The role of N-acetylglucosaminyltransferase III and V in the post-transcriptional modifications of E-cadherin

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It has long been recognized that E-cadherin dysfunction is a major cause of epithelial cell invasion. However, very little is known about the post-transcriptional modifications of E-cadherin and its role in E-cadherin mediated tumor progression. N-acetylglucosaminyltransferase III (GnT-III) catalyzes the formation of a bisecting GlcNAc structure in N-glycans, and has been pointed as a metastasis suppressor. N-acetylglucosaminyltransferase V (GnT-V) catalyzes the addition of β1,6 GlcNAc branching of N-glycans, and has been associated to increase metastasis. The regulatory mechanism between E-cadherin expression and the remodeling of its oligosaccharides structures by GnT-III and GnT-V were explored in this study. We have demonstrated that wild-type E-cadherin regulates MGAT3 gene transcription resulting in increased GnT-III expression. We also showed that GnT-III and GnT-V competitively modified E-cadherin N-glycans. The GnT-III knockdown cells revealed a membrane de-localization of E-cadherin leading to its cytoplasmic accumulation. Further, the GnT-III knockdown cells also caused modifications of E-cadherin N-glycans catalyzed by GnT-III and GnT-V. Altogether our results have clarified the existence of a bidirectional crosstalk between E-cadherin and GnT-III/GnT-V that was, for the first time, reproduced in an in vivo model. This study opens new insights into the post-transcriptional modifications of E-cadherin in its biological function, in a tumor context.

INTRODUCTION

The remodeling of cell surface glycoproteins oligosaccharides by glycosyltransferases has been revealed to be crucial for several functional aspects of glycoproteins. These oligosaccharides play important roles in cell physiology, such as differentiation, adhesion, cell proliferation and in pathological conditions such as cancer where it has been shown to contribute to cell invasion and metastases (1). The oligosaccharides of glycoproteins are produced via catalysis by several glycosyltransferases and most of the cancer-associated changes of sugar chains of glycoproteins are due to the changes of glycosyltransferases. An increasing body of evidence suggests that phenotypic changes observed in cancer cells and tissues seem to be due to a direct effect of glycosyltransferases genes or an indirect one (1,2). Functional glycomics, which uses sugar remodeling by glycosyltransferases, is a promising tool for the understanding and characterization of glycan roles (3).

N-glycosylation represents an important protein post-translational modification, where N-glycans are attached to asparagine residues within the consensus sequence NX(S/T), where X can be any amino acid other than proline (4). N-glycans can be divided in three major groups: High Mannose-type; Complex-type and Hybrid-type (5). Two of the major glycosyltransferases involved in N-glycan biosynthesis are N-acetylglucosaminyltransferase III (GnT-III) and N-acetylglucosaminyltransferase V (GnT-V).

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GnT-III is a glycosyltransferase, product of the human MGAT3 gene, which transfers N-acetylglucosamine (GlcNAc) in β4-linkage to the core mannose of complex or hybrid N-glycans forming a bisecting GlcNAc linkage. The addition of this bisecting GlcNAc residue alters not only the composition, but also the conformation of the N-glycan (6). GnT-III is considered a key glycosyltransferase in N-glycan biosynthetic pathway since the introduction of the bisecting GlcNAc residue results in the suppression of further processing and elongation of N-glycans, precluding the formation of N-glycan (6). GnT-III is a glycosyltransferase in N-glycan biosynthesis, but also the conformation of the N-glycan (6). GnT-III is a glycosyltransferase in N-glycan biosynthesis, but also the conformation of the N-glycan (6). GnT-III has been shown to be a metastases suppressor. When GnT-III gene was transfected into melanoma B16 cells with high metastatic potential, the sugar chains on the cell surface were remodelled and the metastatic potential impaired. Additionally, it was shown that lung colonization after mice intravenous administration of the GnT-III transfectants was significantly decreased (9).

Contrary to the function of GnT-III, GnT-V activity and its product, the β1,6 branched N-glycans, were significantly increased in highly metastatic tumors (10). Consistently, in GnT-V knockout mice cancer metastasis is greatly suppressed (11). Thus, the enzymatic activity of GnT-III has been proposed to have an antagonistic effect to the one of GnT-V (7).

