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Trisomy 1 and 8 occur frequently in hepatocellular carcinoma but not in liver cell adenoma and focal nodular hyperplasia. A fluorescence in situ hybridization study

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Abstract Conventional cytogenetic studies revealed gains and structural aberrations of chromosome 1 to be the most consistent chromosomal aberrations in hepatocellular carcinoma (HCC). We investigated touch preparations of eight HCC, five cholangiocellular carcinomas (CCC), five liver cell adenomas (LCA), four focal nodular hyperplasias (FNH) as well as nine specimens of normal liver tissue using fluorescence in situ hybridization (FISH) with centromere specific probes for chromosomes 1 and 8. Polysomies of chromosome 1, especially trisomy 1, were found in five of eight HCC and four of five CCC but in no normal liver tissue or benign tumour. Only three of seven cases of HCC revealed trisomy 8 whereas the five benign liver tumours and all normal liver tissues examined had disomy 8. Our results confirm conventional cytogenetic findings in terms of chromosome 1 aberrations in HCC although they are not specific for these types of malignant liver tumours. Since α-satellite probes were used in our study, only gains or losses including the centromeric regions of the chromosomes 1 and 8 could be detected. Nevertheless, our findings suggest that FISH may help in the differential diagnosis of malignant versus benign neoplasms of the liver.

Key words Hepatocellular carcinoma · Cholangiocellular carcinoma · Cytogenetics · Chromosome 1 · Fluorescence in situ hybridization (FISH)

Introduction

Hepatocellular carcinomas (HCC) constitute the majority of primary malignant liver tumours [2, 16]. Several attempts have been undertaken to characterize chromosomal abnormalities in HCC, most of them analysing transformed HCC cell lines by conventional cytogenetics [6, 18–20, 30]. Consistent findings of these studies and of the few successful direct cytogenetic preparations of HCC [4, 22, 23, 28] are polysomy and structural aberrations of chromosome 1 maintaining the centromeric region. The importance of chromosome 1 abnormalities in HCC is also suggested by loss of heterozygosity (LOH) on chromosome 1p in a considerable number of cases [22, 31]. Among the most important differential diagnoses of HCC are benign liver tumours [liver cell adenoma (LCA) or focal nodular hyperplasia (FNH)], but there are no cytogenetic data from these lesions.

Karyotyping of HCC is mainly limited by difficulties in culturing of the tumour cells [13]. Additionally, in vitro culture may select for a non-representative subgroup of tumour cells with a growth advantage and can induce changes in karyotype [3]. Fluorescence in situ hybridization (FISH) makes it possible to circumvent these problems by analysing chromosomal abnormalities of interphase nuclei [17]. Determination of chromosome copy numbers is easy to perform with this technique and can be applied to a wide variety of cell preparations, including paraffin sections [15, 26] or nuclear preparations [1, 9]. Using tissue imprints offers the advantage of intact, non-cut nuclei combined with preserved cell morphology [24].

The aim of this study was to verify numerical aberrations of chromosome 1 detected by conventional cytogenetics of cell lines and short term cultures of HCC [4, 6, 18–22, 28, 30], using FISH as a new method for direct visualization of chromosome copy numbers in nuclei of cytological samples. Chromosome 8 is suggested to be at least partly multiplicated in HCC [10]. For this reason, the chromosome 8 copy number was determined simultaneously. The significance of chromosome anomalies in malignant liver neoplasms versus normal tissue and benign tumours was explored.

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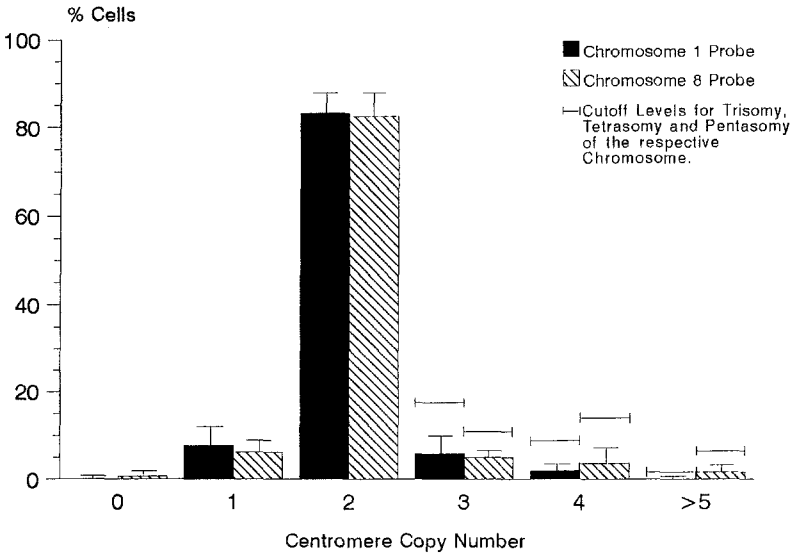

