Differences in CD75s- and iso-CD75s-ganglioside content and altered mRNA expression of sialyltransferases ST6GAL1 and ST3GAL6 in human hepatocellular carcinomas and nontumoral liver tissues

Jamal Souady, Marcel Hülsweig, Ute Distler, Jörg Haier, Axel Denz, Christian Pilarsky, Norbert Senninger, Klaus Dreisewerd, Jasna Peter-Katalinić, and Johannes Muthing

1Institute of Medical Physics and Biophysics, University of Münster, Robert-Koch-Str. 41, D-48149 Münster, Germany; 2Department of General and Visceral Surgery, University Hospital Münster, D-48149 Münster, Germany; and 3Department of Visceral, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, D-01307 Dresden, Germany

Received on July 6, 2010; revised on November 11, 2010; accepted on December 1, 2010

The sialic acid-specific cytotoxic lectin viscumin and its recombinant equivalent rViscumin specifically bind to CD75s-gangliosides with terminal Neu5Acα2,3Galβ4GlcNAc sequence. We, therefore, comparatively analyzed the content of CD75s-gangliosides and closely related iso-CD75s-gangliosides (terminated by Neu5Acα3Galβ4GlcNAc sequence) and the gene expression of associated β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) and β-galactoside α-2,3-sialyltransferase 6 (ST3GAL6), respectively, in 35 hepatocellular carcinoma (HCC) patients. Ganglioside structures were identified in lipid extracts of matched pairs of malignant and nonmalignant liver tissues by thin-layer chromatography immunodetection coupled with infrared matrix-assisted laser desorption/ionization orthogonal time of flight mass spectrometry. CD75s- and iso-CD75s-gangliosides were found to be deregulated in tumor tissues and showed an elevated occurrence in 35 and 41% of HCCs, respectively, compared with nontumoral liver tissues. Statistical analysis revealed a correlation between enhanced iso-CD75s-ganglioside amount and a poor histopathological differentiation (τ = 0.317, P = 0.045) and a significant association of CD75s- and iso-CD75s-ganglioside levels in nontumorous (τ = 0.392, P = 0.003) and in tumorous tissues (τ = 0.650, P < 0.001). Quantitative real-time polymerase chain reaction gene expression analysis of sialyltransferases exhibited no difference in ST6GAL1 expression in cancerous and adjacent noncancerous tissues. Interestingly, the ST3GAL6 expression was significantly diminished in HCCs (P = 0.003). The results indicate that the occurrence of CD75s- and iso-CD75s-gangliosides in tumor tissues is largely independent of the transcriptional expression of ST6GAL1 and ST3GAL6, respectively. Thus, further experiments are required to explore the rationale behind the differential ganglioside level and to validate the applicability of CD75s- and iso-CD75s-gangliosides as targets for individual HCC therapies.

Keywords: IR-MALDI-o-TOF-MS / sialyltransferases / targeted therapies / TLC immunostain / tumor-associated gangliosides

Introduction

Gangliosides are glycosphingolipids (GSLs) containing one or more sialic acid residues (Stults et al. 1989; Levery 2005; Muthing and Distler 2010). They are ubiquitous constituents of the plasma membrane of animal cells and located with the ceramide moiety embedded within the lipid layer of microdomains and the oligosaccharide facing the extracellular environment (Sonnino et al. 2006). They act, for instance, in cell-surface recognition and membrane protein regulation (Schnaar 1991; Lopez and Schnaar 2009) and as receptors for toxins (Müthing et al. 2009), bacteria (Karlsson 2000) and viruses (Miller-Podraza et al. 2000; Campanero-Rhodes et al. 2007; Childs and Elling 2009). Lectin-carbohydrate-mediated recognition systems (Sharon 2007) where GSLs are involved are among the most challenging topics in cell biology (Feizi 2000; Nimrichter et al. 2008) and, based on the knowledge gained, a variety of medical applications of lectins may be envisaged (Sharon 2008). Moreover, many studies have shown that neoplastic transformation is often accompanied by changes in the composition of cell-surface gangliosides (Feizi 1985; Hakomori 1996; Igarashi and Kannagi 2010; Yin et al. 2010) and that gangliosides can serve as candidate targets for anti-cancer therapies (Fredman et al. 2003). The terminally α-2,6-
sialylated ganglioside Neu5Acα6Galβ4GlcNAcβ3Galβ4Glcβ1Cer, previously identified in a liver metastasis of a human pancreatic adenocarcinoma (Månsson et al. 1985) and now renamed as CD75s-ganglioside (Mason et al. 2002), represents such a promising target. Furthermore, the terminally α-2,3-sialylated isomeric ganglioside with Neu5Acα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer structure, analogously named as iso-CD75s-ganglioside, may also play a role in oncological changes (Distler, Souady, et al. 2008). Significantly, CD75s-gangliosides are the targets of viscumin and its recombinant counterpart rViscumin (Müthing et al. 2002, 2004). As a potential anticancer drug, rViscumin has successfully passed clinical phase I trials (Schöffski et al. 2004, 2005; Bergmann et al. 2008) and is currently in clinical development phase II.

