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ORIGINAL ARTICLE

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Expression of MUC1, Thomsen-Friedenreich antigen, Tn, sialosyl-Tn, and α2,6-linked sialic acid in hepatocellular carcinomas and preneoplastic hepatocellular lesions

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Abstract The expression of epithelial mucins and Thomsen-Friedenreich-related antigens in preneoplastic and neoplastic hepatocellular lesions was systematically investigated using an in situ immunohistochemical staining approach. MUC1, MUC2, TF, sialosyl-TF, Tn, sialosyl-Tn, α2,3-linked sialic acid, and α2,6-linked sialic acid were examined in normal and cirrhotic human liver and in human hepatocellular carcinomas (HCCs) and cholangiocarcinomas. Normal hepatocytes and preneoplastic foci of altered hepatocytes did not express MUC1, MUC2, TF, Tn, s-Tn, or α2,6-linked sialic acid. In contrast, HCCs showed positive reactions for MUC1, TF, Tn, s-Tn, and α2,6-linked sialic acid. MUC2 was absent in normal biliary epithelial cells, but present in cholangiocarcinomas. The staining of MUC1, or s-Tn and α2,6-linked sialic acid in human normal liver tissues and various liver diseases did not change after specific treatments such as periodate oxidation or saponification, indicating that their expression in HCC does not result from incomplete glycosylation or low *O*-acetylation, respectively. MUC1, TF, Tn, s-Tn, and α2,6-linked sialic acid may be useful as indicators of progression of HCC in tissue sections, and perhaps also as targets for diagnostic and therapeutic approaches in vivo.

Key words Liver · Mucin · Thomsen-Friedenreich-related antigens · Carbohydrates · Cytokeratin

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Introduction

Hepatocellular carcinoma (HCC) is one of the major cancers of Man, causing at least 250,000 deaths annually throughout the world. In its early stages, it is usually asymptomatic and not accompanied by physical signs, and coexisting with cirrhosis, thus making diagnosis difficult at the time when a cure is still possible. Early detection, accurate distinction of HCCs from benign hepatocellular lesions and improved monitoring of HCCs are urgently needed. The identification of serum tumour markers is a potential method for early clinical recognition of HCC. α-Fetoprotein is one marker used for the diagnosis of HCC, but both false-negative and false-positive results occur [14] and more reliable markers are necessary.

Thomsen-Friedenreich-related histo-blood group antigens (TF/Tn system, including TF, Galβ1–3GalNAcα-*O*-; sialosyl-TF, NeuAcα2–3Galβ1–3GalNAcα-*O*- and Galβ1–3(NeuAcα2–6)GalNAcα-*O*-; Tn, GalNAcα-*O*-; sialosyl-Tn, NeuAcα2–6GalNAcα-*O*-) constitute a group of important carbohydrate epitopes (glycotopes) of glycoconjugates. The precursor of TF is Tn. While Galβ1–3GalNAcα-*O*- and GalNAcα-*O*-saccharide are ubiquitous core structures found in a cryptic manner in many glycoproteins, their expression as TF and Tn glycotopes is restricted to specific carrier proteins in specific cells and organs. Sialosyl-Tn is also a glycotope in biosynthesis-linked TF and Tn. TF, Tn, and s-Tn glycotopes occur in only limited amounts in normal adult human tissues [4] but have been reported as pancarcinoma antigens expressed on cancer cells [15]. In epithelial cells TF and Tn glycotopes are mainly carried by mucins, a family of highly glycosylated, high-molecular-weight glycoproteins present on the surfaces of many glandular epithelial cells and in their secretion products [8]. Mucins can be subdivided into two types, secretory and membrane-bound mucins, the former providing in large part the viscous gel that covers most mucosal surfaces. Membrane-bound mucins are major components of the apical surfaces of epithelial cells that contain a hy-

drophobic stretch of amino acid residues, anchoring the long filamentous molecules in the plasma membrane.

