Glycobiology vol. 14 no. 1 pp. 39–49, 2004
DOI: 10.1093/glycob/cwh002

Expression of β-galactoside α2,6 sialyltransferase and of α2,6-sialylated glycoconjugates in normal human liver, hepatocarcinoma, and cirrhosis

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Received on July 22, 2003; revised on September 2, 2003; accepted on September 2, 2003

β-Galactoside α2,6-sialyltransferase (ST6Gal.I) mediates the addition of α2,6-linked sialic acid to glycoproteins. ST6Gal.I is strongly expressed by the liver and is up-regulated in several cancers, but little is known of its regulation in human liver diseases. We have investigated the expression of ST6Gal.I and its product, the α2,6-sialylated lactosamine, in normal human liver, hepatocarcinoma (HCC), and cirrhosis. We found that both ST6Gal.I activity and mRNA can undergo up- or down-regulation in different HCC patients. At the mRNA level, the groups of specimens showing the highest expression were HCC of grade 2, HCC developed without preexisting cirrhosis, and HCC of male patients. The lectin from *Sambucus nigra* (SNA) reveals a significative overexpression of α2,6-sialylated glycoconjugates in HCC tissue homogenates and their intracellular accumulation in HCC histological sections, even though in a few cases the extent of α2,6-sialylation dramatically decreases. Transcription of the gene occurs through at least two different promoters, resulting in two differentially expressed mRNA species. RNA *in situ* hybridization reveals that the ST6Gal.I mRNA can be expressed at a quantitatively heterogeneous level among the neoplastic cells. Neither ST6Gal.I expression nor α2,6-sialylation are altered in cirrhosis. These data indicate that neoplastic transformation but not cirrhosis can alter the process of α2,6-sialylation of liver glycoproteins.

Key words: cirrhosis/hepatocarcinoma/liver/*Sambucus nigra* agglutinin/sialyltransferases

Introduction

The liver continuously synthesizes a huge amount of proteins, whose principal fates are to be secreted into the blood stream or inserted into the cell membranes. For both secreted and membrane proteins, glycosylation represents one of the most frequently occurring posttranslational modifications, and in some cases the sugar chains of glycoproteins play highly specific roles, such as the mediation of cell–cell and cell–matrix interactions and the binding of microorganisms and toxins (Varki, 1993). In several pathological conditions and especially in cancer, glycosylation undergoes profound changes, which in some cases are specific for a given malignancy (Dall'Olio, 1996).

Sialic acids are sugars bearing a negative electric charge at physiological pH values, frequently terminating the sugar chains of glycoproteins and glycolipids, whose presence profoundly affects the chemicophysical as well as the biological properties of the glycoconjugates (Schauer, 2000). A determinate number of principal sialyl linkages has been described so far: sialic acid may be linked either through an α2,3- or an α2,6-bond to subterminal galactose; through an α2,6-bond to N-acetylgalactosamine (GalNAc); or through an α2,8-bond to another sialic acid, forming polysialic acid. The different sialyl linkages are elaborated by different members of the sialyltransferase family, a class of glycosyltransferases sharing the CMP–sialic acid as donor substrate but differing for the glycosidic structure on which they act and for the type of glycosidic linkage they form (Dall'Olio and Chiricolo, 2001; Harduin-Lepers *et al.*, 2001).

β-Galactoside α2,6-sialyltransferase I (ST6Gal.I according to the nomenclature proposed by Tsuji *et al.*, 1996) has long been thought to be the only sialyltransferase able to catalyze the α2,6-sialylation of galactose (Weinstein *et al.*, 1982a,b, 1987; Taatjes *et al.*, 1988). A second β-galactoside α2,6-sialyltransferase gene has been recently cloned (ST6Gal.II), but because this enzyme sialylates mainly oligosaccharides (Takashima *et al.*, 2002; Krzewinski-Recchi *et al.*, 2003), ST6Gal.I remains the only α2,6-sialyltransferase acting on glycoproteins so far identified. The gene encoding ST6Gal.I maps in chromosome 3 (q21–q28) (Wang *et al.*, 1993), spans at least 145,000 bp of genomic DNA and is made up of at least 9 exons (Figure 1).

Studies performed in the past decade have revealed that at least three major mRNA species, sharing exons I–VI but differing in the 5′-untranslated regions (UTRs), can be generated in a tissue-specific manner through the use of different promoters and a differential assemblage of 5-UT sequences (Svensson *et al.*, 1990; Wang *et al.*, 1990) (Figure 1). The first mRNA form, whose cDNA was cloned from a placenta cDNA library (Grundmann *et al.*, 1990), contains the 5′-UT exons Y and Z (YZ form), is transcribed through the P_3 promoter and is expressed by many tissues (Wang *et al.*, 1993). The second species, characteristic of mature B-lymphocytes, contains the 5′-UT exon X (X form) (Stamenkovic *et al.*, 1990) and is transcribed through the P_2 promoter. The third type of transcript (H form), cloned

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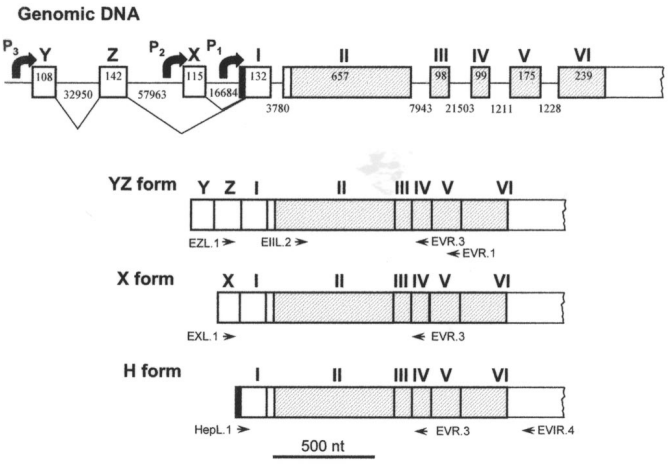

Fig. 1. Schematic representation of the genomic organization of the gene encoding ST6Gal.I and of the three major ST6Gal.I transcripts so far identified in human tissues. Details on the genomic organization has been deduced from public genomic databases. The numbers inside the exons (boxed) indicate the exon length; the numbers between exons indicate intron length. The shaded portion represents the protein coding region. The gene is made up of at least nine exons: exons Y, Z, and X are expressed only in specific transcripts, and exons I–VI are common to all known isoforms. Transcription can be initiated by at least three different promoter regions (P_1 to P_3), indicated by arrows. Through alternative promoter usage and alternative splicing, three major transcripts differing for a portion of the 5′ UTRs are generated. The YZ and X forms are characterized by the presence of the 5′ untranslated exons YZ and X, respectively. The first form is thought to be ubiquitous, and the second is expressed by mature B lymphocytes. The H form is characterized by the presence of a short sequence (solid), absent in the other two transcripts, which is contiguous to exon I in genomic DNA. The H form has been cloned from the HCC cell line HepG2 and among tissues it has so far been detected only in normal and neoplastic colon. In all the three transcripts, translation initiates within exons II and stops within exon VI, generating identical polypeptides. The approximate position of the primers used for PCR amplification is indicated by arrowheads.

from the hepatocarcinoma cell line HepG2, lacks exons Y, Z, or X but contains a short specific sequence in front of exon I (Aas-Eng *et al.*, 1995; Lo and Lau, 1996); this sequence is not separated from exon I by introns in genomic DNA and consequently cannot be defined as an exon. The presence of the H transcript, which is the product of P_1 promoter, although reported in normal and neoplastic colonic tissues (Dall'Olio *et al.*, 2000) and cell lines (Dall'Olio *et al.*, 1999) has never been reported in authentic human hepatic tissues.