Fig. 1 Copy number of chromosomes 1 and 8 in normal liver tissue. Mean values of nine cases

Materials and methods

Twenty-two liver tumour samples – eight HCC, five cholangiocellular carcinomas (CCC), five LCA and four FNH were obtained from patients undergoing surgical resection in the Medizinische Hochschule Hannover, Germany, between 1990 and 1993. Additionally, normal liver tissue was obtained from nine of these patients, three with cirrhosis. Touch preparations were made onto poly-L-lysine coated slides using a newly cut surface of the fresh, unfixed biopsy material. Cell preparations were then fixed in paraformaldehyde (4% in phosphate-buffered saline) at room temperature for 10 min. Diagnoses were made histopathologically from haematoxylin and eosin stained sections according to Edmondson and Steiner [8].

Metaphase spreads from phytohaemagglutinin-stimulated peripheral blood cells of a healthy male were taken as a control in each hybridization to verify that the probe showed a specific signal.

FISH

Slides were treated in an RNAse solution (0,1%) at 37 °C for 1 h before dehydration with graded ethanols. Samples were then predigested by 0,025% proteinase K (Merck, Darmstadt, Germany) for 15 min at 37 °C to enhance the accessibility of the probe. DNA was denaturated in 70% formamide, 2X standard saline citrate (SSC) at 70 °C for 2 min and preparations were then dehydrated again.

Biotinylated alpha satellite probe at a concentration of 1 µg/100 µl (D1Z5 and D8Z1 detecting chromosomes 1 and 8, respectively, Oncor/Amersham-Buchler, Braunschweig, Germany) was mixed with 30 µl of hybridization mixture (Hybrisol VI, Oncor/Amersham-Buchler, Braunschweig, Germany, containing 65% formamide, 2X SSC and blocking DNA), denaturated by heating in a 70 °C water bath for 5 min and chilled in a 4 °C ice bath. Slides were then incubated with the probes overnight at 37 °C under sealed coverslips.

Probe detection

After washing with 65% formamide, 2X SSC, pH 7,0 at 43 °C, followed by 2X SSC, pH 7,0 at 37 °C, 60 µl of fluorescein isothiocyanate (FITC)-labelled avidin was added for 20 min at 37 °C. The signal was amplified by incubation with anti-avidin antibody for 20 min and a second round of FITC-avidin. Finally, nuclei were counterstained with 25% propidium iodide in antifade solution and slides were covered with a glass coverslip. Specimens were viewed under a Leitz Dialux 20 fluorescence microscope with oil immersion (magnification ×1,000). Results were recorded using Kodak Ektachrome 400 ASA colour slide films.

Evaluation of FISH results

The specimens were evaluated by determining cytomorphology, staining intensity and presence of background staining. The hybridization results were documented as revealing 0, 1, 2, 3, 4, 5 or more than 5 specific signals per nucleus examining at least 100 nuclei per slide. Only intact, non-overlapping nuclei were counted. Signals had to have a bright and approximately equal staining intensity in one nucleus. They were only included if they were completely separate from each other. Paired spots were counted as one signal. Minor hybridization sites and background staining were excluded.

Statistical analysis

Descriptional analysis was performed using mean, range and standard deviation. Signal distribution and polysomy were analysed with the Mann-Whitney-U-test and the χ^2 test, respectively.

Results

The accuracy of the method was confirmed by simultaneous evaluation of lymphocyte metaphase preparations obtained from a healthy donor. In each experiment both metaphase chromosomes 1 or 8 showed a bright hybridization spot in the centromeric region. Strong signals were also obtained in the interphase nuclei of these specimens which showed no statistically significant day-to-day variation in signal distribution.