MUC1 (previously labelled episialin, EMA, MAM-6, HMFGP, CA15–3) is a membrane-bound mucin. Its protein backbone (apomucin) consists of an extracellular domain of 20 amino acid residues repeated many times in tandem, a transmembrane sequence, and a 69-amino acid-long cytoplasmic tail. Expression of MUC1 has been described in the normal bile duct, breast, pancreas, gastrointestinal tract, and other gland-epithelial tissues [5, 8, 15]. MUC2 is a secretory mucin with a central region composed of an extended set of tandem repeat sequences of 23 amino acids each. It is present in normal gastrointestinal secretion products and epithelia, and in some tumours [10]. Alterations in glycosylation and cellular distribution of Thomsen-Friedenreich-related antigens glycoconjugates and mucins are some of those changes in cellular phenotypes that emerge during malignant transformation and may lead to altered cellular behaviour. In the clinical context, these tumour-associated glycoconjugate changes promise to be useful markers for diagnosis and targets for immunotherapy [30].

Overexpression of apomucins and Thomsen-Friedenreich-related antigens in human cholangiocarcinomas has been shown previously [34]. Binding of some lectins in cirrhotic liver and HCC has also been demonstrated [21, 35]. However, few systematic studies addressing MUC1, MUC2, and Thomsen-Friedenreich-related antigens in hepatocellular carcinomas and preneoplastic hepatic lesions have been performed. The reactivity of anti-MUC2, anti-TF, anti-Tn, amaranthin (*Amaranthus caudatus* agglutinin, ACA, binding Galβ1–3GalNAcα-*O*- or NeuAcα2–3Galβ1–3GalNAcα-*O*-), and *Sambucus nigra* agglutinin (SNA, binding Neu5Ac(α2–6)Gal/GalNAc) in HCC and preneoplastic hepatic lesions has not been investigated previously.

Liver tissue contains several types of cells, such as hepatocytes, biliary epithelial cells, endothelial cells, Kupffer cells, and Ito cells. To study the expression of the antigens mentioned above in different cell types we used an in situ immunohistochemical approach to label these antigens in normal human liver and in various liver diseases. Cytokeratin 19 (CK19), an intermediate filament protein preferentially occurring in cholangiocytes under normal conditions, was demonstrated in the same human tissues for comparison. Expression of these antigens in foci of altered hepatocytes, which are considered to be preneoplastic lesions frequently associated with cirrhosis and neoplasia [2, 28], was also examined.

Materials and methods

A total of 87 explanted and resected livers were studied. Samples from 8 donor livers were used as normal tissue. Pathologically altered tissues were taken from 42 hepatocellular carcinomas, 33 cases of liver cirrhosis (15 posthepatitic, 8 alcoholic, 4 cryptogenic, 6 biliary), 2 hepatic glycogenoses, and 2 cholangiocellular carcinomas. The study protocol was approved by the Ethics Commission of the Medical Faculty of the University of Heidelberg. All samples were fixed in Carnoy's solution or in 10% buffered formalin and embedded in paraffin. Preneoplastic foci of altered hepatocytes, particularly glycogenotic clear cell and mixed cell foci, were identified and classified in tissue sections (treated with haematoxylin-eosin and periodic acid-Schiff) according to the criteria of Su et al. [28]. Hepatocellular carcinomas were diagnosed histologically and graded according to the classifications of the World Health Organisation [7] and Kojiro [16].

The antibodies A78-G/A7 (anti-TF-α and TF-β [13]) and A76-A/C7 (anti-MUC1, which recognises the peptide epitope APDTRP of the MUC1 tandem repeat [6]), and A53-B/A2 (anti-CK19 [12]) were described previously; CCP58 (anti-MUC2 [33]) was kindly provided by Dr. P-X. Xing (Heidelberg, Victoria, Australia); HMFG-2 (anti-MUC1, which recognises the epitope DTR), HBTn (anti-Tn), and B72.3 (anti-s-Tn) were purchased from Dianova (Hamburg, Germany), DAKO (Copenhagen, Denmark), and Biogenesis (Bournemouth, UK), respectively. Biotinylated preparation of the lectins amaranthin (*Amaranthus caudatus* agglutinin, ACA, which recognises the Galβ1–3GalNAcα-*O*- or NeuAcα2–3Galβ1–3GalNAcα-*O*- [23]), and *Sambucus nigra* agglutinin (SNA, which recognises the Neu5Ac(α2–6)Gal/GalNAc [26]) were obtained from Vector Laboratories (Burlingame, Calif.).