ST6Gal.I is expressed at a dramatically different level among tissues, and the liver is the tissue displaying the highest level of expression (Paulson *et al.*, 1989; Kitagawa and Paulson, 1994; Kaneko *et al.*, 1995). Furthermore, ST6Gal.I is one of the most frequently up-regulated glycosyltransferases in human cancers (reviewed in Dall'Olio, 2000). In some malignancies, such as colon, the increased expression regards nearly 100% of the patients (Dall'Olio *et al.*, 1989, 2000), whereas in other cancers, such as breast cancer, only a subset of patients display this modification (Recchi *et al.*, 1998). Further interest in the expression of hepatic ST6Gal.I stems from the observation that in rat (Kaplan *et al.*, 1983) and mouse (Dalziel *et al.*, 1999) it behaves like an acute phase protein in that during inflammation it is dramatically up-regulated and a large amount of the cell-bound enzyme is released in the blood stream as a soluble, enzymatically active fragment.

Despite the importance of ST6Gal.I in the glycosylation machinery of the liver cells, very little is known on the regulation of human liver ST6Gal.I in normal and pathological conditions. Sometimes conflicting investigations in experimental systems have suggested that the expression of hepatic ST6Gal.I can be profoundly affected by neoplastic transformation (Miyagi *et al.*, 1988; Jain *et al.*, 1993; Pousset *et al.*, 1997) and differentiation (Shah *et al.*, 1992; Vertino-Bell *et al.*, 1994). In this study, we investigate the expression of ST6Gal.I and of its cognate oligosaccharide structure, the sialyl α2,6-lactosaminyl epitope, in normal human liver, as well as in hepatocarcinoma (HCC) and cirrhosis.

Results

Specimens

The features of the 21 HCC cases utilized in this study are reported in Table I. In 11 cases the tumor developed on preexisting cirrhosis, and in the remaining 10 cases cirrhosis was not present. Owing to the fact that the cirrhotic tissue cannot be referred to as normal, we defined *normal* as only the non-HCC tissue of noncirrhotic patients, whereas the non-HCC tissues of both cirrhotic and noncirrhotic patients is referred to as *noncancer* (see also Table II). The male to female ratio (61% to 39%) is grossly representative of HCC sex ratio. Fourteen out of 21 cases are grade 2 HCC; the other cases are of higher degree.

Expression of ST6Gal.I enzyme activity

The ST6Gal.I enzyme activity expressed by the 42 specimens is reported in Table I. The range of activity displayed by nonneoplastic liver is relatively narrow (from 0.44 to 1.82 nmoles h^{-1} mg $prot^{-1}$), whereas that displayed by HCC tissues is much wider (from 0.10 to 2.51), although the mean values of noncancer and HCC tissues are very close (1.15 versus 1.20, respectively, Table II). Data reported in Table I reveal that ST6Gal.I activity may undergo dramatic up- or down-regulations as a consequence of neoplastic transformation in some patients, but in others it shows little or no changes. For example, in cancer tissue of case 8 the activity is about fourfold higher than in normal tissue, whereas in cancer tissue of case 4 it is nearly undetectable. It should be noted that the patients showing the most remarkable increase of activity (cases 1, 8, 16, and 17) are all males, and of the four patients showing the most remarkable decrease of activity (cases 4, 6, 10, 15), three are female. As a consequence, among HCC samples the average ST6Gal.I activity is about 30% lower in females than in males (0.99 versus 1.32), and among noncancer samples the activity is about 30% higher in females than in males (1.33 versus 1.03). Table II shows also that the ST6Gal.I activity is only marginally affected by the histological grade of the tumor (1.25 in G2 HCC versus 1.08 in G3–4), and it is not different in normal and cirrhotic tissues (1.14 versus 1.15). However, HCC developed on cirrhosis

Table I. Clinical features of the patients, ST6Gal.I expression, and SNA reactivity of the specimens

