.86	1.03	1.01	1.00	0.99	0.97	0.47	0.49	1.05	5	1.96
N77	wd	2.12	1.90	1.82	2.18	2.06			2.02	1.05	0.94	0.90	1.08	1.02				0	2.02
N20	wd-md	1.80	1.70	2.04	1.72	2.16			1.88	0.96	0.90	1.08	0.91	1.15				11	1.88
Mean value		1.82	1.73	1.78	1.77	1.88	0.88	0.92	1.80									5	
N15	md	2.40	1.76	2.04	1.72	1.60	1.36	1.02	1.90	1.26	0.92	1.07	0.90	0.84	0.71	0.54	0.75	44	
N13	md	2.04	1.98	1.98	1.88	1.78			1.93	1.06	1.02	1.02	0.97	0.92				0	
N1	md	2.20	1.80	2.20	2.34	1.68	0.92	1.50	2.04	1.08	0.88	1.08	1.14	0.82	0.45	0.73	1.63	7	
N9	md	2.46	2.24	2.76	1.88	1.46	0.88	0.82	2.16	1.14	1.04	1.28	0.87	0.68	0.41	0.38	0.93	10	
N62	md	3.14	1.92	2.70	2.16	1.92	1.82	1.04	2.37	1.33	0.81	1.14	0.91	0.81	0.77	0.44	0.57	13	2.73
N80	md	2.06	2.42	2.92	3.76	1.78	0.96	0.86	2.59	0.80	0.94	1.13	1.45	0.69	0.37	0.33	0.90	0	2.23
N69	md	2.08	3.24	2.50	2.52	2.64	1.55	1.67	2.60	0.80	1.25	0.96	0.97	1.02	0.60	0.64	1.08	41	2.36
N87	md	2.88	1.96	1.96	4.22	1.98	1.68	0.98	2.60	1.11	0.75	0.75	1.62	0.76	0.65	0.38	0.58	50	
N37	md	2.58	1.76	2.86	3.06	3.18	1.34	0.00	2.69	0.96	0.65	1.06	1.14	1.18	0.50	0.00	0.00	51	
N74	md	3.30	3.16	3.16	3.16	3.87	1.08	1.04	3.33	0.99	0.95	0.95	0.95	1.16	0.32	0.31	0.96		
Mean value		2.51	2.22	2.51	2.67	2.19	1.29	0.99	2.42									24	
N121	ld	4.08	3.04	3.84	7.64	2.60			4.24	0.96	0.72	0.91	1.80	0.61				9	
N45	ld	2.26	2.00	1.60	2.40	1.86			2.02	1.12	0.99	0.79	1.19	0.92				15	2.25
N86	ld	2.92	2.36	2.00	3.58	1.92	1.66	0.88	2.56	1.14	0.92	0.78	1.40	0.75	0.65	0.34	0.53	17	
N91	ld	3.02	2.78	2.18	2.36	2.76	2.14	0.00	2.62	1.15	1.06	0.83	0.90	1.05	0.82	0.00	0.00	25	
N71	ld	3.14	2.34	1.88	4.44	1.94	1.14	1.02	2.75	1.14	0.85	0.68	1.62	0.71	0.41	0.37	0.89	30	
N90	ld	3.18	3.94	3.74	3.48	2.90	1.78	0.98	3.45	0.92	1.14	1.08	1.01	0.84	0.52	0.28	0.55	57	3.92
N40	ld	3.84	2.78	4.48	5.38	2.80	1.10	1.66	3.86	1.00	0.72	1.16	1.40	0.73	0.29	0.43	1.51	20	
N47	ld	3.26	4.00	3.26	3.86	2.80	2.40	1.14	3.44	0.95	1.16	0.95	1.12	0.81	0.70	0.33	0.48	0	3.49
N122	ld	4.12	2.04	1.92	2.30	2.02	1.18	1.02	2.48	1.66	0.82	0.77	0.93	0.81	0.48	0.41	0.86	2	
N82	ld	3.60	2.80	2.86	2.84	2.86	0.96	0.94	2.99	1.20	0.94	0.96	0.95	0.96	0.32	0.31	0.98	26	
N123	ld	2.86	3.68	3.50	3.42	3.34			3.36	0.85	1.10	1.04	1.02	0.99				90	
N16	ld	3.68	3.92	3.44	2.94	3.36			3.47	1.06	1.13	0.99	0.85	0.97				100	
Mean value		3.33	2.97	2.89	3.72	2.60	1.55	0.95	3.10									33	

