Thomsen-Friedenreich antigen expression in gastric carcinomas is associated with MUC1 mucin VNTR polymorphism

F. Santos-Silva1,2, A. Fonseca2, T. Caffrey3, F. Carvalho4, P. Mesquita2, C. Reis2, R. Almeida2, L. David4, and M. A. Hollingsworth3

1To whom correspondence should be addressed; e-mail: fsilva@ipatimup.pt

Introduction

Mucins are high-molecular-weight glycoproteins ubiquitously expressed on the surface of epithelia that form a protective and selective barrier, undertake receptor-ligand interactions, and communicate information about external cell conditions through signal transduction (Hollingsworth and Swanson, 2004). There are known variations in the structure of the mucin core proteins that result from allelic variability and from alternate splicing of mucin gene transcripts. In addition, there is substantial variation in post-translational processing of mucins, especially glycosylation, among different normal organ sites. Mucins produced by diseased epithelia are often glycosylated or otherwise modified in ways that are distinct from their normal counterparts, and these alterations are believed to contribute to the pathogenesis and progression of these diseases.

Alterations of mucins during the pathogenesis of cancer have been well documented (Hakomori, 2002). In addition to increased expression/secretion of mucins, neoplastic tissues frequently show aberrant glycosylation of mucins that includes expression of tumor-associated carbohydrate antigens (TACAs), which are often comprised of simple mucin-type carbohydrate antigens and increased sialylation of terminal structures (Hakomori, 2002). Principal among these are Tn, sialyl-Tn, and T (Thomsen-Friedenreich) epitopes, which have been described as pancarcinoma antigens (Ho and Kim, 1991). It is believed that Tn and T structures on mucin peptide cores expressed by adenocarcinomas contribute to adhesion, cell aggregation, invasion, and metastasis (Gendler, 2001; Glinsky et al., 2003). Expression of high levels of these TACAs are associated with poor prognosis in several cancer models (Amado et al., 1998; Byrd and Bresalier, 2004; Kannagi and Hakomori, 2001; Nakagoe et al., 2002; Werther et al., 1996).

The membrane-bound mucin MUC1 is expressed in normal mucosa, and aberrant expression of underglycosylated forms have been reported in carcinomas from different tissues. Underglycosylated forms of MUC1, detected by SM3 antibody, were identified in gastric carcinomas, mainly in aggressive cases (Reis et al., 1998). O-linked glycosylation of MUC1 (and other mucins) is a sequential and concerted process, controlled by glycosyltransferases, and influenced by the primary sequence of peptide core and by the position and structure of newly synthesized O-glycans (Hanisch et al., 2001). The underglycosylation of tumor-associated MUC1 results in the creation of cryptic carbohydrate core structures (Tn, sialyl-Tn, and T) (Taylor-Papadimitriou et al., 1999).

Because MUC1 glycosylation depends on the basic sequence of the peptide core, and gastric tumor-associated MUC1 shows abnormal underglycosylation, we hypothesized that the polymorphism of length in the MUC1 tandem repeat (TR) region (VNTR) may be associated with alterations of MUC1 glycosylation, namely, the appearance of the TACAs Tn, sialyl-Tn, and T in gastric cancer cells.

In this study, we investigated the association of different MUC1 genotypes (that were grouped according to expression of large and small TR alleles) and the expression of TACAs (Tn, sialyl-Tn, and T) in a series of 77 gastric carcinomas.
We also established a human gastric carcinoma cell line model (GP202) expressing recombinant full-length MUC1 constructs carrying different TR regions with 0, 3, 9, and 42 TR units, to evaluate the impact of MUC1 TR length variability on the expression of simple-mucin type carbohydrates. Our results show a significant association of T antigen expression and homozygosity for large TR alleles in a series of gastric carcinomas, whereas there was no significant association for Tn and sialyl-Tn antigens. Similar results were observed in the gastric carcinoma cell line model, as transfectants expressing MUC1 with longer TR regions showed increased expression of T antigen. Taken together, these in vivo and in vitro results show that abnormal glycosylation that produces T antigen epitopes is significantly associated with the length polymorphism in the MUC1 TR region.