Staining of tissue sections was performed by the avidin–biotin peroxidase complex method with a commercial kit (Vectastain ABC Elite Kit, Vector Laboratories) as follows. Paraffin sections 4 µm thick were deparaffinized in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was eliminated by treatment with 3% H_2O_2 in methanol for 30 min at room temperature. Nonspecific binding sites were blocked with normal rabbit serum. After washing with phosphate-buffered saline, sections were incubated overnight at 4°C with mAbs or biotinylated lectins at appropriate dilutions. The thoroughly washed sections (except those stained with biotinylated lectins) were treated with biotinylated anti-mouse immunoglobulin antiserum for 30 min at room temperature, and thereafter with the avidin–biotin peroxidase complex. Colour development during incubation with the peroxidase substrate (diaminobenzidine) was controlled under a microscope. Counterstaining was performed with haematoxylin. Positive controls were normal colon for MUC2 and colon carcinomas for the other antigens. Negative controls were incubated with a comparable dilution of an IgM and IgG from a mouse plasmocytoma (Sigma) instead of the mAb.

For the immunohistochemical detection of sialosyl-TF using anti-TF mAb, sections were incubated with neuraminidase from *Vibrio cholerae* (Serva, Heidelberg, Germany) at a concentration of 0.02 U/ml in phosphate-buffered saline containing 0.01 M Ca^{2+} for 1 h at room temperature to remove NeuAc, washed, and reacted with anti-TF mAb. Erythrocytes present in vessels in the tissue sections functioned as an internal positive control for sialosyl-TF.

Pretreatment by partial deglycosylation was carried out on the sections before incubation with anti-MUC1 and anti-MUC2 mAbs as previously described [5]. Sections were incubated for 30 min at room temperature with 20 mM periodic acid in acetate buffer, 0.05 M, pH 5. After three rinses in phosphate-buffered saline, the sections were neutralised with 1% glycine for 30 min, then further rinsed in phosphate-buffered saline. Staining of normal colon epithelium by A76-A/C7 (anti-MUC1) after the periodic acid treatment was used as positive control for periodate oxidation.

O-Acetyl substituents were removed from sialic acid with KOH pretreatment before incubation with anti-s-Tn, ACA, and SNA as described by Jass et al. [11]. Sections were immersed in 0.5% KOH in 70% alcohol for 30 min at room temperature. Thereafter, sections were rinsed in 70% alcohol and washed in tap water for 10 min. Staining of normal colon epithelium with B72.3 (anti-s-Tn) served as a positive control for saponification.

The extent of the immunohistochemical reaction in neoplastic and preneoplastic hepatocellular lesions was evaluated as follows: –: all cells negative; +: <30% of the cells positive; 2+: 30%–60% of the cells positive; 3+: >60% of the cells positive. The percentage of positive cells was estimated with a ×10 objective lens.

Data were analysed with Fisher's exact probability test [24]. Differences were considered significant if the probability was less than 0.05.

Results

The results of staining for anti-MUC1, anti-MUC2, anti-TF, anti-TF after neuraminidase treatment, anti-Tn, anti-s-Tn, ACA, and SNA in human donor liver and various liver diseases including cirrhosis, metabolic diseases, hepatocellular carcinomas, and cholangiocarcinomas are documented in Fig. 1 and summarised in Table 1. The results obtained with HMFG-2 and A76-A/C7 were similar, and were combined. Foci of altered hepatocytes particularly glycogenotic clear cell foci (glycogen-storing foci, 55 cases) and mixed cell foci (80 cases), observed in posthepatitic or alcoholic liver cirrhosis did not express MUC1, MUC2, TF, Tn, s-Tn, or α2,6-linked sialic acid. In contrast, HCCs showed positive reactions for MUC1, TF, Tn, s-Tn, and α2,6-linked sialic acid. The frequency and extent of positive reactions in HCC ranked as follows (Fig. 2): anti-TF with neuraminidase pretreatment and ACA >anti-Tn >anti-MUC1>anti-TF >anti-s-Tn >SNA. At least one of the five antigens (MUC1, TF, Tn, s-Tn, α2,6-linked sialic acid) was expressed in about 70% of HCCs. An interesting observation was that 12 cases of HCC (29%) were positive for Thomsen-Friedenreich-related antigens but negative for MUC1. In HCCs and cholangiocarcinomas that were positive for the antigens, the reaction was predominantly observed in the cytoplasm of the tumour cells. In 2 cholangiocarcinomas and 7 HCCs containing adenoid (pseudoglandular) formations, membrane staining was also seen. In several cases of chronic liver disease, strict apical membrane localisation of TF, Tn, or s-Tn antigens was observed in some but not all proliferating biliary epithelial cells. One case of a carcinoid was positive for anti-MUC1, anti-TF, anti-Tn, ACA, SNA, and anti-CK 19, and negative for anti-MUC2 and anti-s-Tn.