Case	Age	Sex	Tumor grade[a]	Cirrhosis	N or T	ST6Gal.I[b] activity	SNA reactivity[b]	ST6Gal.I mRNA[b]		
								Coding region	YZ	H
1	60	M	G2	No	N	0.97 ± 0.07	0.26 ± 0.02	1.53 ± 0.02	ND[c]	0.33 ± 0.07
					T	2.00 ± 0.14	0.29 ± 0.05	0.91 ± 0.05	ND	1.30 ± 0.64
2	65	M	G2	Yes	N	0.44 ± 0.01	1.32 ± 0.04	1.61 ± 0.05	0.25 ± 0.02	1.17 ± 0.04
					T	0.39 ± 0.05	1.87 ± 0.70	1.68 ± 0.23	0.15 ± 0.04	0.90 ± 0.22
3	70	M	G2	No	N	0.74 ± 0.16	1.10 ± 0.39	1.43 ± 0.10	ND	0.69 ± 0.27
					T	0.84 ± 0.06	2.56 ± 0.70	1.03 ± 0.12	ND	0.78 ± 0.40
4	60	F	G3-G4	No	N	1.42 ± 0.07	0.89 ± 0.12	1.31 ± 0.07	ND	0.68 ± 0.33
					T	0.10 ± 0.02	0.02 ± 0.01	0.98 ± 0.03	0.11 ± 0.03	0.21 ± 0.04
5	71	M	G2	No	N	1.15 ± 0.02	1.88 ± 0.19	1.48 ± 0.16	ND	0.54 ± 0.22
					T	1.48 ± 0.01	2.34 ± 0.30	1.08 ± 0.17	ND	0.61 ± 0.10
6	60	F	G2	No	N	1.82 ± 0.14	2.07 ± 0.35	0.99 ± 0.41	0.11 ± 0.04	0.81 ± 0.25
					T	1.02 ± 0.14	2.48 ± 0.19	0.71 ± 0.22	0.08 ± 0.02	0.58 ± 0.18
7	70	F	G2	No	N	1.01 ± 0.12	2.03 ± 0.59	4.15 ± 0.85	0.03 ± 0.01	0.64 ± 0.24
					T	0.88 ± 0.07	1.17 ± 0.57	3.71 ± 1.00	0.08 ± 0.02	0.84 ± 0.32
8	77	M	G2	No	N	0.64 ± 0.16	2.03 ± 0.69	0.39 ± 0.14	0.04 ± 0.01	0.22 ± 0.07
					T	2.51 ± 0.59	2.71 ± 0.79	2.61 ± 0.70	ND	0.89 ± 0.20
9	79	M	G3-G4	Yes	N	1.18 ± 0.06	2.34 ± 0.33	5.14 ± 3.22	ND	4.21 ± 1.52
					T	1.51 ± 0.10	1.72 ± 0.72	1.37 ± 0.29	0.10 ± 0.08	0.88 ± 0.09
10	74	F	G2	Yes	N	1.45 ± 0.06	0.94 ± 0.48	1.29 ± 0.13	0.05 ± 0.04	0.28 ± 0.04
					T	0.52 ± 0.02	0.46 ± 0.14	1.47 ± 0.13	0.11 ± 0.02	0.61 ± 0.20
11	54	M	G2	Yes	N	1.54 ± 0.24	0.50 ± 0.21	2.28 ± 0.59	ND	0.42 ± 0.09
					T	1.35 ± 0.23	2.11 ± 0.20	2.15 ± 0.65	0.03 ± 0.01	1.20 ± 0.30
12	62	M	G3-G4	Yes	N	1.06 ± 0.02	0.46 ± 0.09	2.59 ± 0.38	ND	1.22 ± 0.36
					T	1.00 ± 0.14	1.21 ± 0.49	1.02 ± 0.12	0.25 ± 0.06	0.77 ± 0.05
13	73	M	G3	No	N	1.79 ± 0.23	2.77 ± 0.38	1.12 ± 0.3	ND	ND
					T	1.38 ± 0.24	2.31 ± 0.14	3.58 ± 0.70	ND	0.86 ± 0.18
14	56	F	G2	Yes	N	1.34 ± 0.16	2.06 ± 0.66	1.8 ± 0 .94	ND	ND
					T	1.04 ± 0.02	2.66 ± 1.01	1.17 ± 0.12	ND	0.40 ± 0.15
15	56	M	G3	Yes	N	0.84 ± 0.30	3.20 ± 0.44	0.55 ± 0.03	ND	0.30 ± 0.12
					T	0.17 ± 0.02	6.23 ± 1.10	2.38 ± 0.90	ND	0.52 ± 0.09
16	70	M	G2	No	N	0.55 ± 0.18	2.04 ± 0.15	1.30 ± 0.59	ND	ND
					T	1.34 ± 0.06	2.75 ± 0.42	8.42 ± 3.56	0.29 ± 0.12	2.51 ± 0.75
17	70	M	G2	No	N	1.31 ± 0.52	2.24 ± 1.70	0.69 ± 0.04	ND	ND
					T	2.03 ± 0.33	2.99 ± 0.12	3.33 ± 0.86	0.09 ± 0.04	0.87 ± 0.22
18	65	M	G2	Yes	N	1.23 ± 0.28	2.10 ± 0.39	1.57 ± 0.13	ND	0.40 ± 0.07
					T	1.22 ± 0.08	2.10 ± 0.55	1.82 ± 0.25	ND	0.86 ± 0.35
19	64	F	G2	Yes	N	1.19 ± 0.02	2.39 ± 0.22	0.86 ± 0.22	ND	ND
					T	0.96 ± 0.11	3.83 ± 0.10	1.05 ± 0.15	ND	0.21 ± 0.06
20	69	F	G2-G3	Yes	N	1.18 ± 0.09	1.99 ± 0.81	1.31 ± 0.20	0.42 ± 0.15	0.45 ± 0.10
					T	1.57 ± 0.06	2.87 ± 0.25	1.00 ± 0.02	ND	0.25 ± 0.03
21	69	F	G3	Yes	N	1.26 ± 0.04	1.80 ± 1.04	0.51 ± 0.15	0.19 ± 0.10	0.22 ± 0.07
					T	1.83 ± 0.01	4.06 ± 0.62	0.75 ± 0.22	ND	ND

[a]According to Edmonson.
[b]Data are the mean ± SD of at least three independent determinations.
[c]ND, not detectable with a detection limit of 50 fg of target cDNA.

Table II. ST6Gal.I expression and SNA reactivity of groups of specimens

Groups of specimens[a]	Number of specimens	ST6Gal.I activity ± SD	SNA reactivity ± SD	ST6Gal.I RNA ± SD
Noncancer	21	1.15 ± 0.36	1.73 ± 0.76	1.60 ± 1.13
HCC	21	1.20 ± 0.60	2.32 ± 1.34	2.00 ± 1.70
Grade 2	14	1.25 ± 0.59	2.16 ± 0.93	2.22 ± 1.93
Grade 3–4	7	1.08 ± 0.69	2.63 ± 1.88	1.58 ± 0.95
Normal	10	1.14 ± 0.44	1.73 ± 0.71	1.43 ± 0.97
Cirrhosis	11	1.15 ± 0.03	1.74 ± 0.81	1.77 ± 1.23
HCC w/o cirrhosis	10	1.36 ± 0.66	1.96 ± 1.02	2.64 ± 2.25
HCC on cirrhosis	11	1.05 ± 0.50	2.65 ± 1.51	1.44 ± 0.49
Noncancer male	13	1.03 ± 0.39	1.71 ± 0.88	1.76 ± 1.16
Noncancer female	8	1.33 ± 0.24	1.77 ± 0.52	1.41 ± 0.30
HCC male	13	1.32 ± 0.64	2.40 ± 1.30	2.41 ± 1.93
HCC female	8	0.99 ± 0.54	2.19 ± 1.40	1.35 ± 0.92

[a]All normal and cirrhotic specimens are referred to as noncancer. HCC developed without or with preexisting cirrhosis are referred to as HCC w/o cirrhosis and HCC on cirrhosis, respectively.

display a lower ST6Gal.I activity than HCC developed without underying cirrhosis (1.05 versus. 1.36).

Expression of α2,6-sialylated sugar chains in tissue homogenates

The α2,6sialyl-specific lectin from *Sambucus nigra* (SNA) is a widely used probe for the detection of α2,6-linked sialic acid, which is the product of ST6Gal.I (Shibuya *et al.*, 1987). A representative SNA dot-blot analysis of the 42 liver specimens is provided in Figure 2, and the intensity of the dots (mean of at least three experiments) is reported in Table I. As shown, the reactivity of neoplastic tissues is in some cases markedly increased (cases 3, 11, 12, 21), but in a few cases it is dramatically decreased (patients 4 and 10). Statistical analysis (*t* test for paired samples) reveals that the SNA reactivity of HCC samples is significatively higher than that of noncancer samples ($p = 0.014$). Analysis of SNA reactivity by groups of specimens (Table II) suggests that the grade 3–4 specimens are slightly more reactive than grade 2 specimens (2.63 versus 2.16). Regarding the relationship between the expression of ST6Gal.I and that of α2,6-linked sialic acid, data reported in Table I indicate that the changes observed in SNA reactivity often correlate with the ST6Gal.I changes, but in some cases there is a dramatic discrepancy. The most striking example is provided by patient 15, in which the level of ST6Gal.I activity in HCC decreased, whereas the level of SNA reactivity strongly increased. A plot of the ST6Gal.I activity versus the SNA reactivity of the 42 specimens (Figure 2B) indicates the lack of a simple linear relationship between the two parameters ($r = 0.057$; $p = 0.72$); this is consistent with the notion that the regulation of α2,6-sialylation of glycoproteins is multifactorial and is not simply a function of the ST6Gal.I level.