Results

**TACAs (Tn, sialyl-Tn, and T) expression in gastric carcinomas and MUC1 VNTR polymorphism in gastric carcinoma patients**

We studied the expression of TACAs (T, Tn, and sialyl-Tn) in samples of 77 gastric carcinomas of individuals with different MUC1 VNTR genotypes. The gastric carcinomas showed frequent expression of Tn (74 of the 77 cases) and sialyl-Tn (69 of the 77 cases), whereas the expression of T was less frequent (21 of the 77 cases). The frequency of T antigen expression in carcinomas is significantly different among cases with different genotypes for MUC1 VNTR length ($p = 0.003$) (Table I). Cases homozygous for MUC1 larger VNTRs present a higher frequency of T antigen expression (83.3%), whereas cases homozygous for MUC1 smaller VNTRs and heterozygotes showed a lower frequency of T antigen expression (27.5% and 16.1%, respectively). No significant associations were found between Tn and sialyl-Tn antigens expression ($p = 0.81$ and $p = 0.61$) and MUC1 VNTR genotypes (Table I).

**Expression of underglycosylated forms of MUC1 and TACAs (Tn, sialyl-Tn, and T) in GP202 gastric carcinoma cell line MUC1 transfectants**

We analyzed whether variability of MUC1 TR length affected the glycosylation of MUC1 molecules with respect to expression of underglycosylated forms and TACAs (Tn, sialyl-Tn, and T). We evaluated by immunofluorescence microscopy the presence of underglycosylated MUC1, Tn, sialyl-Tn, and T in different clones of the GP202 gastric carcinoma cell line expressing FLAG-MUC1 protein (Figure 1, Table II). Immunofluorescence analysis with SM3 monoclonal antibody showed low levels of underglycosylated forms in clones expressing FLAG-MUC1 with 0, 3, and 9 TRs, similar to those found in clones with the vector and in the parental line (GP202). High expression of underglycosylated MUC1 was detected with SM3 in the clone with 42 TR (Figure 1, Table II). The evaluation of TACAs showed de novo expression of T antigen for clones expressing FLAG-MUC1 with 42 and 9 TR units, whereas this simple mucin type carbohydrate was not present in clones expressing FLAG-MUC1 with 0 and 3 TRs, in clones with the vector, and in the parental line (GP202) (Figure 1). The transfectants expressing FLAG-MUC1 with 42 TRs showed the higher expression levels of T antigen (Figure 1, Table II).

Immunofluorescence microscopy analysis of the simple mucin type carbohydrate antigens (Tn, sialyl-Tn) showed that clones with 0, 3, 9, and 42 TRs have a higher expression of Tn antigen when compared with the parental line and the clone with the vector. No significant differences for Tn antigen expression levels were found between the clones with 0, 3, 9, and 42 TRs. Reduced expression levels of sialyl-Tn were found in all the analyzed clones and parental cell line (Table II).