The staining of anti-MUC1 and anti-MUC2 in human tissues was not significantly enhanced by analogous periodate treatment. Saponification with KOH did not increase the reactions of anti-s-Tn, ACA, or SNA with human normal or altered cells.

Comparing the expression of MUC1 and CK19 in human normal and pathologically altered liver, we observed that normal hepatocytes did not express either MUC1 or CK19, while biliary epithelial cells expressed both antigens at different sites (MUC1 was localised in the apical membrane and CK 19 was diffusely distributed in the cytoplasm). Oval cells in cirrhosis expressed CK19 strongly and MUC1 not at all. Preneoplastic clear and mixed cell lesions were negative for MUC1, but amphophilic cell populations displayed positive staining for CK19; the expression of MUC1 and CK19 was not correlated in HCCs. Expression of MUC1 and CK19 was negative in 22 of 42 HCCs; in 7 HCCs MUC1 was positive but CK19 was negative; a negative reaction for MUC1 and a positive reaction for CK19 was found in 4 HCCs; finally, in 9 HCCs both MUC1 and CK19 were positive.

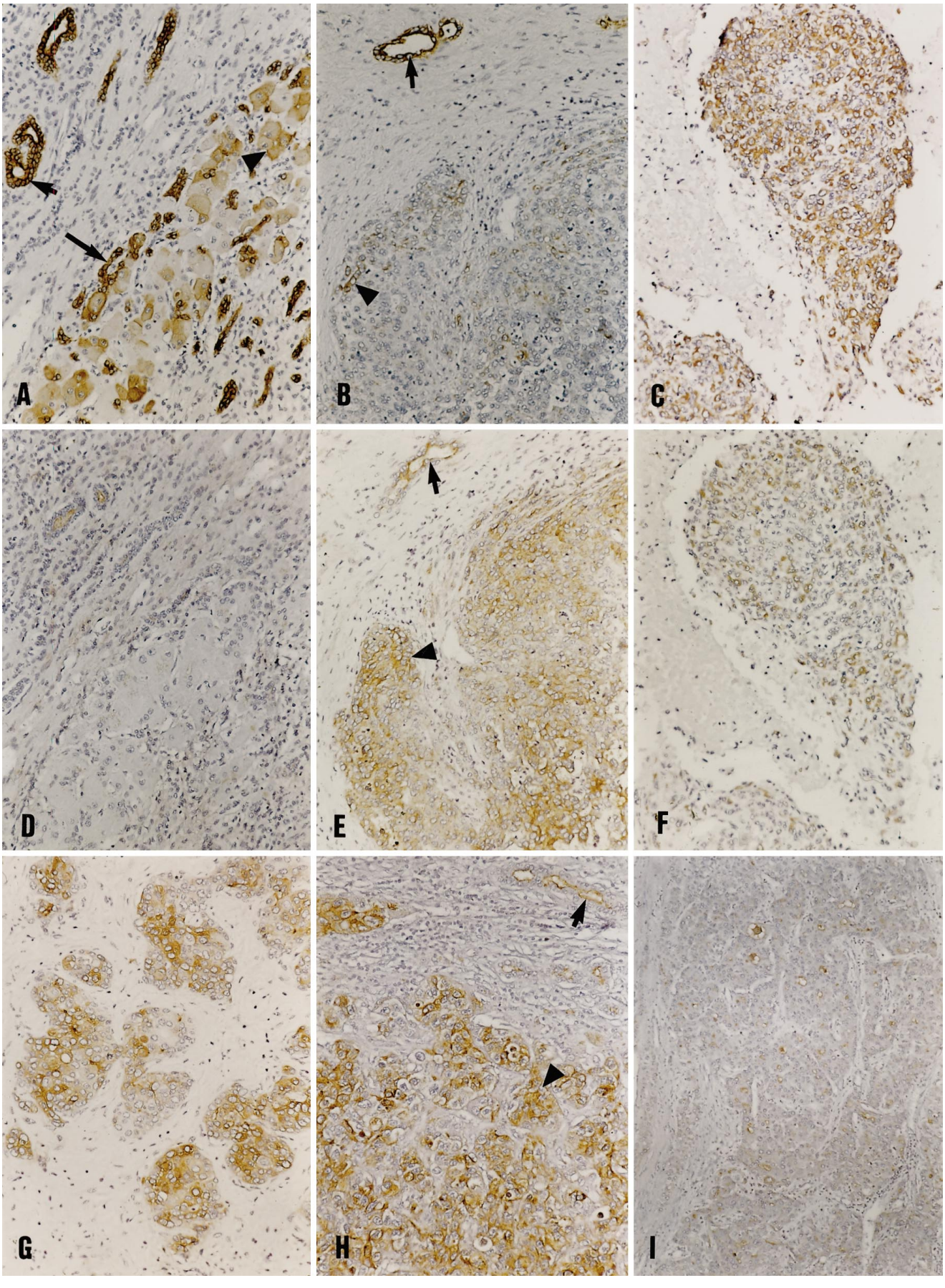
Discussion

The expression of Thomsen-Friedenreich-related antigens, which are considered to be oncodevelopmental markers, and of epithelial mucins in tumours correlates closely with the phenotypic changes that occur during neoplastic transformation. These antigens appear to be involved in the fundamental functional changes that occur during transformation of an ordinary epithelial cell into a carcinoma cell [9, 10, 15]. Clinically, quantitative and/or qualitative alterations in Thomsen-Friedenreich-related antigens and epithelial mucins have been widely