We recently revealed evidence for an enhanced occurrence of CD75s-gangliosides in a random collection of gastrointestinal tumors (n = 3), including hepatocellular carcinomas (HCCs; Müthing et al. 2005). As an extension of our preliminary findings, we now report on a comprehensive investigation of CD75s-gangliosides and closely related iso-CD75s-gangliosides and gene expression analysis of associated sialyltransferases in a cohort of clinically characterized HCCs.

Results

Lipid extracts from matched pairs of malignant versus adjacent nonmalignant tissues were probed for the abundance of CD75s- and iso-CD75s-gangliosides using the thin-layer chromatography (TLC) overlay technique in conjunction with infrared matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometry (IR-MALDI-o-TOF-MS) followed by statistical evaluation. We also performed quantitative real-time polymerase chain reaction (qRT-PCR) to elucidate the transcriptional expression of β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) and β-galactoside α-2,3-sialyltransferase 6 (ST3GAL6), which execute the terminal α-2,6- and α-2,3-sialylation of CD75s- and iso-CD75s-gangliosides, respectively.

TLC immunodetection of tumor-associated CD75s-gangliosides

GSL extracts equivalent to 2 mg tissue wet weight of cancerous and corresponding noncancerous tissues, respectively, were separated by TLC followed by immunostain of CD75s-gangliosides with the antibody AB2-6 which specifically binds to the Neu5Acα6Galβ4GlcNAc epitope. The tumors applicable to TLC densitometry (see Supplementary data, Table S1) were ranked from 1 to 29, where rank 1 indicates the highest and rank 29 the lowest ganglioside content.

| Table I. Ranking and categories of CD75s-1- and iso-CD75s-1-ganglioside content in HCC tissues |
|-----------------|-----------------|-----------------|
| Categorya % Rankb Patient | Categorya % Rankb Patient |
| I 20.7 1 6 | I 10.3 1 7 |
| 2 19 | 2 26 |
| 3 7 | 3 19 |
| 4 26 | II 31.0 4 4 |
| 5 20 | 5 3 |
| 6 5 | 6 5 |
| II 13.8 7 28 | 7 28 |
| 8 3 | 8 23 |
| 9 16 | 9 8 |
| III 20.7 11 23 | 11 15 |
| 10 4 | 10 20 |
| 12 25 | 12 18 |
| 15 13 | III 20.7 13 27 |
| 14 14 | 14 13 |
| 15 15 | 15 10 |
| 16 27 | 16 25 |
| IV 44.8 17 10 | 17 16 |
| 18 17 | 18 6 |
| 19 8 | IV 37.9 19 9 |
| 20 9 | 20 12 |
| 21 2 | 21 29 |
| 22 29 | 22 17 |
| 23 22 | 23 1 |
| 24 12 | 24 14 |
| 25 11 | 25 11 |
| 26 18 | 26 2 |
| 27 24 | 27 21 |
| 28 21 | 28 22 |
| 29 1 | 29 24 |

Table I. Ranking and categories of CD75s-1- and iso-CD75s-1-ganglioside content in HCC tissues

aBased on the ranking data, HCCs were grouped as tumors with high (I), moderate (II), equal (III) and lowered (IV) ganglioside content compared with nontumorous tissues.

bThe 29 tumors applicable to TLC densitometry (see Supplementary data, Table S1) were ranked from 1 to 29, where rank 1 indicates the highest and rank 29 the lowest ganglioside content.

and 44.8% diminished occurrence (see Table I, left column). The antibody AB2-6, viscumin and rViscumin displayed identical CD75s specificity (see controls in Figure 1A).