The enzymatic competition between GnT-III and GnT-V, where GnT-III has been shown to exhibit a priority activity, contributes to the suppression of cancer metastasis. In fact, overexpression of GnT-III in highly metastatic melanoma cells reduced β1,6 GlcNAc branching in cell surface N-glycans, which has been shown to be accompanied with an increase of bisecting GlcNAc N-glycans (9,12). Along with this enzymatic competition between GnT-III and GnT-V, cells expressing GnT-III showed an enhanced cell—cell adhesion through the prolonged turnover of E-cadherin on the cell surface (13). Conversely, GnT-V overexpression, accompanied with an increase of β1,6 branched structures, resulted in a decrease in the rates of N-cadherin mediated cell—cell adhesion (14). Overall, these results suggest the existence of a putative co-modulation of distinct cadherins controlled by glycosyltransferases GnT-III and GnT-V.

It is a long-lasting interest of our group to identify molecular mechanisms underlying E-cadherin dysfunction in cancer development and progression. Most studies mainly focus on the role of epi/genetic alterations in its promoter region and in its coding sequence or in its transcriptional repressors (15–20). However, N-glycans have been shown to be essential for E-cadherin folding and to assure its biological functions (21,22). Since E-cadherin is a glycoprotein that can be post-transcriptionally modified by phosphorylation, O-glycosylation and N-glycosylation, in the present work, we aimed to elucidate the role of GnT-III and GnT-V glycosyltransferases and its products, in E-cadherin expression.

We found that GnT-III knockdown resulted in a remarkable modification of cell morphology accompanied by a membranar de-localization of E-cadherin leading to an increase of aberrant cytoplasmic expression. Further, we verified that E-cadherin regulates the transcription levels of GnT-III which through competition with GnT-V glycosylate E-cadherin molecule. In primary gastric carcinoma, we validate our in vitro model and showed the mutual regulatory mechanism between E-cadherin and GnT-III/GnT-V.

RESULTS

Stable transfection of E-cadherin wild-type in MDA-MB435 cells induced alterations in GnT-III mRNA transcription levels

The stable transfection of a full-length human E-cadherin in MDA-MB435 cells, that endogenously lack E-cadherin, induces an increase in transcription levels of MGAT3 gene (Fig. 1A). Quantitative real-time PCR analysis further confirmed that GnT-III mRNA transcription levels in MDA-MB435 transfected with E-cadherin wild-type (wt) were ~2-fold higher than those in MDA-MB435 mock cells (Fig. 1B). The same was not verified for GnT-V mRNA expression levels since no alterations of this enzyme were verified, upon stable transfection of wt E-cadherin (data not shown).

Increase GnT-III mRNA expression after E-cadherin transfection altered the overall protein glycosylation in general and E-cadherin glycosylation in particular

Due to the significant increase of GnT-III mRNA expression mediated by E-cadherin transfection in MDA-MB435 cells, we evaluated the N-linked glycosylation in the total cell lysates from MDA-MB435 E-cadherin negative mock cells and from MDA-MB435 transfected with wt E-cadherin and on E-cadherin immunoprecipitated from these later MDA-MB435 cells. L-PHA lectin, that specifically recognizes β1,6 branched structures (product of GnT-V), and E-PHA lectin, which recognizes bisecting GlcNAc structure (product of GnT-III), were used in order to analyze the alterations in the N-glycosylation pattern of glycoproteins and on E-cadherin immunoprecipitated. Figure 2A shows that the introduction of E-cadherin in MDA-MB435 cells resulted in a notable decrease of GnT-V product (β1,6 branched structures) in the whole glycoprotein lysate, as revealed by decrease L-PHA reactivity in MDA-MB435 wt E-cadherin derived cells. The differences in GnT-III product (E-PHA blot) were not easily detected between mock and transfected cells, however, a slight increase in E-PHA reactivity in MDA-MB435+E-cadWT cells could be observed.