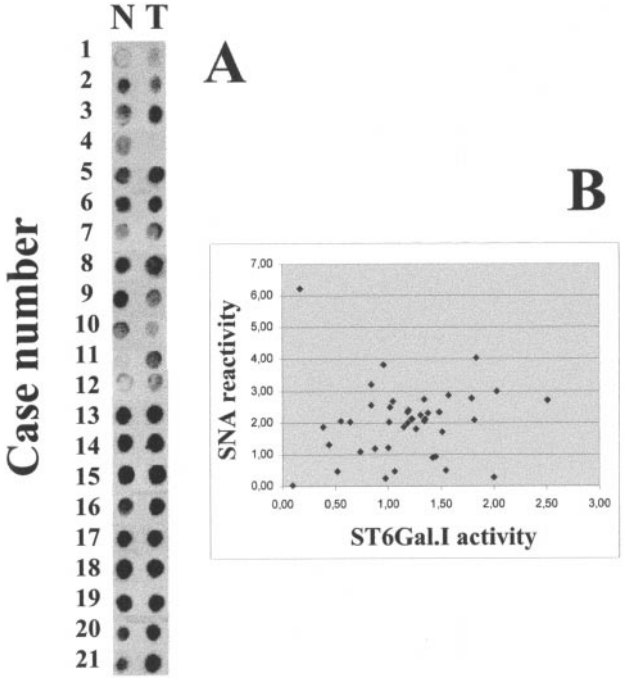

Fig. 2. (**A**) SNA dot-blot analysis of homogenates. Ten micrograms of total homogenates from noncancer (N) and HCC (T) tissues were spotted on a nitrocellulose filter and probed with digoxigenin-conjugated SNA, as detailed in *Material and methods*. (**B**) The intensity of the dot (mean of at least three experiments) of each specimen was plotted against the relative ST6Gal.I activity to evaluate the relationship between the two parameters. Statistical analysis ($r = 0.057$, $p = 0.72$) reveals the lack of a linear relationship.

To investigate the qualitative changes of SNA reactivity associated with neoplastic transformation, SNA-lectin blot analysis was performed on some representative cases (Figure 3). It should be noted that the pattern of staining is usually well conserved in noncancer and cancer samples in the region below 35 kDa. Nevertheless, specific bands appears or disappear in cancer tissues. For example, in sample 6N there are SNA-reactive bands of about 120, 68,

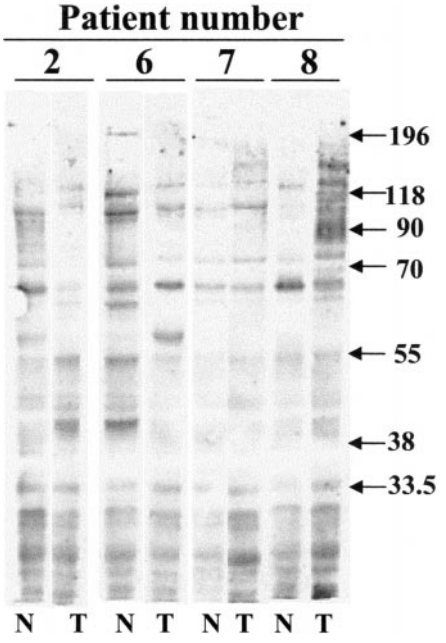

Fig. 3. SNA-lectin blot analysis of noncancer (N) and HCC (T) specimens. One hundred micrograms of total homogenate were electrophoresed, blotted, and probed with SNA-dig, as detailed in *Materials and methods*. Neoplastic transformation often results in qualitative changes consisting in the appearance of new SNA-reactive bands. Arrows indicate the migration position of molecular weight standards.

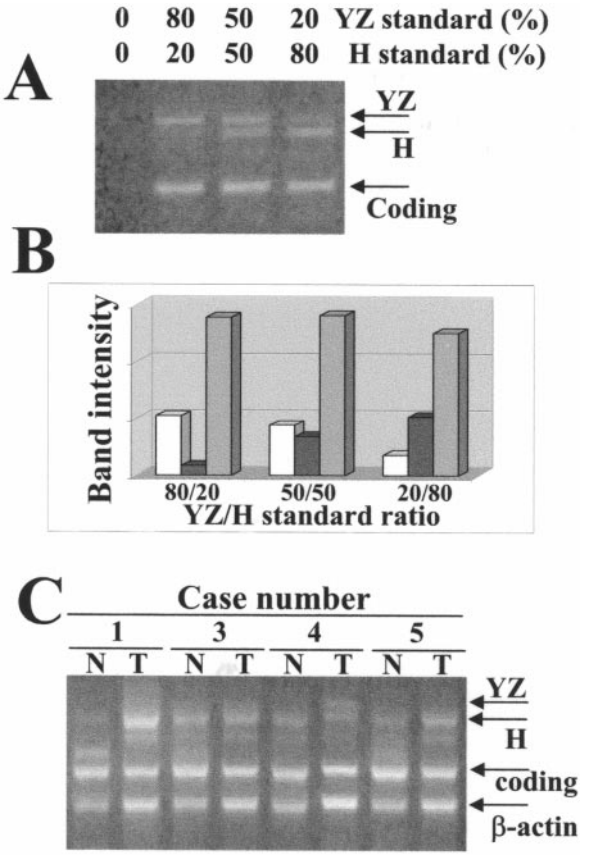

Fig. 4. Multiplex RT-PCR analysis of ST6Gal.I transcripts. (**A**) Amplification of different proportions of the YZ and H cDNA standards. Each reaction mixture contained a total of 250 fg of cloned YZ form and H form ST6Gal.I cDNAs, in the indicated proportions (from 8:2 to 2:8) and the primers designed for the specific amplification of the YZ and H forms as well as of a part of the coding region that is common to both isoforms. The three arrows indicate, respectively, the migration position of the YZ band (primers EZL.1/EVR.3, size 1053 nt), the H band (primers HepL.1/EVR.3, size 982 nt), and the coding region band (primers EIIL.2/EVR.3, size 742 nt). (**B**) Quantification of the bands of **A** (white histograms: YZ; black histograms: H; gray histograms: coding region) indicates a good relationship with the amount of standard cDNA used. (**C**) Multiplex RT-PCR analysis of some representative liver samples. Each sample was amplified with the same primer mixture used for the amplification of standard cDNAs containing, in addition, the primers for the amplification of β-actin at a 10-fold lower concentration.

55, and 40 kDa, which are absent in sample 6T, whereas a strong band around 60 kDa is present in sample 6T but absent in its normal counterpart. In sample 8T, there is marked accumulation of high molecular weight (80–150 kDa) SNA-reactive bands that are not present in sample 8N. These data demonstrate a markedly heterogeneous expression of α2,6-sialylated glycoproteins in HCC specimens.