In normal liver cells, the majority of nuclei revealed two signals for chromosome 1 (mean 83,1%, range 74–92%, Fig. 1) and 8 (mean 82,5%, range 76–89%, Fig. 1), respectively (Table 1). In a minor proportion of interphase nuclei non-specific losses or gains of both chromosomes were noticed (Table 1) being not significantly different from those observed in normal lymphocytes (Table 4).

Table 1 Fluorescence in situ hybridization analysis of malignant and benign liver tumours (*LCA* liver cell adenoma, *FNH* focal nodular hyperplasia, *HCC* hepatocellular carcinoma, *CCC* cholangiocellular carcinoma, – not determined)

Case number	Tissue	Grade[a]	Histological type	Chromosome 1							Chromosome 8						
				Signals per nucleus (percentage of cells with indicated signal frequency)							Signals per nucleus (percentage of cells with indicated signal frequency)						
				0	1	2	3	4	5	>5	0	1	2	3	4	5	>5
1	Normal liver tissue			0	10	74	15	1	0	0	–	–	–	–	–	–	–
2	Normal liver tissue			0	7	83	7	3	0	0	–	–	–	–	–	–	–
3	Normal liver tissue			0	9	89	2	0	0	0	2	8	84	4	1	0	1
4	Normal liver tissue			0	13	80	5	2	0	0	–	–	–	–	–	–	–
5	Normal liver tissue			0	5	92	2	1	0	0	0	8	85	6	1	0	0
6	Normal liver tissue			1	4	81	7	8	0	0	2	5	76	8	7	1	1
7	Cirrhosis			0	11	83	5	1	0	0	0	4	76	6	9	5	0
8	Cirrhosis			0	12	84	4	0	0	0	0	3	89	3	3	1	1
9	Cirrhosis			0	9	82	6	2	1	0	1	10	85	3	1	0	0
10	LCA			0	5	86	8	1	0	0	–	–	–	–	–	–	–
11	LCA			0	5	91	3	1	0	0	–	–	–	–	–	–	–
12	LCA			0	19	79	2	0	0	0	–	–	–	–	–	–	–
13	LCA			0	7	86	6	1	0	0	0	7	91	2	0	0	0
14	LCA			2	5	84	6	3	0	0	1	7	86	5	1	0	0
15	FNH			0	7	90	3	0	0	0	5	3	76	1	14	0	1
16	FNH			0	7	88	4	1	0	0	2	5	84	4	5	0	0
17	FNH			7	8	80	3	2	0	0	0	5	88	4	3	0	0
18	FNH			0	8	91	1	0	0	0	–	–	–	–	–	–	–
19	HCC	IV	Trabecular	0	7	49	44	0	0	0	0	6	34	47	11	1	1
20	HCC	II	Trabecular	0	8	86	6	0	0	0	0	3	39	9	42	1	6
21	HCC	IV	Pseudoglandular	0	7	69	24	0	0	0	–	–	–	–	–	–	–
22	HCC	III	Trabecular	0	10	80	10	0	0	0	0	2	27	12	25	21	13
23	HCC		Fibrolamellar	0	2	63	23	11	1	0	0	9	71	9	7	1	3
24	HCC	II	Compact/trabecular	0	9	84	6	0	1	0	1	5	82	6	6	0	0
25	HCC	III	Trabecular	0	5	57	27	5	3	3	2	2	68	9	13	1	5
26	HCC	II	Trabecular	0	3	64	28	4	0	0	0	0	52	20	26	0	2
27	CCC	II	Glandular	0	15	65	16	0	0	0	–	–	–	–	–	–	–
28	CCC	II	Glandular	0	3	40	57	0	0	0	–	–	–	–	–	–	–
29	CCC	II	Glandular	0	8	50	25	15	2	0	–	–	–	–	–	–	–
30	CCC	III	Adenosquamous	9	9	53	25	3	1	0	–	–	–	–	–	–	–
31	CCC	III	Glandular	2	4	57	27	9	1	0	–	–	–	–	–	–	–
32	Lymphocytes			0	4	89	7	0	0	0	–	–	–	–	–	–	–
33	Lymphocytes			0	6	90	4	0	0	0	–	–	–	–	–	–	–
34	Lymphocytes			0	10	86	4	0	0	0	–	–	–	–	–	–	–
35	Lymphocytes			0	6	87	6	1	0	0	2	11	81	5	1	0	0
36	Lymphocytes			1	8	83	6	2	0	0	–	–	–	–	–	–	–
37	Lymphocytes			1	10	86	3	0	0	0	–	–	–	–	–	–	–
38	Lymphocytes			0	6	89	5	0	0	0	–	–	–	–	–	–	–
39	Lymphocytes			–	–	–	–	–	–	–	4	10	84	1	1	0	0