Whereas the mean number of signals for all chromosomes was not significantly increased in wdHCC, a significant increase was seen in mdHCC and ldHCC, respectively. Accordingly, the ratio of chromosomes to each other was disturbed only in wdHCC in 3/25 (12%) ratios, whereas this number increased to 25/50 (50%) and a very similar value of 35/60 (58%; disturbed ratios in light grey fields). However, 4 ratios only, in dark grey fields, revealed a loss or gain of at least 50% of a chromosome relative to the other chromosomes. For gonosomes X and Y this pattern varied with 6/18 ratios <0.5 or >1.5 in two samples with complete loss of chromosome Y.

Table 4. Detailed FISH analysis of chromosome 8

Patient No.	Histological grade	FISH					CGH gain of 8q and/or loss of 8p
		cen8	8q24	8p22	ratio of 8q24/cen8	ratio of 8q24/8p22	
N120	wd	1.64	1.88	1.18	1.15	1.59	X
N59	wd	1.70	1.90	1.32	1.12	1.44	
N25*	wd	1.98	2.24	1.66	1.13	1.35	X
N77	wd	2.34	2.48	2.10	1.06	1.18	
N20	wd-md	1.68	1.90	1.66	1.13	1.14	
Mean		1.87			1.12	1.34	
N15*	md	1.74	1.88	1.50	1.08	1.25	X
N13*	md	1.92	1.84	1.10	0.96	1.67	X
N1*	md	2.60	2.56	2.20	0.98	1.16	X
N9*	md	1.76	2.52	1.24	1.43	2.03	X
N62	md	2.08	2.42	2.04	1.16	1.19	
N80*	md	3.52	3.82	1.20	1.09	3.18	X
N69	md	2.76	2.94	1.98	1.07	1.48	
N87*	md	4.34	7.18	1.34	1.65	5.36^{amp}	X
N37*	md	3.36	4.26	1.24	1.27	3.44^{amp}	X
N74*	md	3.58	4.06	1.66	1.13	2.45	X
Mean		3.51			1.18	1.80	
N121	ld	8.12	9.44	1.56	1.16	6.05^{amp}	X
N45*	ld	2.34	3.62	1.70	1.55	2.13	X
N86*	ld	3.48	5.28	1.80	1.52	2.93^{amp}	X
N91*	ld	2.72	2.96	1.62	1.09	1.83	X
N71*	ld	4.08	6.84	1.68	1.68	4.07^{amp}	X
N90*	ld	3.96	4.02	2.28	1.02	1.76	X
N40*	ld	5.30	5.48	1.58	1.03	3.47	X
N47*	ld	4.30	5.90	2.26	1.37	2.61	X
N122	ld	2.56	3.06	2.12	1.20	1.44	X
N82*	ld	2.70	3.98	2.52	1.47	1.58	X
N123	ld	3.62	5.30	2.22	1.46	2.39	X
N16*	ld	3.12	2.24	1.98	0.72	1.13	X
Mean		3.26			1.27	2.04	

In addition to 18 samples already analyzed in a previous study (patient No. marked by an asterisk), the remaining 9 cases were examined with the same probes to get a complete data set. In all samples ratios of 8q24/8p22 were disturbed (threshold: 1.11, samples in light grey fields). Moreover, ratios >1.5 were seen in 17/27 cases (dark grey fields). When comparing these data with CGH results, all five samples with a high-level amplification of 8q (marked with 'amp') also had FISH ratios >1.5. We then looked for the occurrence of losses of 8p, gains of 8q, or both. In wdHCC only 1/5 cases revealed at least one of these two aberrations. In contrast, in mdHCC one or both of these chromosome arms were affected in 7/10, and in ldHCC in 12/12 tumours, respectively.