Expression of ST6Gal.I transcripts in surgical specimens

The expression of ST6Gal.I transcripts in surgical specimens was investigated by a semiquantitative multiplex reverse transcriptase polymerase chain reaction (RT-PCR) approach. After a preliminary investigation that ruled out the expression of the X form (data not shown), the study was focused on the YZ and H forms. An estimation of the total amount of ST6Gal.I transcripts and of the amount of the YZ and H forms was obtained through a comparison with the parallel amplification of known amounts of the YZ form and H form ST6Gal.I cDNAs. In Figure 4A is shown the multiplex amplification of the cloned YZ and H forms, mixed in the indicated proportions and used as a quantitative standard. The contemporary amplification of the two types of cDNA gives rise to two closely spaced but clearly distinguishable isoform-specific bands (indicated by the two upper arrows) and to one band resulting from the amplification of the coding region. In Figure 4B is the quantification of the three PCR bands in the three standard amplification reactions. It should be noted that the intensity of the YZ (white bar) and H products (black bar) grossly reflects the relative amount of template used. Consistent with the fact that the total amount of cDNA in the three samples is the same, the intensity of the coding region signal in the three samples is very similar (gray bar).

A representative example of the patterns obtained with the cDNAs from normal liver and HCC of some patients is provided in Figure 4C. In this case, an housekeeping gene (β-actin) was coamplified to control for RNA quality. Beside a β-actin and coding region signals of comparable intensity, all these specimens show the presence of the H transcript, indicating that this mRNA species is expressed by authentic normal and HCC liver tissues. The YZ form is expressed only in trace amounts by both N and T samples, with the remarkable exception of sample 4T, whose YZ expression is comparable with that of the H form. Interestingly, the

same specimen show a nearly complete down-regulation of enzyme activity and SNA reactivity (Table I). However, a comparison between the intensity of the H and the coding region bands, reveals striking differences among specimens. In fact, although in sample 1T the intensity of the H and the coding region bands is similar, suggesting that nearly 100% of the transcript is of the H type, in sample 1N the low amount of H transcript does not appear to be sufficient to account for the intensity of coding region band detected. The contemporary absence of the YZ transcript and of the B-lymphocyte-specific X transcript (data not shown) could be suggestive of the presence of other mRNA species whose presence will be investigated by 5′-rapid amplification of cDNA ends (RACE) (see later discussion).

Table I reports the intensity of the coding region as well as of the YZ and the H bands, normalized for the internal β-actin standard obtained with the 42 specimens. As shown, ST6Gal.I transcripts dramatically accumulate in HCC tissue of several patients (cases 8, 13, 15, 16, 17). However, in two patients a marked reduction is observed (cases 9 and 12), whereas in the other cases the level of transcript shows little or no changes. A comparison of the mean ST6Gal.I mRNA levels expressed by groups of specimens (Table II) indicates a tendency toward an accumulation in grade 2 (2.22 versus 1.60), but not in grade 3–4 HCC (1.58 versus 1.6) and in HCC developed without underlying cirrhosis (2.64 versus 1.43), but not in HCC developed on preexisting cirrhosis (1.44 versus 1.77). As observed for ST6Gal.I enzyme activity, the mRNA expression is also lower in female HCC tissues, compared with male HCC tissues (1.35 versus 2.41). The most remarkable increase of transcript expression is observed in patient 8, which shows also the most dramatic elevation of ST6Gal.I activity. Nevertheless a comparison of the ST6Gal.I activities with the respective level of mRNA also reveals marked inconsistencies. For example, in case 15 the transcript level is markedly increased in cancer tissue, but the enzyme activity is reduced. This reveals that the level of ST6Gal.I transcript may not directly correlate with the enzyme activity and suggest a multifactorial control. Multiplex RT-PCR analysis reveals that the H form is the major transcript expressed in all the 42 specimens examined (Table I). The expression of the YZ form is, in many cases, below the detection limit of this technique (50 fg cDNA); in other cases it is clearly detectable but expressed as a minor form; and in three cases (4T, 20N, and 21N) the H and YZ forms are expressed at a similar level. As already observed for sample 1N, in many cases the total level of ST6Gal.I transcript (coding region) does not appear to be supported by an adequate amount of either of the two forms. For this reason the nature of the sequences 5′-flanking exon I in sample 1N was investigated.

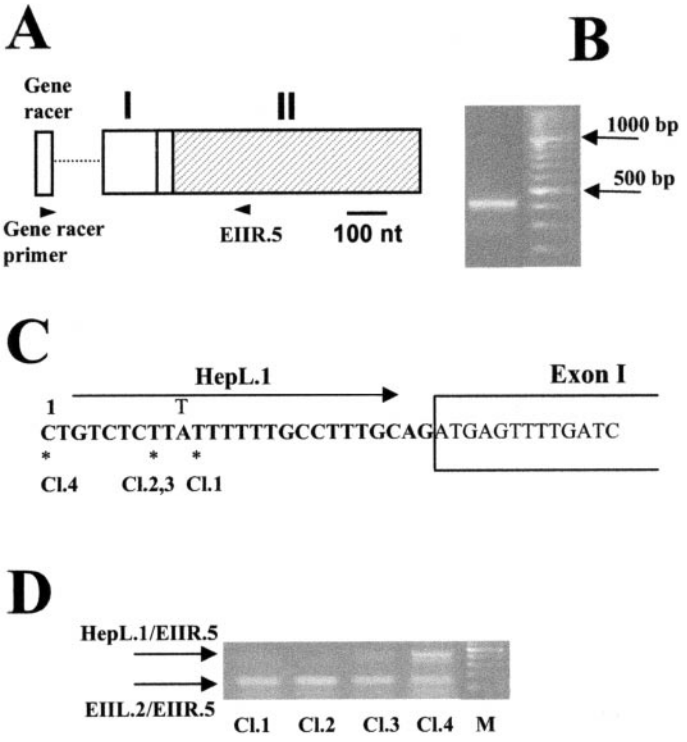

Fig. 5. 5′-RACE analysis. (**A**) Total RNA was reverse transcribed and a short oligonucleotide (GeneRacer) was linked to the 5′ ends of the obtained cDNAs. The 5′-terminal portion of ST6Gal.I cDNAs was specifically PCR amplified using GeneRacer primer as forward primer and EIIR.5 as reverse primer. A single PCR product (**B**) of about 420 nt was obtained. Sequencing of the cloned PCR product revealed the presence (**C**) beside a sequence identical to that previously cloned from HepG2 cells (clone 4), of shorter product probably derived from different transcription initiation points (marked with an asterisk). Clones 2 and 3 start at position +8 (position +1 is the first nucleotide of the longest form), and clone 1 starts at position +11. Moreover, clone 2 exhibited an A/T polymorphism at position +10. (**D**) PCR amplification of the cloned sequences revealed that although all four clones acted as good templates for the primer pair EIIL.2/EIIR.5, only clone 4 and to a much lesser extent clone 3 acted as good templates for primer pair HepL.1/EIIR.5.