**Table I. Expression of TACAs in gastric carcinoma according to patients MUC1 VNTR genotypes**

<table>
<thead>
<tr>
<th>MUC1 recoded genotypes</th>
<th>TACAs</th>
<th>T</th>
<th>Negative cases (%)</th>
<th>Positive cases (%)</th>
<th>Tn</th>
<th>Negative cases (%)</th>
<th>Positive cases (%)</th>
<th>Sialyl-Tn</th>
<th>Negative cases (%)</th>
<th>Positive cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (16.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0 (0)</td>
<td></td>
<td></td>
<td></td>
<td>1 (16.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 (83.3)</td>
<td></td>
<td></td>
<td></td>
<td>6 (100.0)</td>
<td></td>
<td></td>
<td></td>
<td>5 (83.3)</td>
<td></td>
</tr>
<tr>
<td>LS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 (3.2)</td>
<td></td>
<td></td>
<td></td>
<td>2 (6.5)</td>
<td></td>
</tr>
<tr>
<td>26 (83.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 (96.8)</td>
<td></td>
<td></td>
<td></td>
<td>29 (93.5)</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 (5.0)</td>
<td></td>
<td></td>
<td></td>
<td>5 (12.5)</td>
<td></td>
</tr>
<tr>
<td>29 (72.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38 (95.0)</td>
<td></td>
<td></td>
<td></td>
<td>35 (87.5)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 (3.9)</td>
<td></td>
<td></td>
<td></td>
<td>8 (10.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>74 (96.1)</td>
<td></td>
<td></td>
<td></td>
<td>69 (89.6)</td>
<td></td>
<td></td>
<td></td>
<td>69 (89.6)</td>
<td></td>
</tr>
<tr>
<td>$p$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.003</td>
</tr>
</tbody>
</table>

512
The occurrence of alterations in mucin glycosylation is a ubiquitous phenomenon in all types of experimental and human cancers (Hakomori, 2002). Recent studies revealed crucial roles that these membrane glycoproteins play, namely, mediating (1) interactions between cells and exogenous carcinogens (Van de Bovenkamp et al., 2003), (2) interactions between normal and neoplastic cells (Kaldoyanidi et al., 2003), and (3) interactions between neoplastic cells (Glinsky et al., 2003). The relevance of the TACAs in cell invasion and metastatic potential have also been demonstrated (Nemoto-Sasaki et al., 2001).

In the present study, profiling of TACAs expression in gastric carcinomas showed a high prevalence of Tn (96.1%) and sialyl-Tn (89.6%) glycans, whereas T antigen was less frequent (27.3%). These results are in accordance with previous reports that showed frequent expression of Tn and sialyl-Tn in gastric carcinoma and precursor lesions (intestinal metaplasia) of the human stomach (David et al., 1992; Sakamoto et al., 1997; Yonezawa et al., 1992). The results show that in gastric carcinomas, the biosynthetic pathways of simple mucin type carbohydrate synthesis favor the expression of Tn and sialyl-Tn instead of T antigen (Figure 2). These findings can be explained by different expression levels or catalytic efficiency of ST6GalNAcI and C1GalT enzymes, because both enzymes compete for Tn substrate to form either sialyl-Tn or T antigens (Brockhausen et al., 2001). Another hypothesis is that competition from enzymes adding GlcNAc to form core 2 structures or sialyltransferases adding sialic acid to galactose may be responsible for the lower levels of T antigen (Dalziel et al., 2001; Kudo et al., 2002). In our series of gastric carcinomas, 53.2% of the cases (data not shown) express sialyl-T antigen, indicating that sialylation may contribute to the low frequency of cases with T antigen expression.

The evaluation of MUC1 VNTR polymorphism in our series of gastric carcinoma revealed a high frequency (51.9%) of homozygotic patients for short MUC1 VNTRs and lower frequencies for heterozygotes (40%) and homozygotic patients for long MUC1 VNTRs (7.8%). The association of short MUC1 VNTR with gastric carcinoma patients and patients with precursor lesions has been previously reported (Carvalho et al., 1997; Silva et al., 2001; Vinall et al., 2002).

In gastric carcinoma patients, the expression of Tn and sialyl-Tn is not associated with polymorphism in length of the MUC1 VNTR. Our data show that in gastric carcinomas, TR length appears to have a limited if any influence in the initial steps of MUC1 O-glycosylation, namely, Tn and sialyl-Tn synthesis. Similar findings have been reported in a colorectal adenocarcinoma cell line model transfected with MUC1 recombinant chimeric mucins with TR sequences from MUC2, MUC4, MUC5AC, or MUC5B (Silverman et al., 2001). Considering the complexity of the glycosylation process, further studies should be undertaken to investigate whether the expression and activity of gastric cell line glycosyltransferases are independent of MUC1 sequence (or TR length) or if the expression/activity of those enzymes is adjusted to compensate for substrate sequence requirements (Brockhausen, 2003; Marcos et al., 2003).