The gains and losses of chromosomal arms 1q, 4q, 8p, 8q and 13q detected by c/aCGH were correlated with the number of numerical aberrations detected by FISH. There was no correlation between the gain of 1q and the number of numerical aberrations. Loss of 4q was strictly correlated with the total number of aberrations detected by c/aCGH ($p < 0.001$; Mann-Whitney test) and the number of aberrations detected by FISH ($p < 0.020$). Loss of 13q was also strictly correlated with an increased total number of structural changes seen in c/aCGH ($p < 0.005$), whereas a significant correlation with the numerical aberrations detected by FISH was not observed. A gain of

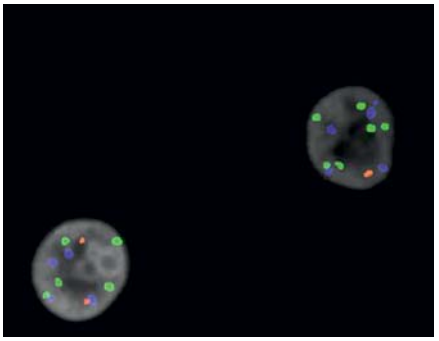

Fig. 2. FISH analysis for centromere 8 (blue signals), 8q24 (green signals), and 8p22 (red signals) in a tissue section of case N87. As indicated by the blue signals, aneuploidy of chromosome 8 occurred in this sample. Moreover, the relative number of signals for 8q24 was higher than for centromere 8, whereas the signal number of 8p22 was clearly diminished. In this case, aCGH revealed an amplification of the complete arm of chromosome 8 paralleled with a loss of 8p.

8q was correlated with an increasing number of numerical aberrations ($p < 0.031$) but not with the number of structural changes. Within the cases that showed a gain of 8q, the subset of tumours with high amplification of 8q did not differ significantly with respect to aberrations detected by FISH. Loss of 8p in c/aCGH was correlated with the number of structural imbalances seen in c/aCGH ($p < 0.048$), but not with the number of numerical changes seen in FISH.

Correlation of FISH and Immunohistochemistry

Immunohistochemical stainings for p53 revealed a mean number of positively stained nuclei of 5% in wdHCC, 24% in mdHCC, and 33% in ldHCC. When comparing the mean number of chromosomes detected in the nuclei by FISH and the percentage of nuclei positive for p53 (table 3), there was a significant correlation ($p = 0.033$, Spearman's rho test, two-sided).

Correlation of FISH and mRNA Expression

mRNA expression of CMYC (fig. 3) was evaluated based on data published earlier [15]. Statistical analysis by Spearman's rho test (two-sided) revealed a highly significant correlation between the expression of CMYC and the histological grading ($p < 0.002$). For the number of FISH signals only a trend was seen ($p < 0.1$).

Cytometry

Nine cases were exemplarily analyzed by cytometry to prove FISH results. Three tumours were wdHCC, 3 mdHCC, and 3 ldHCC, respectively (table 3). 271–963 cells were measured with the exception of N80. Since a small amount of tissue was left only 145 cells were measured in this sample. The number of control cells was in the range of 26–39. DNA content was seen between 1.88 and 3.92, which was very similar to the mean number of chromosome signals found in the tumour cells.

Discussion

Cytogenetic aberrations in HCC are a well-known phenomenon that has been reported in more than 800 cases investigated by CGH analysis to date. The most frequently observed structural aberrations included gains of 1q, 8q, 17q and 20q, and losses of 4q, 6q, 8p, 13q, 16q and 17p [20–23]. In addition to the correlations reported between genetic aberrations and clinical characteristics from LOH experiments [8], a close correlation has been shown between increasing chromosomal instability and morphological grading [12]. Furthermore, a close association between the increasing number of structural aberrations detected by aCGH and the histological grading has been reported [14]. In particular, there was a close association between losses of 4q and 13q and ldHCC. However, the cytometric evaluation of DNA content in HCC with respect to clinicopathological findings was not conclusive [24–26]. In the present study, we focused on the correlation between structural and numerical aberrations as detected by CGH and FISH.

First, a significant correlation was detectable between the histological grading and the number of aberrations detected in FISH and CGH, respectively. The increase in the aberrations detected by FISH occurred in a coordinated manner with the ratio of the chromosomes to each other relatively stable. Signal ratios for chromosomes greater than 1.5, indicating a polysomy of a single chromosome in comparison to the other chromosomes, were detected in only five samples (3.7% of all calculated ratios, all affecting chromosome 8). We therefore suggest that the increase of numerical aberrations is based on polyploidization in the nuclei rather than being due to amplification of single chromosomes alone.