Table 1 Expression of MUC1, MUC2, cytokeratin 19, and Thomsen-Friedenreich-related antigens in donor liver tissues and various liver lesions. Cases for which a specific location of staining is not noted were mainly diffusely stained. No. of cases reactive/total no. of cases examined (percentages)

Tissues	MUC1	MUC2	TF	N+G/A7[a]	ACA	SNA	Tn	s-Tn	CK19
Donor liver									
Hepatocytes	0/6	0/6	0/6	3/6(50)[b]	3/6(50)[b]	0/6	0/6	0/6	0/6
Cholangiocytes	6/6(100)[c]	0/6	0/6	6/6(100)[c]	6/6(100)[c]	0/6	0/6	0/6	6/6(100)
Glycogenosis Ia									
Hepatocytes	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Cholangiocytes	2/2(100)[c]	0/2	0/2	2/2(100)[c]	2/2(100)[c]	0/2	0/2	0/2	2/2(100)
Cirrhosis									
Hepatocytes	0/33	0/33	0/33	17/33(52)[b]	17/33(52)[b]	0/33	0/33	0/33	12/33(36)
Cholangiocytes	33/33(100)[c]	0/33	6/33(18)[c]	33/33(100)[c]	33/33(100)[c]	0/33	6/33(18)[c]	4/33(12)[c]	33/33(100)
Hepatocellular carcinoma	16/42(38)	0/42	16/42(38)	24/24(57)	24/42(57)	9/42(21)	20/42(48)	9/42(21)	13/42(31)
Well differentiated	1/7(14)	0/7	2/7(29)	2/7(29)	2/7(29)	0/7	2/7(29)	1/7(14)	0/7
Moderately differentiated	13/31(42)	0/31	13/31(42)	21/31(68)	21/31(68)	8/31(26)	16/31(52)	7/31(23)	11/31(35)
Poorly differentiated	2/4(50)	0/4	1/4(25)	1/4(25)	1/4(25)	1/4(25)	2/4(50)	1/4(25)	2/4(50)
Cholangiocarcinoma	2/2	1/2	2/2	2/2	2/2	1/2	2/2	1/2	1/2