There was a statistically significant association of T antigen expression with large TR alleles of MUC1. The expression of T antigen in our series of gastric carcinomas is not associated with the expression of other TACAs (Tn and sialyl-Tn antigens) (data not shown), suggesting that the
synthesis of T antigen is not limited by the abundance of GalNAc or SA\(^ {\alpha} \)\(_ {2.6}\)GalNAc residues. In fact, one possibility that would explain these findings is that the stoichiometric power (Hollingsworth and Swanson, 2004) of large TR alleles create a locally high concentration of T antigen acceptor substrates for the C2GnT or the ST3Gal1 enzymes expressed by these cells, which results in incomplete extension of the O-linked structures because enzyme concentration or donor substrates become rate-limiting. Another possibility is that the long alleles of MUC1 exhibit regulatory activity on the C2GnT or the ST3Gal1 enzymes, either through direct protein interactions, or by signal transduction mechanisms that regulate the expression of these glycosyl transferases (Kohlgraf \textit{et al.}, 2003).

The generation of the gastric carcinoma cell line model, expressing FLAG-MUC1 with different TR lengths, was crucial to test the association of particular TACAs with MUC1 VNTR polymorphisms. The immunofluorescence analysis showed expression of T antigen in clones with 9 and 42 TR units, and these findings were also found in immunoprecipitates of FLAG-MUC1 from the same clones (data not shown). The results in the cell line model are in accordance with the association between MUC1 polymorphism (large TR) and T antigen expression found in our series of carcinomas. The immunofluorescence analysis showed no differences for sialyl-Tn and Tn expression between MUC1 clones. The levels found in FLAG-MUC1 clones were similar to the parental cell line (GP202) for sialyl-Tn antigen and were higher for Tn antigen. The impact of endogenous MUC1 in the glycosylation machinery of the cell line requires further studies with cells in which endogenous MUC1 has been reduced by RNAi or other methods.

The association of MUC1 length polymorphisms with the expression of TACAs is indirectly supported by the high expression of underglycosylated MUC1 epitopes, detected by SM3 monoclonal antibody in FLAG-MUC1 clone with 42 TRs. To a certain extent, the high reactivity of the clone with 42 TRs for SM3 antibody may depend on a higher proportion of TR units. However, in agreement to a recent publication, recognition by SM3 is only weakly dependent on the TR length, which indicates that at least partly the higher SM3 reactivity depends on higher levels of underglycosylation (Karsten \textit{et al.}, 2004).

In summary, our study showed that in a series of gastric carcinomas the expression of T antigen is associated with large TR domains in MUC1 gene, and this finding is reinforced by a gastric carcinoma cell line model in which different length alleles of MUC1 were expressed. Further studies are required to clarify the molecular processes that govern this association, but considering the relevance of this TACA for tumor progression (Baldus \textit{et al.}, 2001; Flucke \textit{et al.}, 2001; Reis \textit{et al.}, 1998), MUC1 polymorphism may be a potential factor in the identification of subgroups of gastric cancer patients susceptible to develop more aggressive carcinomas expressing T antigen.
**T antigen and MUC1 VNTR polymorphism in gastric cancer**

**Material and methods**

**Tissue samples**

Surgical specimens from 77 gastric carcinomas and adjacent mucosa consecutively resected at the Medical Faculty, Hospital S. João, were included in this study. Tissue fragments from the primary tumors fixed in formalin and embedded in paraffin were available from each case. Tissue samples from non-neoplastic gastric mucosa were collected immediately after surgery, frozen in liquid nitrogen, and stored at −70°C until DNA extraction.