5′-RACE analysis of normal liver tissue

RACE allows the PCR amplification of the unknown terminal portions of a cDNA whose central part is known. The RNA from sample 1N was reverse-transcribed and a short oligonucleotide of known sequence (Generacer, Figure 5A) was linked to the 5′ ends of the obtained cDNAs. The ST6Gal.I cDNAs were then PCR amplified, using Generacer primer, complementary to Generacer sequence as forward primer, and primer EIIR.5, complementary to exon II, which is shared by all known ST6Gal.I mRNAs, as reverse primer. The result of this PCR amplification is shown in Figure 5B. A single product of about 420 nt was obtained. This size is consistent with the presence of a very short sequence between Generacer and exon I. This product was cloned, and the several clones that were sequenced were all found to contain, beside exon I, the 5′-UT sequence of the H form. However, the different clones differed for the length of the hepatic sequence (Figure 5C), revealing that transcription through the P_1 promoter can initiate in at least three different points (marked with an asterisk in Figure 5C). Clone 1 starts at position +11 (position 1 is the first nucleotide of the longest form), clones 2 and 3 start at position +8, and clone 4 starts at position +1. Moreover, clone 2 contained a T instead of A at position +10, revealing the existence of a possible polymorphism at this position, which generates a stretch of nine T residues: PCR amplification of the four cloned sequences with left primers HepL.1 and EIIL.2 (Figure 5D) reveals that although all

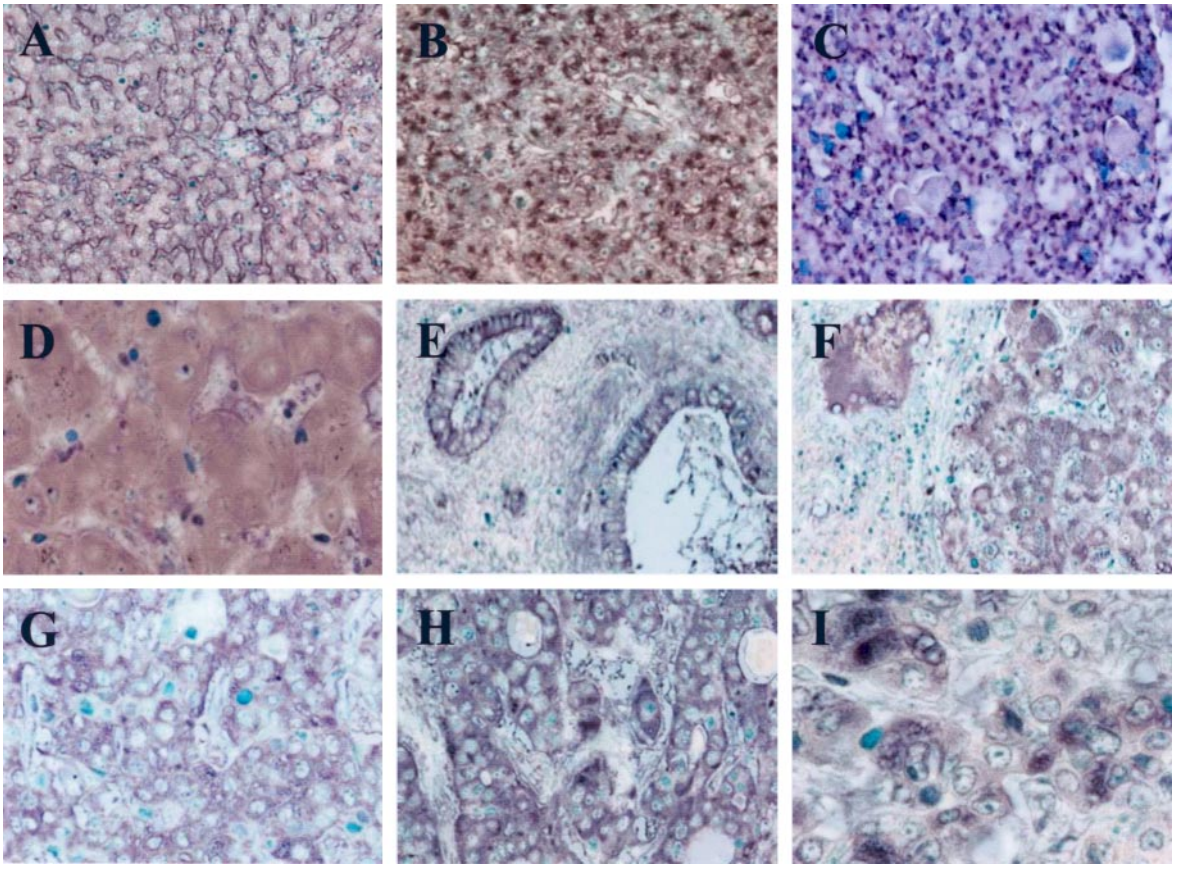

Fig. 6. SNA-dig analysis on histological sections and *in situ* RNA hybridization. α2,6-Sialylated glycoconjugates were detected in histological sections of normal (**A**) and HCC tissues (**B** and **C**) by the α2,6-sialyl-specific lectin SNA. In normal liver, α2,6-sialylated glycoconjugates are evenly expressed by sinusoid cells. In HCC, this organization is often lost, and SNA reactivity is mainly associated with intracytoplasmatic deposits, suggesting an alteration of the sorting mechanisms. ST6Gal.I mRNA was detected in histological sections by *in situ* mRNA hybridization. Normal liver provides a weak and even cytoplasmic reactivity associated with hepatocytes (**D**), whereas a stronger reactivity is expressed by the bile ducts (**E**). In cirrhosis, reactivity is associated with the regenerating hepatocytes but not with connective tissue (**F**). In HCC specimens (**G**–**I**), the reactivity is often heterogeneously expressed by the neoplastic cells, as evident in **I**. (**A**, **B**, **C**, **E**, **F**) Original magnification 25×; (**D**, **G**, **H**, **I**) original magnification 40×.

the four clones were efficiently amplified with primer pair EIIL.2/EIIR.5, only clone 4 and at a much lesser extent clone 3 were amplified with primer pair HepL.1/EIIR.5. Because primer HepL.1 overlaps completely only with the longest form (clone 4), it is possible that the poor amplification yield given by some samples with HepL.1 reflects a higher proportion of shorter forms. Moreover, the difference between the amplification yields between clones 2 and 3 (which are of identical length but differ for the A/T substitution at position +10) suggests that also this difference (which creates a mismatch with primer HepL.1) contributes to a lower amplification efficiency with the H-specific primer in some samples. These data conclusively demonstrate the expression by authentic human normal liver of the H form and indicate that these transcripts are heterogeneous with respect to the length of their 5′-ends.

Expression of α2,6-linked sialic acid and of ST6Gal.I mRNA in histological sections

In Figure 6A–C, the SNA-staining patterns of histological sections from normal liver (A) and from HCC (B and C) are compared. In normal liver (A), SNA reactivity is mainly associated with sinusoids, whereas in HCC specimens this staining pattern is completely lost and the SNA reactivity appears as dense aggregates in the cytoplasm of the neoplastic hepatocytes (B, C). This pattern is shared by many of the HCC specimens examined and is consistent with an intracellular accumulation of α2,6-sialylated glycoconjugates probably because of an alteration of the apical/basolateral sorting mechanisms in cancer cells.

The tissue expression of ST6Gal.I mRNA, as determined by *in situ* hybridization, also reveals marked differences between normal and neoplastic tissues (D–I). In normal liver parenchyma (D), *in situ* hybridization provides a weak diffuse cytoplasmic staining evenly distributed in the tissue, whereas the cells of the bile ducts show a stronger level of expression (E). In cirrhotic tissue (F), the regenerating liver cells show a strong, uniform mRNA expression, and the surrounding connective tissue is unreactive. In HCC specimens, a variable degree of tissue heterogeneity is often evident (G–I). The specimen shown in Figure 6I shows an extreme heterogeneity, with some cells expressing very high levels of ST6Gal.I mRNA in close proximity with

cells showing weak expression. Together, these data indicate that on neoplastic transformation, ST6Gal.I expression may undergo marked up- or down-regulation among the cells forming the tumor.