[a] G/A7 after neuraminidase treatment
[b] Present in the supranuclear cytoplasm or Golgi region
[c] Present along the luminal surface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◀ **Fig. 1A–I** Immunohistochemical findings for MUC1, TF, Tn, s-Tn, α2,6-linked sialic acid and CK19 in human cirrhosis, hepatocellular carcinoma (HCC), and cholangiocarcinomas. **A**, **D** Serial sections through a cirrhotic liver. **A** A53-B/A2 (anti-CK19) stains the cytoplasm of hepatocytes (*arrowhead*), biliary epithelial cells (*shorter arrow*), and ductular (oval) cells (*longer arrow*) diffusely; **D** A76-A/C7 (anti-MUC1) stains the apical membrane of biliary epithelial cells. **B**, **E** Serial sections through an HCC. **B** A53-B/A2 stains the cytoplasm of some tumour cells (*arrowhead*) and all biliary epithelial cells (*arrow*) diffusely; **E** A76-A/C7 stains the cytoplasm of most tumour cells (*arrowhead*) diffusely, but only the apical membrane of biliary epithelial cells (*arrow*). **C**, **F** Serial sections through an HCC. **C** HBTn (anti-Tn) and **F** B72.3 (anti-s-Tn) stain the cytoplasm of many tumour cells diffusely. **G** In a case of HCC, A78-G/A7 stains the cytoplasm of most tumour cells diffusely. **H** In a case of cholangiocarcinoma, A76-A/C7 stains the apical membrane of biliary epithelial cells (*arrowhead*) and the cytoplasm of tumour cells (*arrow*). **I** In a case of HCC, SNA (binding α2,6-linked sialic acid) stains the plasma membrane and the cytoplasm of most tumour cells weakly. **A–H** ×200, **I** ×100

used as indicators in diagnosis and as targets for immunotherapy [30]. Thus, a basic comprehensive knowledge of the occurrence of mucins, Thomsen-Friedenreich antigen and its biosynthetically linked carbohydrate antigens (s-TF, Tn, and s-Tn) in normal liver tissues and various liver diseases, including cirrhosis, metabolic diseases, and hepatocellular and cholangiocellular neoplasia, is of interest for both basic research and clinical understanding.

In this study, 38% and 20% hepatocellular carcinomas from human beings expressed MUC1 and s-Tn, which is in line with the results of Ma et al. [18]. The occurrence of TF in HCC has already been demonstrated by biochemical assays with lectins in a few cases [22]. For our immunohistochemical approach, a new anti-TF mAb with higher specificity was used in a total of 42 HCCs, 38% of which expressed this antigen. The expression of MUC1, MUC2, TF, Tn, s-Tn, and α2,6-linked sialic acid in human normal liver and chronic liver diseases including HCC has not been investigated previously. None of these antigens were demonstrable by immunohistochemistry in human normal liver parenchyma or benign hepatocellular lesions, including preneoplastic foci altered hepatocytes in the present study. In contrast, MUC1, TF, Tn, s-Tn, and α2,6-linked sialic acid were present in HCCs. When the five antigens were combined, 70% of the HCC showed a positive reaction. Thus, these antigens may be useful as indicators of progression of HCC in tissue sections and, perhaps, also as targets for diagnostic and therapeutic approaches in vivo. However, a larger number of cases has to be investigated to permit final conclusions. The antigens studied are also expressed at the surface of cancer cells and may, hence, be released into the blood. It remains to be seen whether some or all of these antigens are released and whether they have any potential for clinical application in early detection and/or serological determination of progression of HCC.

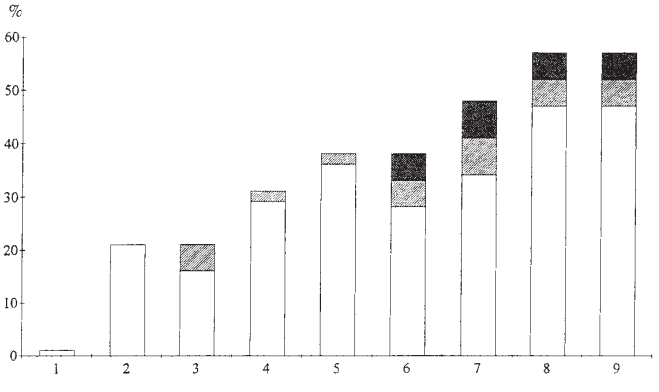

Fig. 2 Percentage of positively reacting cases and extent of reaction with *1* anti MUC2, *2* SNA, *3* anti-s-Tn, *4* anti-CK19, *5* anti-TF, *6* anti-MUC1, *7* anti-Tn, *8* anti-TF with neuraminidase pretreatment, *8* and *9* ACA in HCCs (□ <30% of the cells positive; ▨ 30%–60% of the cells positive; ■ >60% of the cells positive)