**Expression of TACAs in gastric carcinomas**

Sections from formalin-fixed paraffin-embedded carcinoma material were used for evaluation of Tn, sialyl-Tn, and T antigens expression. Immunostaining with primary antibodies (Table III) specific for Tn, sialyl-Tn, and T antigens expression were performed as previously described (David et al., 1987). Scoring of the staining reactions was made using a semi-quantitative approach: −, no positive cells; +, a few positive cells (<5%); ++, well-defined areas of positive staining (5–50%); ++++, extensive areas of positively stained tumor cells (>50%). The staining intensity was homogeneous in all positive cases, and therefore it was not scored.

**MUC1 VNTR polymorphism analysis in gastric carcinoma patients**

High-molecular-weight DNA was isolated from non-neoplastic gastric mucosa using a salt-chloroform extraction method (Mullenbach et al., 1989). DNA samples were digested with EcoRI that recognizes restriction sites in the regions flanking the TRs of the MUC1 gene. Southern blotting analysis, allele identification, and recoding into large (L) and small (S) categories was done as previously described (Carvalho et al., 1997).

---

**Table III. Monoclonal antibodies used to study underglycosylated MUC1 and TACAs (Tn, sialyl-Tn, and T) expression in gastric carcinomas and in human gastric carcinoma cell line transfectants**

<table>
<thead>
<tr>
<th>Monoclonal antibody (isotype)</th>
<th>Specificity</th>
<th>Dilution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1E3 (IgG2A)</td>
<td>Tn</td>
<td>Undiluted</td>
<td>Clausen and Hakomori unpublished data</td>
</tr>
<tr>
<td>HB-Tn (IgM)</td>
<td>Tn</td>
<td>1:15</td>
<td>Dako</td>
</tr>
<tr>
<td>TKH2 (IgG1)</td>
<td>Sialyl-Tn</td>
<td>1:5</td>
<td>Kjeldsen et al. (1988)</td>
</tr>
<tr>
<td>HB-STn (IgG1)</td>
<td>Sialyl-Tn</td>
<td>1:8</td>
<td>Dako</td>
</tr>
<tr>
<td>HH8 (IgM)</td>
<td>T</td>
<td>1:5</td>
<td>Clausen et al. (1988)</td>
</tr>
<tr>
<td>HB-T (IgM)</td>
<td>T</td>
<td>1:10</td>
<td>Dako</td>
</tr>
<tr>
<td>SM3 (IgG1)</td>
<td>Underglycosylated-MUC1</td>
<td>Undiluted</td>
<td>Burchell et al. (1987)</td>
</tr>
</tbody>
</table>

---

**Gastric carcinoma cell line**

We used a human diffuse gastric carcinoma cell line—GP202, previously established in our laboratory (Gartner et al., 1996).

**Cell culture**

The cells were cultured in 150 cm² flasks at 37°C in the presence of 5% CO₂ with complete medium (RPMI 1640, supplemented with Glutamax-I, 25 mM HEPES, 10% fetal bovine serum and 50 µg/ml gentamicin). Media was changed every 3–4 days, and the cells were passaged when they reached 80–90% confluence using 0.05% trypsin–0.53 mM ethylenediamine tetra-acetic acid in Hank’s balanced salt solution. Cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

**Generation of transfectant cell lines with epitope-tagged MUC1 constructs**

The GP202 cell line was transfected with a eukaryotic expression vector pHb-APr1-neo alone or containing previously subcloned epitope-tagged MUC1 (FLAG-MUC1) cDNAs with different number of TR units (0, 3, 9, and 42 repeats) (Burdick et al., 1997). GenePORTER2 transfection reagent was used according to the manufacturer’s instructions (Gene Therapy Systems, San Diego, CA). After transfection, 600 µg/ml G418 was added to the medium to select stable transfected cells. Nontransfected cells died within 8 days; single colonies were picked with cloning rings and expanded for screening.

**Expression of FLAG-MUC1 in GP202 gastric carcinoma cell line transfectants**

GP202 gastric carcinoma cell line transfectants were screened for expression of FLAG-MUC1 by immunofluorescence microscopy and flow cytometry analysis using M2, an anti-FLAG monoclonal antibody from Sigma (St. Louis, MO). The cells were harvested from 80% confluent clones and resuspended in phosphate buffered saline (PBS) to a final concentration of 2 × 10⁶ cells/ml.