[a] HCC were graded according to Edmondson and Steiner [8]

Table 2 Cut-off levels for defining trisomy, tetrasomy and pentasomy of chromosomes 1 and 8

	Chromosome 1	Chromosome 8
	Cut-off level	Cut-off level
Trisomy	>17,6%	>11,0%
Tetrasomy	> 9,2%	>14,2%
Pentasomy	> 1,0%	> 6,9%

Based on the evaluation of copy numbers for chromosomes 1 and 8 on normal liver samples, trisomy, tetrasomy and pentasomy for these chromosomes were defined according to Ward et al. [27] and Takahashi et al. [23] if the percentage of nuclei with three or more signals exceeded the mean plus three standard deviations (Table 2, Fig. 1).

No case of liver cirrhosis or benign liver tumours revealed polysomy 1 or 8 employing these criteria (Table 3, Fig. 2a, 2b). Among HCC, polysomy of chromosome 1 occurred in five of eight cases (Table 3, Fig. 2c), all of them displaying trisomy. Polysomy of chromosome 8 was noticed in four of seven HCC, two of which had both trisomy and tetrasomy, whereas the other cases only revealed trisomy or tetrasomy, respectively (Table 3, Fig. 2d). Four of five CCC had polysomy for chromosome 1 (Table 3).

Statistical evaluation of the results revealed significant differences in chromosome copy numbers for chromosome 1 only. The occurrence of trisomy 1 in HCC

Table 3 Number of cases with polysomy of chromosomes 1 and 8 in liver touch preparations

Tissue	Chromosome 1				Chromosome 8			
	Polysomy[a]	Trisomy	Tetrasomy	Pentasomy	Polysomy[a]	Trisomy	Tetrasomy	Pentasomy
Normal liver tissue	0/6	0/6	0/6	0/6	0/3	0/3	0/3	0/3
Cirrhosis	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
LCA	0/5	0/5	0/5	0/5	0/2	0/2	0/2	0/2
FNH	0/4	0/4	0/4	0/4	0/3	0/3	0/3	0/3
HCC	5/8	5/8	1/8	1/8	4/7	3/7	3/7	1/7
CCC	4/5	4/5	1/5	1/5	–	–	–	–