Second, an increasing number of structural aberrations detected by CGH correlated with losses of 4q, 8p and 13q. These aberrations have been reported previously: in HCC a loss of 13q is correlated with low differentiation of HCC [9]. A loss of 4q is correlated with hepatitis B [27] and a loss of 8p with development of metastases [28]. To date, only a few genes that are located on these

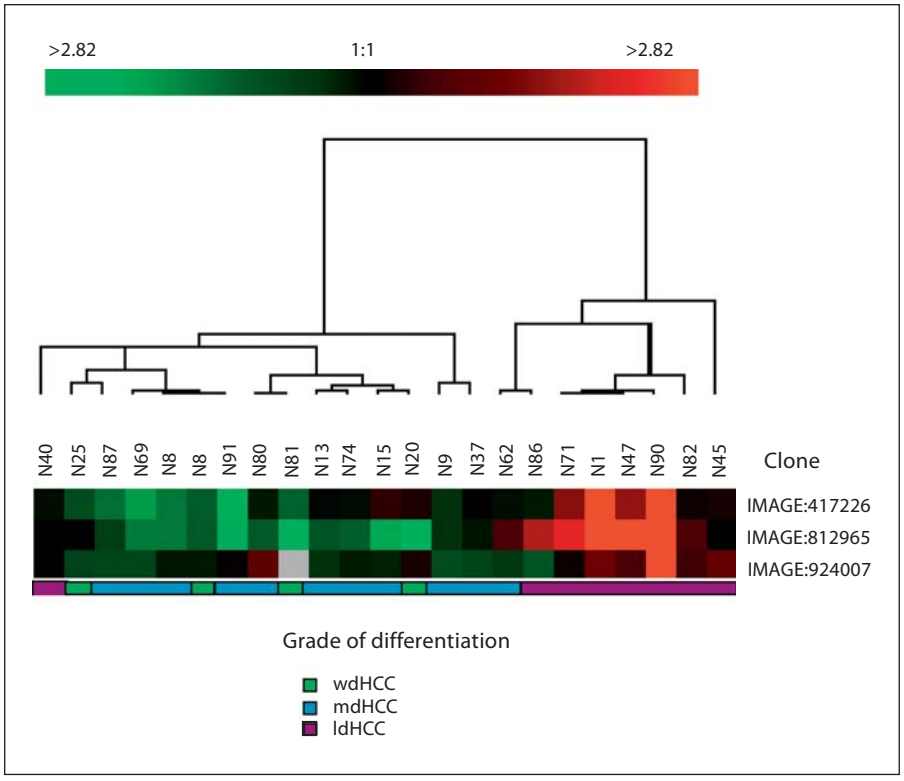

Fig. 3. mRNA expression of CMYC as revealed by earlier array-based analyses. Three clones covering different parts of mRNA were included. Presentation of expression data is given based on supervised hierarchical clustering. A correlation with the histological grading is evident as proven by statistical analysis ($p < 0.002$, Spearman's rho test, two-sided). Specimens N8 and N86 were not included in the actual study since FISH data were not available for these cases.

chromosome arms and are possibly involved in the process of dedifferentiation have been identified. Interpretation is additionally complicated by the influence of epigenetic factors such as microRNA (miRNA). This has been shown to be the case for 13q: aberrant expression was reported for miRNA localized to this chromosome arm in chronic lymphatic leukemia [29]. As a further example miRNA 17-5p is located on 13q and involved in the development of polyploidization. This is caused by downregulation of CMYC and E2F1, respectively. In case of a deletion of miRNA 17-5p this negative control is lost and polyploidization is enforced by repeated S phases without following mitoses. For 4q at the present time 10 miRNAs are known and for 8p there are 7 miRNAs, respectively (http://microrna.sanger.ac.uk/). Although the detailed function of these miRNAs is not known yet, it is worthwhile to get more information about the impact of these regulative factors on the development of dedifferentiation and chromosomal instability, respectively.