Discussion

In the present study we found that neoplastic transformation of hepatocytes can result in opposite effects on ST6Gal.I expression. In fact, we observed a different modulation of the enzyme in different groups of patients, and *in situ* hybridization shows that even within the same tumor the ST6Gal.I transcript can be up- or down-regulated by different groups of cells. These findings are not surprising in the light of the contradictory conclusions reached by the studies on experimental systems published in the past years. Beside studies reporting a positive relationship between ST6Gal.I and neoplastic transformation, others report an opposite conclusion. For example, a specific elevation of ST6Gal.I has been reported in a rat model of chemically induced hepatoma (Miyagi *et al*., 1988), whereas an increase of ST6Gal.I activity and an accumulation of the mRNA were observed in HCCs spontaneously developed by transgenic mice expressing the large T antigen under the control of an hepatocyte-specific promoter (Pousset *et al*., 1997). On the other hand, ST6Gal.I expression was found to be strongly down-regulated, rather than increased, in a rat HCC cell line compared with normal tissue (Jain *et al*., 1993), and a positive relationship with organ differentiation is indicated by the fact that during ontogenesis of rat liver the level of ST6Gal.I mRNA, which is very low in newborns, dramatically increases (Vertino-Bell *et al*., 1994). The molecular bases of liver carcinogenesis are very heterogeneous in that many oncogenes and tumor suppressor genes may be involved (Ozturk, 1999); thus the opposite modulation of ST6Gal.I may reflect different pathways of oncogenic activation. In this light, it is interesting to note that the expression of the large T antigen, which induces HCCs overexpressing ST6Gal.I (Pousset *et al*., 1997), leads to the functional inactivation of the Rb protein and that the inactivation of Rb is a relatively frequent mutagenic event (20–25% of the cases) in liver carcinogenesis (Ozturk, 1999).

Although the number of cases examined in this article does not allow us to reach a definitive conclusion, we observed that ST6Gal.I expression in HCC is influenced by the grade of the tumor, by the underlying presence of cirrhosis, and by the sex of the patient. A lower ST6Gal.I expression by the less differentiated tumors, a tendency more evident at the mRNA level, is in agreement with a recently published article that reports a down-regulation of the ST6Gal.I enzyme protein in less differentiated HCC specimens (Cao *et al*., 2002). The fact that cirrhosis does not alter the expression of ST6Gal.I is unexpected because inflammatory conditions are well known to induce an up-regulation of this enzyme through a mechanism that, in mice, involves an IL-6-mediated transcription through the P_1 promoter (Dalziel *et al*., 1999). Moreover, in HCC developed without preexisting cirrhosis the average ST6Gal.I mRNA is increased, but in those developed on a preexisting cirrhosis this tendency is not observed. This difference could be explained if it is hypothesized that different pathways of transformation are preferentially activated in the two conditions and that ST6Gal.I is modulated only by given pathways. ST6Gal.I expression, both at the enzyme activity and mRNA level, is lower in female than in male HCC specimens, although in noncancer specimens this tendency is less evident. In considering this observation, it should be kept in mind that ST6Gal.I can be modulated by steroids, such as dexamethasone, in rat hepatoma cells (Wang *et al*., 1989), suggesting that transcription of the gene might be affected by sexual steroid hormones.

In this study we have also demonstrated that in both normal and HCC tissues ST6Gal.I transcription occurs mainly through the P_1 promoter, resulting in the H form as the major mRNA species. However, as occurs in colonic tissues (Dall'Olio *et al*., 1999, 2000), the P_3 promoter can also be utilized by liver tissues and the contribution of the H and YZ transcripts to the total mRNA pool is variable. As previously observed in HepG2 cells (Lo and Lau, 1996), transcription through the P_1 promoter can start in different points, giving rise to 5′-UTRs of different lengths. Even though the biological significance of a differential use of P_1 and P_3 promoters in human liver remains to be investigated, a recent study has shown that knockout mice specifically unable to express ST6Gal.I through the P_1 promoter show a reduced ST6Gal.I expression beside a surprisingly normal level of α2,6-sialylation of serum glycoproteins (Appenheimer *et al*., 2003). Interestingly, on challenge with *Salmonella typhimurium* these mice show a greater accumulation of neutrophils in the peritoneal space and an increased bacterial burden in liver and spleen, suggesting that the P_1-driven ST6Gal.I expression can play a role in antimicrobial defense mechanisms.

The expression of α2,6-sialylated glycoconjugates has been studied using SNA as a probe. The specificity of this lectin has been reported to include the α2,6-sialylated lactosamine (the ST6Gal.I product) as well as the sialyl-Tn (sialic acid α2,6-linked to N-acetylgalactosamine), which is the product of different sialyltransferases (Shibuya *et al*., 1987). However, histochemical studies have revealed that the contribution of sialyl-Tn to SNA reactivity of tissue sections is negligible (Murayama *et al*., 1997) and SNA can be considered a specific tool for the ST6Gal.I product. α2,6-Sialylated glycoconjugates show a significative tendency to accumulate in HCC tissue specimens, even though in a minority of patients we observed a lower expression, and histochemical studies reveal that the tissue distribution of these compounds in HCC can be markedly altered. In fact, in normal liver α2,6-sialylated glycoconjugates are mainly expressed along the sinusoids, but in HCC they often accumulate as dense aggregates in the cytoplasm of neoplastic hepatocytes. This latter observation is consistent with that obtained with another α2,6-sialyl-specific lectin, the CD22 molecule (Cao *et al*., 2002) and suggests an alteration of the mechanisms of intracellular transport in HCC. These data demonstrate that factors other than the ST6Gal.I level can influence the accumulation of α2,6-sialylated glycoconjugates and explain why we observed that the relationship between the expression of ST6Gal.I and that of α2,6-sialylated glycoconjugates is often loose.

Although transfection studies have convincingly demonstrated that the expression of the ST6Gal.I cDNA is by itself sufficient to induce the appearance of α2,6-sialylated glycans on the cell surface (Dall'Olio *et al*., 1995; Lee *et al*., 1989), studies aimed at establishing the quantitative relationship between ST6Gal.I expression and α2,6-sialylation have revealed that such a relationship may be very loose not only in tissues (Dall'Olio *et al*., 2000; Kaneko *et al*., 1995) but also in cell lines transfected with the ST6Gal.I cDNA under the control of a constitutive promoter (Dall'Olio *et al*., 2001). In this strictly controlled artificial system, the amount of α2,6-linked sialic acid on the cell surface depends strongly on the cell line used, not only on the level of ST6Gal.I enzyme activity.

In conclusion, our study demonstrates that the expression of ST6Gal.I and of α2,6-sialylated glycoconjugates can undergo dramatic variations as a consequence of neoplastic transformation of hepatocytes. The identification of the molecular events at the basis of such alterations will require further work.