Structure characterization of tumor-associated CD75s-gangliosides

The AB2-6 antibody-positive upper and lower bands of tumor and nontumorous tissues were investigated directly on the TLC plates by IR-MALDI-o-TOF-MS. All CD75s-1-gangliosides were detected as singly-charged sodiated species. Results are exemplarily shown for patient 7 (tumor category I) in Figure 2. Intense ion signals corresponding to IV6Neu5Acα-ne-Lc4Cer (d18:1, C24:1/C24:0) and IV6Neu5Acα-ne-Lc4Cer (d18:1, C22:0) were observed in the tumor tissue (Figure 2C), whereas relatively low abundance of these ions was found in the nontumor tissue (Figure 2A). Ions corresponding to IV6Neu5Acα-ne-Lc4Cer (d18:1, C16:0) were highly abundant in the lower band, a finding that is consistent with the increased antibody staining of IV6Neu5Acα-ne-Lc4Cer (d18:1, C16:0) in the tumor tissue (Figure 2D) compared with weak signals of this ganglioside in the nontumorous tissue (Figure 2B). A summary of all
Fig. 1. TLC immunodetection of tumor-associated CD75s-gangliosides in HCCs. (A) Lipid extracts equivalent to 2 mg wet weight of nontumoral (N) and tumor tissues (T) were simultaneously separated by TLC and CD75s-gangliosides detected with the antibody AB2-6. The synopsis of CD75s-ganglioside distribution in HCCs is provided in Table I. Representative examples are shown for the different amounts of CD75s-gangliosides in malignant versus nonmalignant tissues ranging higher (I), moderately enhanced (II), equal (III) and lower content (IV) in HCCs. Gangliosides from human granulocytes (for structures, see Supplementary data, Table S3) served as positive reference for the orcinol stain (O; 10 µg) and overlay detection using the anti-CD75s-antibody (AB2-6, 1.5 µg), viscumin (V, 10 µg) and rViscumin (rV, 10 µg). (B) Structure of the CD75s-1-ganglioside IV^Neu5Ac-nLc4Cer (d18:1, C16:0).

Fig. 2. Structural characterization of CD75s-1-gangliosides in nontumorous (A, B) and HCC tissues (C, D) by TLC-IR-MALDI-ο-TOF-MS. Lipid extracts equivalent to 2 mg wet weight of nontumoral (N) and tumor tissues (T) of patient 7 (tumor category I, see Table I) were separated by TLC and CD75s-gangliosides detected with the antibody AB2-6. Arrowhead-marked gangliosides were analyzed by TLC-IR-MALDI-ο-TOF-MS. The high abundant [M^2+2Na-H]^+ ions at m/z 1672.91/1674.53 detected in the upper band correspond to IV^Neu5Ac-nLc4Cer (d18:1, C24:1/ C24:0) and IV^Neu5Ac-nLc4Cer (d18:1, C16:0) (D) of the tumor tissue (T). The same species were detected with much lower signal intensities in the corresponding bands (A, B) of the nontumorous liver tissue (N). For further details, refer to Supplementary data, Table S2.
The distribution analysis of iso-CD75s-gangliosides was performed with antibody AB2-3 which specifically binds to the Neu5Acα3Galβ4GlcNAc epitope. The tumors were ranked and grouped into categories I to IV as described in Table I (right column). Four representative immunostains are shown for each tumor category (Figure 3A). In 41.3% of the investigated HCC samples, an enhanced content of iso-CD75s-1-gangliosides (= IV3Neu5Ac-nLc4Cer; for structure, see Figure 3B) was determined. In 20.7% of the cases, an equal amount of iso-CD75s-1-gangliosides was detected in nontumoral and tumor tissues, and a diminished content in HCCs was observed in 37.9% (see Table I, right column).

A structural profile of iso-CD75s-1 species in HCCs was obtained by TLC-IR-MALDI-o-TOF-MS using the AB2-3 antibody as exemplarily shown for patient 7 (tumor category I) in Figure 4. Highly abundant ions detected in the upper band of the tumor evidenced an increase of IV3Neu5Ac-nLc4Cer (d18:1, C16:0) (Figure 4C) compared with low abundant ions of the corresponding ganglioside of the nontumorous tissue (Figure 4A). The intense signals of the lower band of the HCC tissue correspond to IV3Neu5Ac-nLc4Cer (d18:1, C16:0) (Figure 4D); this species was not detected in the nontumorous tissue (Figure 4B). A synopsis of all identified iso-CD75s-1-gangliosides is provided in Supplementary data, Table S2.