Third, amplifications defined as ratios greater than 1.5 in CGH and FISH were rare events and only detected in chromosome 8 according to our previous CGH analysis [14] accompanied by losses of 8p in all these cases. Accordingly, FISH revealed ratios of 8q/8p that were greater than 1.5 in these 5 samples and also in an additional 12 cases (1 wdHCC, 6 mdHCC, 10 ldHCC). Moreover, FISH detected disturbed ratios of 8q24/8p22 in every sample with more aberrant ratios in mdHCC and ldHCC, respectively. We assume that these disturbed ratios of 8q24/8p22 are indicative of the occurrence of isochromosome i(8q). This is underscored by the finding of a more or less balanced ratio of cen8 and 8q24 revealing not only a gain of 8q, but also of the centromere of the chromosome. Isochromosome 8q is the second most common isochromosome in human neoplasia and is frequently found in solid tumours such as adenocarcinoma (lung: 22%, colon: 15%, stomach: 12%) and malignant melanoma (17%) [30], which is similar to our results. So far, i(8q) has been described as a secondary abnormality in hematological neoplasms such as AML and MDS and data for malignant melanoma also suggest that i(8q) is a secondary rather than a primary aberration [30]. In most adenocarcinomas, the presence of i(8q) is associated with monosomy 8p. No data are available to suggest which of the aberrations, gain of 8q or loss of 8p, has the greater effect on tumour progression, but to date gain of 8q seems to be the more important abnormality.

In a multivariate analysis, gain of 8q was the only aberration to be correlated with the number of numerical changes as detected by FISH ($p < 0.031$). This is of interest because of the mechanism that may be involved in tumour progression. The most prominent gene located at 8q24 is CMYC [31]. The CMYC transcription factor modulates the expression of target genes by binding to specific DNA sequences. In the case of p53 dysfunction, cells with damaged DNA do not arrest in G1 but in a G2-like state [32]. In this state, the cells can pass through additional S phases without intervening normal mitoses resulting in polyploidization [33]. Accordingly, in our study we observed a concordant increase in signals for centromere regions of all the chromosomes studied, indicating polyploidization of tumour cells. When comparing these findings with mRNA expression of CMYC for statistical correlations a trend was seen ($p < 0.1$) in regard to the number of FISH signals. In regard to the histological grading a highly significant correlation was detectable ($p < 0.002$). We assume that this is indicative of a mechanism of polyploidization involving CMYC as a possible important factor.

In nonneoplastic liver tissues, polyploidization has been associated with growth and a subsequent increased metabolism [34]. Furthermore, polyploidization has been reported as a mechanism that protects DNA duplication and repair under anaerobic conditions [35] frequently observed in rapidly growing tumours due to insufficient angiogenesis. In addition, polyploid cells improve their capability to repair DNA double-stranded breaks [36]. Polyploidization probably also acts as a protective mechanism in tumour cells to circumvent negative effects of increased metabolism and decreased oxygen. Whether this phenomenon is an adaptive process with upregulation of depending genes enforced by these environmental factors or initiated by an imbalance, e.g. of chromosome 8 and consecutive gene dosage effects, cannot easily be answered yet. Even more additional factors such as miRNAs, acetylation and methylation hamper the detection of underlying mechanisms. Despite these uncertain aspects structural and numerical aberrations as seen here occur only in dedifferentiating malignant cells and are therefore useful as diagnostic tools.

A further exception of chromosomal balance in our study was chromosome Y. Complete loss of chromosome Y was seen by FISH in two samples and ratios less than 0.5 or greater than 1.5 occurred in 6/18 HCCs. Losses of chromosome Y in HCC have also been reported by Park et al. [17]. However, even in nonneoplastic cells, losses of chromosome Y have been reported in the elderly without major impact [37]. Since chromosome Y harbours a limited set of genes that are not known to be directly involved in the cell cycle and differentiation, this finding is not surprising.

In conclusion, we found that losses of 4q, 8p and 13q were closely correlated with an increasing number of aberrations detected by CGH, whereas gains of 8q were observed in the context of polyploidization, the cytogenetic correlate of morphological dedifferentiation. Despite the increase of imbalances and polyploidization, an overall basic chromosomal balance was maintained with 8q being the only exception.

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