[a] Trisomy, tetrasomy or pentasomy

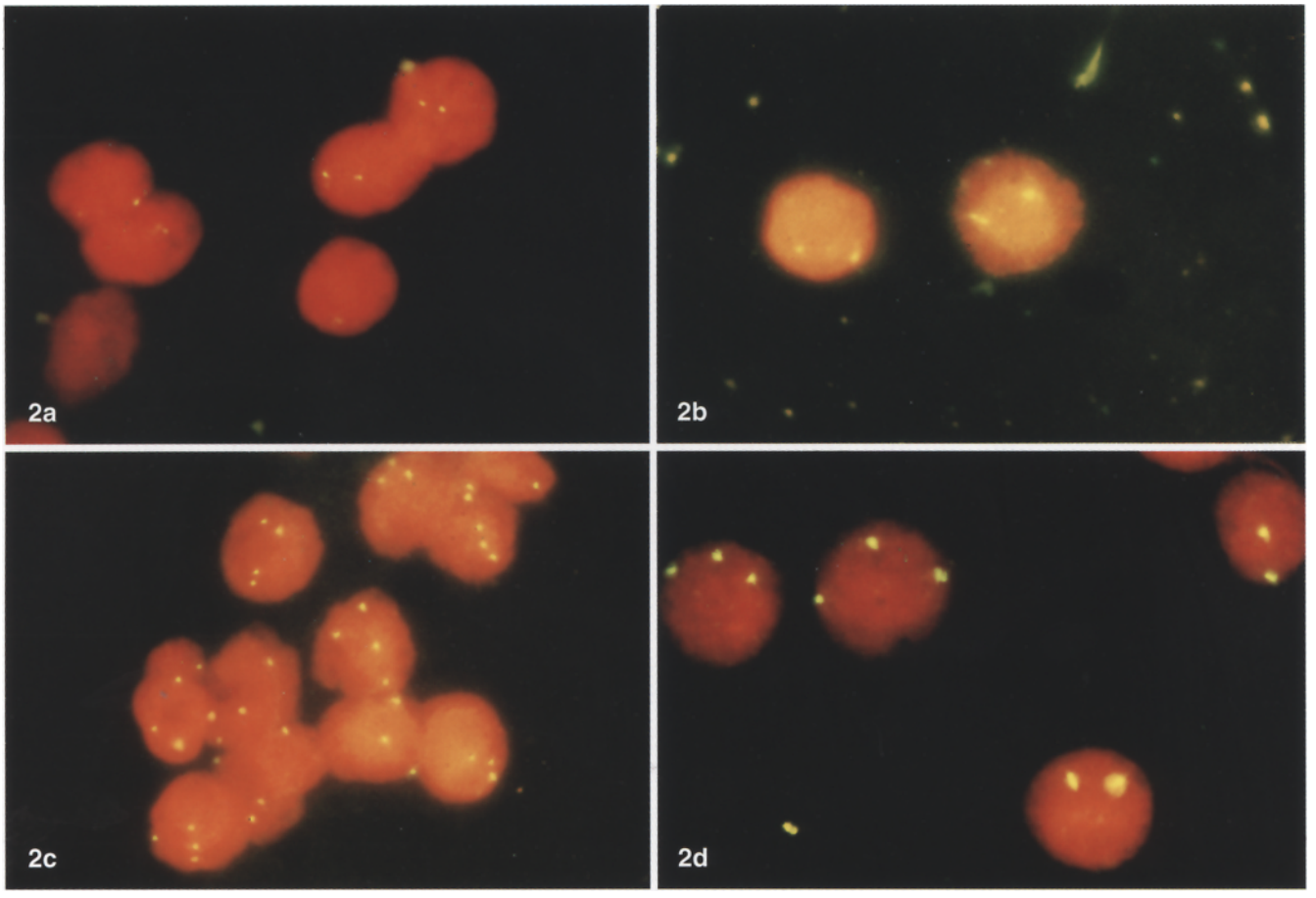

Fig. 2a–d Results of fluorescence in situ hybridization with centromeric probes for either chromosome 1 or 8. **a** Normal liver tissue (case number 8) showing disomy of chromosome 1. **b** Benign liver neoplasm (liver cell adenoma, case number 12) with disomy of chromosome 8. **c** A case of hepatocellular carcinoma (HCC; case number 20) revealing polysomy 1. **d** HCC (case number 26) showing trisomy of chromosome 8. Note that staining with the chromosome 8 (D8Z1) probe reveals brighter and better detectable signals than the chromosome 1 probe (D1Z5). Some of the signals are visible out of the plane of focus. Counterstaining with propidium iodide, original magnification ×1,000

was statistically significant if compared to normal liver tissue ($P \leq 0{,}0048$), LCA ($P \leq 0{,}0242$) and FNH ($P \leq 0{,}0384$, Table 4). Similar results were obtained with trisomy 1 in CCC (Table 4).

For polysomy of chromosome 8, no statistical significant differences could be observed between normal liver tissue, HCC, LCA and FNH (all $P > 0{,}05$, Table 4).

No correlation between grading and numerical changes of both chromosomes could be obtained because of the low number of cases in each group.

Discussion

From conventional cytogenetics it has been suggested that chromosome 1 abnormalities are associated with HCC [4, 6, 18–22, 28, 30]. Since cultivation and generation of metaphases of HCC cells are difficult to perform [13], FISH as a simple and rapid method seems to be the

Table 4 Statistical significance (*P*-values) of polysomy 1 and 8 in normal and cirrhotic liver as well as in benign and malignant liver neoplasms