Statistical analysis of ganglioside distribution
Box-plot diagrams of densitometric CD75s-1- and iso-CD75s-1-ganglioside data (see Table I) and difference values (calculated by subtraction of nontumorous from related tumor tissue values) are shown in Figure 5A and B. No significant difference between the amounts of CD75s-1-gangliosides in nontumoral and tumor tissues was found ($P = 0.596$). However, the distribution of the CD75s-1-gangliosides is altered, showing an increased variance in tumors. The highest CD75s-1-ganglioside amounts determined in this study were found in a few tumors, pointing to a subgroup of patients with considerably enhanced occurrence of this ganglioside in malignant tissues. However, at the same time, a high number of patients showed low content or even absence of CD75s-1 in the tumoral tissues, indicating a heterogeneous and undirected regulation of CD75s-1 upon neoplastic transformation (Figure 5A). Compared with CD75s-1-gangliosides, occurrence of iso-CD75s-1-gangliosides (Figure 5B) is more prevalent in both nontumoral and tumor tissues and shows a similar distribution pattern without a significant difference in the mean content ($P = 0.624$). These observations indicate an undirected regulation of CD75s-1- and iso-CD75s-1-ganglioside biosynthesis upon malignant transformation.

When the tumor types are categorized according to degree of differentiation from G1 (well differentiated) to G2 (moderately differentiated) and G $> 2$ (poorly differentiated to undifferentiated), a correlation between increasing iso-CD75s-1-ganglioside amount and the degree of cell differentiation from well (G1) to undifferentiated (G $> 2$) HCCs was found ($\tau = 0.317$, $P = 0.045$; Figure 5C). No other significant relationship of ganglioside content and clinicopathologic parameters (e.g. size of the primary tumor or grade of cirrhosis) could be observed. This apparent independence of ganglioside...
levels and the differing cirrhotic statuses (see Supplementary data, Table S1) is exemplarily shown in Supplementary data, Figure S1, for CD75s-1-gangliosides (Supplementary data, Figure S1A) and for iso-CD75s-1-gangliosides (Supplementary data, Figure S1B).

However, despite the fact that CD75s-1- and iso-CD75s-1-ganglioside levels in tumors did not correlate with those in adjacent noncancerous tissues (see Figure 5A and B, respectively), the statistical calculations gave evidence for a significant association of CD75s-1- and iso-CD75s-1-ganglioside distribution in nontumorous ($\tau = 0.392, P = 0.003$) and in tumorous tissues ($\tau = 0.650, P < 0.001$) as demonstrated in the box-plot diagrams. In conclusion, the amounts of CD75s-1- and iso-CD75s-1-gangliosides in tumors are not related to those determined in nontumoral tissues and the biosynthesis of both gangliosides is likely to be similarly regulated within the same type of tissues.

**Statistical analysis of sialyltransferase gene expression**

The transcriptional expression analysis of ST6GAL1 and ST3GAL6 by qRT-PCR (see Supplementary data, Table S1) revealed no significant changes of ST6GAL1 in tumors compared with nontumoral tissues ($P = 0.177$), whereas ST3GAL6 expression is significantly decreased in tumor tissues ($P = 0.003$) as shown in the box-plot diagrams of Figure 6A. Comparing the relative expression of sialyltransferases (Figure 6B), approximately 75% of the tumors exhibited a diminished ST3GAL6 expression versus the nontumorous tissues. With the exception of a few patients, the ST3GAL6 expression was downregulated, on average, by a factor of 2. However, no significant association between ST6GAL1 and ST3GAL6 expression levels with tumor size or grade of differentiation could be detected.

Because it is known that inflammatory reactions induce an increase in sialyltransferase reactivities preferentially that of ST6GAL1, changes in the transcriptional expression of sialyltransferases might occur at the differing cirrhosis stages determined in the adjacent noncancerous tissue samples of the patients (see Supplementary data, Table S1). However, the examination of sialyltransferase gene expression in cancerous and noncancerous tissues from patients with no, starting and advanced cirrhosis did not reveal any differences or correlation with the grade of cirrhosis (see Supplementary data, Figure S2). Thus, the observed phenomena are apparently independent of the cirrhotic status and seem to be tumor-specific.