In order to evaluate whether E-cadherin is targeted for modifications by GnT-III and GnT-V, E-cadherin isolated from MDA-MB435 wt E-cadherin transfected cells was immunoprecipitated and probed with L-PHA and E-PHA lectin (Fig. 2B). The Figure 2B shows that E-cadherin was targeted by both GnT-V and GnT-III enzymes, being modified with β1,6 branched structures (L-PHA positive reactivity) and with bisected GlcNAc structures (E-PHA positive reactivity).

Knockdown of GnT-III by siRNA caused no alteration in expression levels of E-cadherin mRNA and protein, but induces alterations on E-cadherin cellular localization in MCF-7/AZ cells

Assuming that E-cadherin has a role in the regulation of GnT-III expression which in turn glycosylates E-cadherin,
we silenced, by siRNA, GnT-III in order to breakdown this loop and in this way evaluate the role of GnT-III on E-cadherin glycoprotein. For that we used MCF-7/AZ cell line which endogenously expresses wild-type E-cadherin. The levels of GnT-III mRNA expression in MCF-7/AZ cells are similar with MDA-MB435 + E-cadWT cells (Fig. 3).

As shown in Figure 4A, there was no alteration in E-cadherin mRNA transcription levels after GnT-III knockdown compared with parental cells (MCF-7/AZ) and with controls cells (mock cells and non-silencing siRNA cells). Moreover, the levels of GnT-V mRNA transcription levels were also not affected (data not shown) by GnT-III knockdown. Additionally, no alteration in E-cadherin protein expression was observed after GnT-III knockdown cells compared with parental and controls cells, as illustrated in Figure 4B.

However, the same was not verified concerning E-cadherin localization at cell membrane when cells were treated with siRNA for GnT-III. E-cadherin cellular localization was evaluated by immunofluorescence with an antibody that recognizes the cytoplasmic domain of E-cadherin. In GnT-III knockdown MCF-7/AZ cells, E-cadherin modified its pattern of expression exhibiting an increase of the cytoplasmic staining, compared with non-silencing cells. The membranar expression was limited only to focal points of intercellular contact. Figure 4C illustrates that GnT-III knockdown leads to a de-localization of E-cadherin membranar expression.

Moreover, MCF-7/AZ cells treated with siRNA for GnT-III showed notable modification of the cellular phenotype. Figure 4C (bottom) shows a disruption of the cell–cell contact with an increase of lamellipodia and filopodia formations. When MCF-7/AZ cell line which endogenously expresses E-cadherin are treated with siRNA for GnT-III, the cells maintain the contact with neighboring cells only by cytoplasmic extensions. In control cells (non-silencing siRNA cells, subjected to the same experimental conditions
as GnT-III knockdown cells), E-cadherin was predominantly expressed in the cell membrane and cells form a compact monolayer (Fig. 4C, top).

**Knockdown of GnT-III caused changes in N-glycosylation in the whole glycoprotein lysate and specifically in E-cadherin**

We evaluated whether GnT-III silencing could modify the N-glycosylation profile in terms of $\beta_{1,6}$ branched structures (GnT-V product) and bisecting GlcNAc structures (GnT-III product) in glycoproteins using total cell lysates and immunoprecipitated E-cadherin. Total cell lysates and immunoprecipitated E-cadherin, from both control cells and GnT-III siRNA cells, were probed with E-PHA or L-PHA lectin (Fig. 5). Figure 5A (top) shows that the GnT-III knockdown resulted in a decrease of bisecting GlcNAc structure (E-PHA reactivity) compared with the control cells (Fig. 5A, left). Along with this observation, there was an increase of $\beta_{1,6}$ branched structures (L-PHA blot) after GnT-III knockdown, compared with control cells (Fig. 5A, right). Taking into account these observations in whole glycoprotein lysates, we further evaluated specifically the alterations in E-cadherin N-glycosylation after GnT-III silencing. Figure 5B (bottom) shows that GnT-III knockdown resulted in a decrease of bisecting GlcNAc structure (E-PHA reactivity) accompanied with an...
increase of β1,6 branched structures (L-PHA reactivity) on E-cadherin glycoprotein when compared with control cells.