The cells to be screened by FACS analysis were fixed in ethanol, washed with PBS and incubated for 1 h at 4°C with M2 monoclonal antibody. The cells were then washed with PBS and incubated with secondary antibody FITC-conjugated donkey anti-mouse (Jackson Immuno Research Laboratories, West Grove, PA). After being washed with PBS, the cells were resuspended and 1 × 10⁵ cells were analyzed on a Dickinson Facs Scan (Coulter Counter Epics XL-MCL).

The cells to be screened by immunofluorescence microscopy were applied to a microscope slide. The cells were fixed in ice cold acetone, then washed twice with TBS and incubated with rabbit nonimmune serum (Dako, Copenhagen, Denmark) for 20 min to eliminate nonspecific staining. Incubation with M2 antibody was carried overnight, followed by an incubation with an fluorescein isothiocyanate (FITC)–conjugated rabbit anti-mouse secondary antibody for 40 min. Slides were washed in Tris-buffered saline (TBS) and mounted in vectashield medium for fluorescence microscopy (Vector Laboratories, Burlingame, CA).
The samples were examined in a fluorescence microscope (Leica DMIRE2). A semi-quantitative approach was used to score the immunofluorescence labeling. Intensity of immunofluorescence labeling was graded as: –, negative; +, weakly positive; ++, moderately positive; ++++, strongly positive. The percentage of labeled cells was also scored. Results are based on three independent assays analyzed by two independent observers.

Expression of MUC1 underglycosylated forms and TACAs in GP202 gastric carcinoma cell line transfectants

GP202 gastric carcinoma cell line transfectants were evaluated for expression of MUC1 underglycosylated forms and TACAs (Tn, sialyl-Tn, and T) by immunofluorescence using monoclonal antibodies (Table III). The cells were harvested from 80% confluent clones and applied to a microscope slide. The cells were fixed in ice-cold acetone, rinsed twice in TBS (pH 7.6) and incubated for 20 min with rabbit nonimmune serum diluted 1:5 in TBS containing 10% bovine serum albumin (BSA). The cells were rinsed in TBS and incubated overnight at 4°C with monoclonal antibodies (Table III) diluted in TBS containing 5% BSA. After incubation the cells were washed with TBS and incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (Code F-261, Dako) diluted 1:70 in TBS containing 5% BSA. Slides were washed in TBS and mounted in vextashield medium for fluorescence (Vector Laboratories).

The samples were examined in a fluorescence microscope (Leica DMIRE2). A semi-quantitative approach was used to score the immunofluorescence labeling. Intensity of immunofluorescence labeling was graded as: –, negative; +, weakly positive; ++, moderately positive; ++++, strongly positive. The percentage of labeled cells was also scored. Results are based on three independent assays analyzed by two independent observers.

Statistical analysis

Associations between MUC1 VNTR polymorphism and the expression of simple-mucin type carbohydrate antigens (Tn, sialyl-Tn, and T) were evaluated using the STATVIEW 4.02 Software (SAS Institute, Cary, NC). A Monte Carlo test was applied (10,000 iterations) whenever expected values were less than 5 as described by Sham and Curtis (1995).

Acknowledgments

We thank Dr. Joy Burchell and Prof. Joyce Taylor-Papadimitriou for providing the SM3 antibody. This work was supported by Fundação para a Ciência e a Tecnologia (grant SFH/BPD/3622/2000), Fundação para a Ciência e a Tecnologia programa POCI co-financed FEDER (project POCI/CBO/44812/2002), Fundação Lusó Luso Americana para o Desenvolvimento (project 173/2002), Fundação Calouste Gulbenkian (project FC-54918), and National Institutes of Health (grant R01-CA57362).

Abbreviations

BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; TACA, tumor-associated carbohydrate antigen; TBS, Tris-buffered saline; TR, tandem repeat.

References