**Discussion**

HCCs represent the third leading cause of cancer death worldwide, and clinical treatment options for HCCs are currently limited (Jemal et al. 2009). The prognosis remains poor because of high tumor recurrence or tumor progression, and systemic therapy with cytotoxic agents provides only marginal benefit. Understanding the molecular biology of HCCs is crucial in developing individualized treatment regimens and...
novel therapies for this highly aggressive and chemoresistant cancer. In view of the lack of effective conventional systemic therapy, treatment of HCCs is now entering the era of molecular targeted therapy (Taieb et al. 2006; Pang and Poon 2007; Zhu 2008).

Searching for GSL targets of HCCs, we employed our newly developed strategy combining TLC with IR-MALDI-o-TOF-MS (Distler, Hülsewig, et al. 2008; Müthing and Distler 2010). This combinatorial technique is particularly useful for the analysis of GSLs within lipid extracts omitting any purification steps (with the exception of saponification of co-extracted phospholipids) or column isolation procedures such as anion-exchange or silica gel adsorption chromatography. We recently refined the range of applications by multiple sequential immunodetection on TLC plates matched with direct IR-MALDI-o-TOF-MS (Souady et al. 2009) being of special advantage for the analysis of
GSLs in lipid extracts obtained from small tissues such as biopsy samples. With respect to pathogen–GSL adhesion, TLC-IR-MALDI-o-TOF-MS was also successfully applied to the structural identification of bacteria-binding GSLs (Müskens et al. 2010). We are currently working on combining an IR-MALDI source with to tandem mass spectrometer which may allow us a more precise structural characterization due to fragmentation capabilities as shown by Li, Teneberg, et al. (2008) and Li, Zhou, et al. (2008) employing ion-trap MS.

Several studies have shown that the majority of tumors exhibit an altered GSL expression compared with healthy tissues (Feizi 1985; Hakomori 1996), and tumor ganglioside metabolism has been implicated in modulating tumor formation and progression (Kaucic et al. 2006; Liu et al. 2010). Inhibition of glucosylceramide synthesis by transfection of an antisense sequence to glucosylceramide synthase and resulting reduction of total ganglioside content caused a striking reduction in melanoma formation in mice, providing experimental support for an enhancing role of gangliosides in tumor formation (Deng et al. 2002). Furthermore, treatment of the host with a novel imino sugar inhibits melanoma tumor growth accompanied with reduced ganglioside content and is thus a promising novel therapeutic approach to inhibit tumor progression (Weiss et al. 2003). On the other hand, gangliosides have engendered great interest as potential target molecules for anticancer therapies (Chu et al. 2000; Fredman et al. 2003; Yin et al. 2006; Kannagi et al. 2010) involving antibodies, toxins or lectins. Promising results have been obtained using two monoclonal antibodies, recently raised against tumor-associated ganglioside epitopes (Durrant et al. 2006; Roque-Navarro et al. 2008). In addition to monoclonal antibodies, the bacterial Shiga toxin from pathogenic Escherichia coli, which specifically binds to the neutral GSL globothriaosylecramide, is currently under investigation as a potential drug for anticancer therapies (Distler et al. 2009; Johannes and Römer 2010). With respect to novel, carbohydrate-based tumor-targeting strategies, the recombinant plant lectin rViscumin has already passed phase I clinical trials wherein its safe administration was demonstrated in patients suffering from different types of solid tumors (Schöffski et al. 2004, 2005; Bergmann et al. 2008). Currently, a phase II study with rViscumin is underway in patients with metastatic melanoma (ClinicalTrials.gov ID: NCT00658437). A second study will be conducted in metastatic colorectal carcinoma patients (ClinicalTrials.gov ID: NCT00932724). These promising approaches prompted us to investigate the rViscumin ganglioside receptors carrying the Neu5Acα6Galβ4GlcNAc (CD75s) epitope in HCC and adjacent nontumoral tissues. Following earlier findings of increased quantities of CD75s-1-gangliosides in HCCs in two out of three patients (Müthing et al. 2005), we report now on heterogeneous distribution of CD75s-1-gangliosides and of the closely related iso-CD75s-1-gangliosides in liver tumors of HCC patients. A high expression of CD75s-gangliosides has been documented in cancerous liver tissues with monoclonal antibodies (Hakomori et al. 1983; Okada et al. 1986; Taki et al. 1990, 1992; Tanno et al. 1993; Kannagi 2000) recognizing the Neu5Acα6Galβ4GlcNAc or Neu5Acα6Gal epitope. In three of these studies, a high accumulation of CD75s-gangliosides could be demonstrated in tissue cryosections (Tanno et al. 1993) and tissue lipid extracts (Hakomori et al. 1983; Taki et al. 1990; Tanno et al. 1993) of HCCs, whereas CD75s-gangliosides were undetectable in the nongenrator liver. Even though Okada et al. (1986) could not find an exclusive association of CD75s-ganglioside expression with neoplastic transformation, an overexpression of CD75s-gangliosides in 5 out of 12 patients indicates an upregulated biosynthesis in almost half of the HCCs investigated. Monoclonal antibodies against the CD75s epitope being extremely helpful in isomer discrimination have been raised and used so far not only for the investigation of rViscumin receptors (Müthing et al. 2005), but also for the differential expression analysis of α2-6 sialylated polyolactosamine structures in human B and T cells (Kniep et al. 1999; Zimring et al. 2003).