Tissue	Compared with (tissue)	Chromosome 1			Chromosome 8		
		Trisomy	Tetrasomy	Pentasomy	Trisomy	Tetrasomy	Pentasomy
Normal liver tissue	Lymphocytes	>0,05	>0,05	>0,05	>0,05	>0,05	>0,05
	Cirrhosis	>0,05	>0,05	>0,05	>0,05	>0,05	>0,05
	LCA	>0,05	>0,05	>0,05	>0,05	>0,05	>0,05
	FNH	>0,05	>0,05	>0,05	>0,05	>0,05	>0,05
	HCC	0,0048	>0,05	>0,05	>0,05	>0,05	>0,05
	CCC	0,0015	>0,05	>0,05	–	–	–
LCA	FNH	>0,05	>0,05	>0,05	>0,05	>0,05	>0,05
	HCC	0,0242	>0,05	>0,05	>0,05	>0,05	>0,05
	CCC	0,0098	>0,05	>0,05	–	–	–
FNH	HCC	0,0384	>0,05	>0,05	>0,05	>0,05	>0,05
	CCC	0,0164	>0,05	>0,05	–	–	–
HCC	CCC	>0,05	>0,05	>0,05	–	–	–

technique of choice for chromosome analysis in this tumour type. This method allows the investigation of a large number of tumour cells in a greater number of cases. The use of touch preparations offers the particular advantage of examining intact, non-cut nuclei in combination with preserved cytomorphology [24] when compared with tissue sections and nuclear preparations.

In the present study, we analysed malignant and benign liver tumours for numerical changes of chromosomes 1 and 8 based upon visualization of centromeric regions of these chromosomes by FISH. Trisomy 1 was found to be significantly increased in malignant liver lesions (HCC and CCC) when compared with benign tumours and normal tissue. Tetrasomy and pentasomy 1 and polysomy 8 was detected in malignant lesions but did not reach statistical significance when compared with benign lesions or normal tissue. Normal liver tissue, cirrhotic liver and benign neoplasms failed to show any significant numerical changes of chromosomes 1 or 8.

The most consistent aberrations detected concerning chromosomes 1 in HCC are polysomy 1 and deletions or translocations involving the short arm of chromosome 1 [4, 6, 21, 22, 28]. However, since trisomy 1 has also been observed in CCC in our studies, the specifity of this chromosome abnormality remains unclear. In general, chromosome 1 abnormalities have been suggested to be associated with advanced stages of malignant neoplasms [3].

Cytogenetic studies concerning chromosome 8 in liver tumours are controversial. Although certain abnormalities have been described in HCC, no constant aberration has been determined by karyotyping. Chen et al. [6] reported trisomy 8 in an HCC cell line, whereas Bardi et al. [4] found monosomy 8 after a short term culture of HCC. Our results showed trisomy 8 in some cases of HCC which did not reach statistical significance when compared with normal liver tissue. From restriction fragment length polymorphism analyses, evidence has risen that parts of chromosome 8, most of them located on the long arm, can be found in multiple copies in HCC [10]. With the approach chosen in the present study, however, only changes in copy numbers including the centromeric region of the chromosomes could be detected, thus isolated multiplication of parts of the long arm might have been missed.

Numerical aberrations of other chromosomes (11 and 13) may also occur in HCC [4, 21, 28]. However, these changes are inconsistent and do not seem to present up to six copies of the respective chromosome as observed with chromosome 1 [28]. Among structural changes, several studies focus on chromosome 4 where LOH has been found [12, 32].

Benign liver neoplasms are still poorly characterized cytogenetically. None of our cases reported herein showed numerical aberrations for chromosome 1 or 8. Compared with normal liver tissue and liver cirrhosis, benign lesions presented no statistical significant difference in chromosome copy numbers. This is consistent with the studies of Deprez et al. [7] who found a diploid DNA content in non-malignant liver tumours and a predominance of diploid cases in liver cirrhosis as characterized by Feulgen staining.

Histological features of liver neoplasms are sometimes difficult to interpret especially if only small amount of tissue is available. Genetic changes, however, have been shown to be characteristic for malignant tumour entities in our study. Because of the fairly low number of cases reported, further evaluation is needed. The introduction of FISH into pathology as an additional diagnostic tool may nevertheless be helpful in the differential diagnosis of hepatic malignancies [29].

We conclude that FISH provides a rapid and simple method to investigate cytogenetic changes in liver neoplasms. However, since the α-satellite probes used in our study are only suitable to detect gains or losses of the centromeric regions of the respective chromosome, further structural abnormalities cannot be excluded. For the additional identification of structural aberrations, specific cosmid probes [5, 14] or multi-colour techniques [11, 25] are needed. As suggested by our results, FISH can help to verify conventional cytogenetic findings in a larger number of cases and may provide a valuable tool in the diagnosis of malignant versus benign liver neoplasms.

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