MUC1 is expressed widely in normal epithelial tissues and carcinomas, such as breast, colon, rectum, stomach, and pancreas carcinomas [5, 10], and has been considered to be a marker for epithelial tissues. Our results clearly show that MUC1 is present in HCCs; similar findings were previously reported for HCCs [18] and hepatoblastomas [1]. Therefore, MUC1 (CA15-3) and mucin-type carbohydrate antigens, which have been used extensively in histological and serological diagnosis of carcinomas, are not useful for distinguishing HCCs from other carcinomas, such as cholangiocarcinomas and gastrointestinal carcinomas.

The alterations in the expression of mucins and Thomsen-Friedenreich-related antigens may be of biological significance. For example, TF antigen on the surface of extrahepatic cancer cells may lead to binding to normal hepatocytes [3]; sialic acid-dependent cell adhesion to collagen IV correlates with in vivo tumorigenicity in a variety of neoplasm [20]; enhanced mucin and α2,6-linked sialic acid strongly reduce the contacts between epithelial cells [9, 19].

The mechanisms underlying the expression of MUC1 and Thomsen-Friedenreich-related antigens in HCCs is not clear. In breast and colon cancers, overexpression of MUC1 is related to up-regulated mucin synthesis [9], incomplete glycosylation [5, 17], and accumulation. Apolar localisation of MUC1 in these tumour types may be due to obstruction of mucin transport within the tumour cells. TF and Tn exposure in colon cancer results from several defects in glycosyltransferase activities [32], and s-Tn expression relates to reduction in sialic acid *O*-acetylation [11]. In this study, we found that the reactions for apomucin or sialic acid were not significantly changed after specific pretreatment procedures such as periodate oxidation or saponification in human normal liver tissues and various liver diseases. This indicates that MUC1, or both s-Tn- and α2,6-linked sialic acid expression in HCCs does not result from incomplete glycosylation or low *O*-acetylation, respectively. It is noteworthy that a number of HCCs expressed only Thomsen-Friedenreich-related antigens and did not possess MUC1, to which

these antigens are usually bound. It remains to be seen which other carrier molecules of Thomsen-Friedenreich-related antigens are available under these conditions.

CK19 is present in early fetal hepatocytes, but disappears at about the 10th week [27]. In biliary epithelial cells, however, it remains and it is also expressed in oval cells, which often proliferate in response to chronic liver injury. MUC1 is not demonstrable in fetal or mature hepatocytes [25], nor yet in the oval cells of cirrhotic livers. However, it is expressed at the apical surfaces of biliary epithelial cells with differentiation and formation of bile ducts. CK19 has previously been shown to be sometimes present in foci of altered hepatocytes and HCCs and has been regarded as a possible marker for a transdifferentiation of hepatocytes to ductular cells during hepatocarcinogenesis [29]. Similarly, the occurrence of MUC1 in HCCs might indicate a transdifferentiation of hepatocytes to ductular cells. MUC1 might be a particularly appropriate marker in this context since, in contrast to CK19, it is not expressed in fetal hepatocytes.

Human normal bile ducts do not show MUC2, and cholangiocarcinomas express this antigen ectopically, as reported previously [34]. One recent study revealed that MUC2 mRNA was detectable in the normal cholangiocytes [31]. But the presence of MUC2 protein was not demonstrable by means of immunohistochemical staining even in combination with mild, carbohydrate-specific periodate pretreatment in the present study.

In conclusion, MUC1, TF, Tn, s-Tn, and α2,6-linked sialic acid are expressed in human HCC, but are not useful for distinguishing HCCs from other carcinomas, such as cholangiocarcinomas and gastrointestinal carcinomas. These antigens are absent from normal liver parenchyma and benign hepatocellular lesions, but emerge increasingly in HCCs with progression. The clinical implications and functional significance of this abnormal expression of MUC1 and Thomsen-Friedenreich-related antigens in HCCs require further study.

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