Materials and methods

Surgical specimens

Clinical information on the 21 patients who underwent surgery for HCC is reported in Table I. Informed consent was obtained from all patients. Samples from nonneoplastic and HCC tissues were removed at surgery. A portion of the tissue was formalin-fixed and paraffin-embedded for routine histopathological examination; other portions of the tissue were frozen in liquid nitrogen and stored at −80°C.

Sialyltransferase assay and blot analysis

Tissues were potter-homogenized in ice-cold distilled water; the protein concentration of the homogenate was measured by the Lowry method and adjusted to 10 mg/ml. ST6Gal.I activity was measured in whole homogenates in the range of linearity with respect to time and enzyme concentration as incorporation of [^{14}C]-labeled sialic acid on asialotransferrin (prepared by mild acid hydrolysis of human transferrin) as detailed elsewere (Dall'Olio *et al*., 1996). For dot-blot analysis, 10 μg of the homogenates was applied to Hybond nitrocellulose membrane (Amersham, Little Chalfont, U.K.). Membranes were probed with 1 μg/ml digoxigenin-conjugated SNA (SNA-dig) (Boehringer) which was detected with horseradish peroxidase–labeled antidigoxigenin antibodies (Boehringer). The reaction was finally developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and detected by autoradiography. Intensity of the dots was quantitated by the Kodak Digital Science 1D software. Details on dot-blot procedure have been reported previously (Dall'Olio *et al*., 2000). For lectin blot analysis, 100 μg of proteins from total tissue homogenates was electrophoresed under reducing conditions on a 10% polyacrylamide gel and electrotransferred to Hybond membrane. After a wash with phosphate buffered saline with Tween, the blot was processed as detailed for dot-blot.

RT-PCR analysis

Total RNA was extracted from normal and neoplastic tissues by RNAZolB B (Biotecx Laboratories, Houston, TX) method. Two micrograms of total RNA were reverse-transcribed using the TaKaRa RT-PCR kit Ver 2.1 (TaKaRa, Shuzo, Japan), using random 9-mers primers, according to the manufacturer's instructions. Two to five microliters of the cDNAs were amplified by a multiplex RT-PCR approach, which allows the simultaneous amplification of the YZ form, the H form, and the coding region of ST6Gal.I as well as of the β-actin cDNA as an internal control. The PCR reaction contained in a final volume of 50 μl: 1× Taq polymerase buffer; 1.7 mM $MgCl_2$; 0.2 mM each dATP, dCTP, dGTP, dTTP; 0.5 U InViTAQ DNA polymerase (Eppendorf, Milan, Italy); and the following concentrations of primers (the approximate position of the primers is indicated in Figure 1):

- 120 nM primer EIIL.2, forward primer for coding region of ST6Gal.I (5′-CTGCGTCCTGGTCTTTCTTC-3′)
- 500 nM primer HepL.1, forward primer for H form (5′-GTCTCTTATTTTTGCCTTTGCAG-3′)
- 250 nM primer EZL.1, forward primer for YZ form (5′-GAGAAGTGGTGAATGTCATGGAG-3′)
- 250 nM primer EVR.3, common reverse primer for ST6Gal.I (5′-TGGGTCCCATACAATTAGGAT-3′)
- 40 nM each of the primers ACTL.3 (5′ GGCATCGTGATGGACTCCG 3′) and ACTR.3 (5′ GCTGGAAGGTGGACAGCGA 3′) for β-actin

After a denaturation step for 1 min at 94°C, amplification was performed for 35 cycles of the following program: denaturing 94°C, 1 min; annealing 60°C, 1 min; elongation 72°C, 2 min. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. The intensity of the bands was quantified by the Kodak Digital Science 1D software and compared with that given by known amounts of YX and H cDNA standards, prepared as follows. The cDNA from HepG2 cells, a cell line known to express both the YZ and the H forms (Dall'Olio *et al*., 1999) was used as a source for the PCR amplification of the two forms. After gel isolation, the PCR products were cloned in pGEM-T easy vector (Promega, Madison, WI). The concentration of the purified plasmids was carefully determined by A_{260} optical density and brought to a concentration of standard cDNA of 25 fg/μl.

5′-RACE

The whole procedure was made according to the instructions of the GeneRacer kit (Invitrogen, Carlsbad, CA). Five micrograms of normal liver total RNA were reverse-transcribed using random primers, and the resulting cDNA was 5′-ligated with GeneRacer oligonucleotide (Invitrogen) and subjected to PCR amplification with GeneRacer primer (Invitrogen) and oligonucleotide EIIR.5 (5′-AATCAGACCCCATGGCCAATTTCC-3′) as follows: preliminary denaturing step: 94°C, 1 min, then 37 cycles of the following program: denaturing 94°C, 1 min; annealing 62°C, 1 min; extension 72°C, 1 min. The resulting PCR product was gel isolated, cloned by using the TOPO TA cloning kit (Invitrogen), and sequenced. All sequence

analysis were performed automatically using a Beckman-Coulter CEQ2000XL DNA analysis system.

SNA staining of histological sections

Sections were deparafinized in xylene, rehydrated in graded ethanol, and subjected to antigen retrieval (boiling in 10 mM citrate buffer, pH 6, for 15 min). Previous experiments had shown that this treatment maximizes SNA reactivity. SNA-dig staining and detection with alkaline phosphatase–conjugated antidigoxigenin antibodies (Boehringer) were as previously described (Dall'Olio and Trere, 1993).

In situ *hybridization*

Nonisotopic *in situ* hybridization was performed essentially as described previously (Fiorentino *et al*., 1999). Digoxigenin-labeled probes were prepared from the linearized pGEM-T easy vector containing the ST6Gal.I cDNA from HepG2 cells (see previous discussion). Sense and anti-sense RNA probes were generated with T3 and T7 RNA polymerase for 2 h at 37°C in 1× transcription buffer; 35 U RNAase inhibitor; 1 mM each ATP, CTP, GTP; and 1 mM of a mixture of unlabeled UTP and digoxigenin UTP (6.5:3.5 ratio) (Boehringer). The size of the probes was reduced to 50–100 nucleotides by means of alkaline hydrolysis. Before hybridization, tissue specimens were digested with 10 mg/ml proteinase K (Sigma, St. Louis, MO) 30 min at 37°C. Hybridization was performed at 46°C overnight with 10 pM of digoxigenin-labeled probe in 25 µl hybridization buffer (50% deionized formamide, 2× saline sodium citrate, 10% dextran sulfate, 1% sodium dodecyl sulfate). Posthybridization washes were at 60°C in 50% deionized formamide 2× saline sodium citrate for 30 min. Antidigoxigenin antibodies 1:500 diluted were applied overnight at 4°C. Detection was accomplished with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate for 3–6 h. Sections were then counterstained in methyl green. Control for specificity was performed with sense probe.

Acknowledgments

This work was supported by grants from the University of Bologna to F.D. and W.F.G., Funds for Selected Research Topics to F.D., and from the MIUR to F.D. and W.F.G.

Abbreviations

HCC, hepatocellular carcinoma; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase polymerase chain reaction; SNA, *Sambucus nigra* agglutinin; ST6Gal.I, β-galactoside α2,6-sialyltransferase I; UTR, untranslated regions.

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