Another approach that is supposed to be useful in assessing the sialylation status in tumor tissues employs qRT-PCR expression analysis of the specific sialyltransferases. Importantly, we did not observe any significant association of ST6GAL1 with the amount of CD75s-1-gangliosides in malignant compared with nontumoral tissues. Reasons for differences between sialyltransferase transcription and final occurrence of ganglioside end product can be manifold and data on the phenotypic effects of ST6GAL1 expression are conflicting and in many cases not conclusive (Dall’Olio and Chiricolo 2001). For example, ST6GAL1 may compete with other sialyltransferases for type 2 chains depending on the background of sialyltransfases expressed by a given cell type. Moreover, different sialylation may result from an altered subcellular localization of sialyltransfases that was previously described for ST6GAL1 in hepatocytes after malignant transformation (Cao et al. 2002) being a possible explanation of our findings. Other possible factors that may have a pronounced effect on the final CD75s-ganglioside content are the altered availability of activated sugars in liver cancer cells or the competition of glycoproteins with glycolipids which are also sialylated by ST6GAL1 and were found to be expressed in higher quantities in HCCs (Cao et al. 1999).

Besides the ubiquitously expressed ST6GAL1, a second α2,6-sialyltransferase was characterized in the early 2000s named ST6GAL2 (Takashima et al. 2002; Krzewinski-Recchi et al. 2003). The substrate specificities of ST6GAL1 and ST6GAL2 are distinct, although both are able to preferentially act on free Galβ4GlcNAc disaccharides. ST6GAL2 exhibited relatively low and no activities toward some glycoproteins and GSLs, respectively, and its expression levels were much lower than those of the ST6GAL1 gene (Takashima et al. 2002). While ST6GAL1 is expressed in a high number of tissue types, ST6GAL2 showed a restricted tissue-specific pattern mainly found in brain tissue, small intestine and colon (Takashima et al. 2002; Krzewinski-Recchi et al. 2003; Lehoux et al. 2010). Furthermore, no ST6GAL2 gene expression could be observed in a variety of tumors (Takashima et al. 2002). All of these render a significant involvement of ST6GAL2, which was not investigated in this study, in the biosynthesis of CD75s-gangliosides in HCC and nontumoral liver tissues rather unlikely. Our data support the
findings of Cao et al. (2002) and Dall’Olio et al. (2004), indicating that ST6Gal1 can undergo up- or down-regulation in different HCC patients resulting in quantitatively heterogeneous levels of enzymes and related sialylglycoconjugates among neoplastic liver cells.

Regarding α-2,3-sialylation, we observed that the transcriptional expression of ST3GaL6 was significantly decreased in HCCs and did not correlate with the amount of iso-CD75s-gangliosides. Because the principal cell types expressing high levels of ST3GaL6 are myeloid lineage cells, reduced expression levels observed in HCCs may be due to a possible concomitant decrease in myeloid dendritic cells or hepatic macrophages also known as Kupffer cells that are present in nonmalignant liver tissues (Crispe 2009; Nemeth et al. 2009). Although the reported substrate specificity of various α-2,3-sialyltransferases remains somewhat unclear (Harduin-Lepers et al. 2001), ST3GaL6 is considered as the main sialyltransferase producing iso-CD75s structures on gangliosides (Okajima et al. 1999). ST3GaL4 represents an alternative candidate possibly mediating this reaction, but previous investigations on its substrate specificity are somewhat contradictory (Sasaki et al. 1993; Kitagawa and Paulson 1994; reviewed by Tsuji 1996) and a study on murine ST3GaL4 suggests a preferred substrate specificity for glycoproteins (Kono et al. 1997). Thus, the impact of ST3GaL4 on the synthesis of iso-CD75s-gangliosides remains elusive and requires further exploration. Interestingly, we observed a correlation between the levels of iso-CD75s-1-gangliosides and poor tumor cell differentiation in HCCs. This result is in consistent with data from Suzuki et al. (1991) who showed preferential binding of a monoclonal anti-iso-CD75s-ganglioside antibody with poorly differentiated gastric adenocarcinoma, suggesting iso-CD75s-gangliosides as potential markers for less differentiated malignancies of the stomach.

We conclude that transcriptional expression of sialyltransferases ST6Gal1 and ST3GaL6 investigated in this study does not reflect the occurrence of CD75s- and iso-CD75s-gangliosides, respectively. The lack of correlation between sialyltransferases and the occurrence of gangliosides therefore underlines the importance of bioanalytical approaches that probe the abundance of glycoconjugates on the metabolic level, such as chromatographic, immunochemical and mass spectrometric techniques. However, further experiments are mandatory to validate the applicability of targeting CD75s-gangliosides for a personalized treatment of individual HCC using rViscumin, which has just entered clinical phase II trials.

Materials and methods

Surgical specimens

The study was performed using HCC tissues from 35 patients who had undergone surgery for their primary tumors at the University Hospital Dresden or the University Hospital Münster (Müthing et al. 2005). Corresponding control specimens were obtained from the same patients at organ sites without macroscopic tumor involvement. Tissue samples were snap-frozen in liquid nitrogen after removal and stored at −80°C until analyzed. Tumor histology and stage were determined according to the criteria of the WHO (Hamilton and Aaldoten 2000) and the Union Internationale Contre le Cancer (Sobin and Wittekind 2002). The wet weights of investigated malignant and adjacent nonmalignant tissues together with the clinicopathologic characteristics of the tumors and annotations to applicable tissue samples for ganglioside and sialyltransfase as well as statistical analysis are provided in Supplementary data, Table S1. The Local Ethical Committees of the Medical Council of Westfalen-Lippe, the Medical Faculty of the University Hospital Münster (Distler, Souady, et al. 2008a, 2009) and the University Hospital Dresden approved the current study.

Preparation of lipid extracts from surgical specimens

Lipid extracts were prepared as previously described (Distler, Souady, et al. 2008, 2009). The extracts were adjusted to defined volumes of chloroform/methanol (2/1, v/v) corresponding to 0.1 mg wet weight per microliter.

Reference gangliosides

The CD75s-1- and CD75s-2-gangliosides IVαNeu5Ac-nLc4Cer and VIαNeu5Ac-nLc6Cer, respectively, and the isomeric iso-CD75s-1- and iso-CD75s-2-gangliosides IVβNeu5Ac-nLc4Cer and VIβNeu5Ac-nLc6Cer, respectively, from human granulocytes served as references (Meisen et al. 2003) (see Supplementary data, Table S3). The nomenclature of GSLs follows the recommendations of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology (Chester 1999).

Anti-ganglioside antibodies, viscinum and rViscumin

The features of the chicken AB2-6 and AB2-3 antibodies, which specifically recognize CD75s- and iso-CD75s-gangliosides, respectively, have been previously described (Meisen et al. 2003; Müthing et al. 2005; Distler, Souady, et al. 2008; Distler, Hülsewig, et al. 2008). The CD75s-binding specificity of viscinum and its recombinant equivalent rViscumin has been described in previous publications (Müthing et al. 2002, 2004).

High-performance TLC and TLC overlay assay

Tissue lipid extracts were separated on high-performance TLC plates (no. 1.05633.0001; Merck, Darmstadt, Germany) and the GSLs were stained with orcinol or immunodetected with primary chicken anti-GSL antibodies in conjunction with secondary alkaline phosphatase-labeled anti-chicken IgY antibodies as detailed previously (Müthing 1998; Meisen et al. 2003; Distler, Hülsewig, et al. 2008). Binding of viscinum and rViscumin toward CD75s-gangliosides was analyzed as described (Müthing et al. 2002, 2004). Bound secondary antibodies were visualized with 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, and bands were scanned in reflectance mode at λ = 630 nm using a CD60 scanner (Desaga, Heidelberg, Germany, software ProQuant®, version 1.06.000). Ganglioside amounts were determined semiquantitatively as relative expression compared with references.
Infrared matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometry

The specifications of the IR-MALDI-o-TOF mass spectrometer (AB SCIEX, Concord, Ont., Canada) and the TLC-IR-MALDI-o-TOF-MS analysis of immunostained gangliosides on the TLC plate have been described in detail (Distler, Souady, et al. 2008; Distler, Hülesewig, et al. 2008; Müthing and Distler 2010).

RNA extraction and cDNA synthesis

Snap-frozen tissue samples were stored in PrepProtect (Milenyi Biotec, Bergisch Gladbach, Germany) at −20°C until use. RNA was extracted including on-column digestion with DNase I (Qiagen, Hilden, Germany) using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA purity and concentration were analyzed spectrophotometrically. Reverse transcription was performed with the QuantiTect Reverse Transcription Kit (Qiagen) following manufacturer’s advice.

Quantitative real-time PCR

qRT-PCR was performed on an ABI Prism 7900 HT (Applied Biosystems, Foster City, CA) using the QuantiTect SYBR Green PCR Kit (Qiagen). After a denaturation and polymerase activation step (15 min at 95°C), 40 cycles of denaturation (15 s at 94°C), annealing (30 s at 58°C) and extension (30 s at 72°C) were run. Specific amplification was assessed by melting curve experiments. All samples were analyzed as triplicates. Primers (Sigma-Aldrich, Hamburg, Germany) were designed using the online software tools Primer3 (Rozen and Skaletsky 2000) and GenScript primer design tool (https://www.genscript.com/ssl-bin/app/primer). Primers of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L13a (RPL13A), ST6GAL1 and ST3GAL6 were used (Supplementary data, Table S4). Ct-values were determined by SDS 2.2 software (Applied Biosystems) and further analyzed using the qBASE software program (Hellemans et al. 2007) and Microsoft Excel (Redmond, WA). For normalization, two housekeeping genes, GAPDH and RPL13A, were used. For each sialyltransferase, normalized values of the nontumorous and tumor samples were set in relation to the common minimum value.

Statistical analysis

The data of the TLC immunostained CD75s- and iso-CD75s-gangliosides and the ST6GAL1 and ST3GAL6 gene expression were compiled with the software package SPSS 14.02 (SPSS Inc., Chicago, IL) for nonparametric statistical analysis. Primary densitometric data of immunopositive gangliosides and secondary difference values calculated by subtraction of nontumorous from related tumor tissue values were employed. The secondary densitometric data were also used to define four categories of tumors compared with the nontumorous tissues by formal cut-point analysis: highly increased (category I, 2000 < x), moderately enhanced (category II, 400 < x < 2000), equal (category III, −400 < x < 400) and lowered content (category IV, x < −400) of CD75s-1-gangliosides and highly increased (category I, 1000 < x), moderately enhanced (category II, 200 < x < 1000), equal (category III, −200 < x < 200) and lowered amount (category IV, x < −200) of iso-CD75s-1-gangliosides. The Wilcoxon signed-rank test was applied to evaluate the association of ganglioside content with neoplastic transformation. The relationship between the ganglioside values with clinicopathologic parameters was analyzed with Kendall’s τ. Gene expression data were analyzed likewise. Kendall’s τ was also calculated to measure the correlation between CD75s-1- and iso-CD75s-1-ganglioside measurements. All tests used were two-tailed, and the level of significance was set to P = 0.05.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Conflict of interest statement

None declared.

Funding

This work was supported by the Deutsche Krebshilfe (grant numbers DKH 106742, 108502); and the Deutsche Forschungsgemeinschaft (grant number DR416-5/1).

Acknowledgements

We thank Dr. Joanne Yew and Dr. Iris Meisen (both University of Münster) for critical reading of the manuscript and stimulating discussions.

Abbreviations

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSL, glycosphingolipid; HCC, hepatocellular carcinoma; IR-MALDI-o-TOF-MS, infrared matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometry; qRT-PCR, quantitative real-time polymerase chain reaction; RPL13A, ribosomal protein L13a; ST6GAL1, β-galactoside α-2,6-sialyltransferase 1; ST3GAL6, β-galactoside α-2,3-sialyltransferase 6; TLC, thin-layer chromatography.

References


J Souady et al.
with strict preference to gangliosides and glycoproteins with terminal Neu5Acα2-6Galβ1-4GlcNAc residues. Biochemistry. 43:2996–3007.