**Relationship between expression of E-cadherin, bisecting GlcNAc and β1,6 branched N-glycans structures expression in sporadic diffuse gastric carcinomas**

In order to examine whether the relationship between E-cadherin, GnT-III and GnT-V observed in vitro was also observed in vivo, we performed E-cadherin, E-PHA and L-PHA histochemistry in a small series of diffuse gastric carcinoma, that harbor in a large percentage of cases alterations of E-cadherin expression. In all cases studied of diffuse gastric carcinoma, there was a significant loss of expression of E-cadherin which varied between <5% and 5–25% of tumor cell expression (Fig. 6, top). E-PHA reactivity, detecting bisecting GlcNAc structures, also shows a lower expression in diffuse gastric carcinomas that varied between <5% and 25–50% of expression. The E-PHA reactivity was mainly observed in the cytoplasm of tumor cells (Fig. 6, middle). On the contrary, L-PHA reactivity was consistently higher in all isolated tumor cells from diffuse gastric carcinomas (50–75% to >75% of L-PHA reactivity). Almost all isolated tumor cells labeled with L-PHA lectin, which stained in the cytoplasm and/or in the cell membrane (Fig. 6, bottom). Figure 6 illustrates in the same case of diffuse gastric carcinoma a low expression level of both E-cadherin and GnT-III product (E-PHA reactivity), and a higher expression level of GnT-V product (L-PHA reactivity), validating our in vitro model system.

**DISCUSSION**

E-cadherin is a cell–cell adhesion molecule that plays a pivotal role in epithelial cell invasion and has been considered the major contributor to cancer progression (23). In spite of a wide variety of studies focusing on the epi/genetic regulation on the E-cadherin-switch-off during tumorigenesis, post-translational mechanisms of regulation of this molecule remain largely unknown (15–20,24).

E-cadherin is a glycoprotein and glycan modifications have been shown to play key roles in the regulation of diverse proteins and also in fundamental cellular processes such as cell differentiation, adhesion and proliferation (25). Recently, the importance of E-cadherin N-glycosylation in the folding, stability and in its adhesive functions has been reported (21,22,26). The aim of the present study was to evaluate the relationship between E-cadherin expression, GnT-III and GnT-V glycosyltransferases and its products, namely, bisecting GlcNAc structures and β1,6 branched structures, respectively. This knowledge will identify new regulatory mechanisms of E-cadherin that are likely to contribute to cancer progression, namely in diffuse gastric carcinoma negative for E-cadherin genomic alterations.

We have demonstrated for the first time that E-cadherin regulates MGAT3 gene transcription and leads to a significant up-regulation of GnT-III mRNA transcription levels. Our studies are in accordance to the reports of Iijima et al. (12) and Akama et al. (27) that suggested that E-cadherin–catenin–actin complex plays an important role in the regulation of GnT-III enzymatic activity, since GnT-III activity increases in confluent culture conditions compared with sparse culture conditions.

Changes in glycosyltransferases expression levels have impact in the remodeling of cell surface glycoproteins oligosaccharides, which may consequently affect several cellular mechanisms such as intercellular adhesion (1–3). Thus, it would be expectable that the increase of GnT-III expression observed after E-cadherin transfection would change the N-glycosylation profile of the glycoproteins. Accordingly, we found that the up-regulation of GnT-III resulted in a significant decrease of β1,6 branched structures, product of GnT-V, on glycoproteins, showing that GnT-III and GnT-V modify the same target glycoprotein in a competitive fashion, as previously shown by Zhao et al. (7).
In fact, when E-cadherin glycoprotein was stably transfected in MDA-MB435 cells, which are E-cadherin negative cells, was N-glycosylated by GnT-III (as demonstrated by presence of bisecting GlcNAc structures) and by GnT-V (presence of β1,6 GlcNAc branched structures). This post-translational modification of E-cadherin by GnT-III is functionally relevant since it has been previously suggested that glycosylation of E-cadherin by GnT-III has a role in metastases suppression (13,28).

Taking into account all above-mentioned observations, we can conclude that E-cadherin up-regulates GnT-III expression which in turn modifies E-cadherin N-glycans, adding bisecting GlcNAc structures suggesting the existence of a functional feedback loop between E-cadherin and GnT-III, illustrated in Figure 7A and B.

In order to clarify the effect of this post-transcriptional modification on E-cadherin carried out by GnT-III, we disrupted this functional loop (Fig. 7A and B) by silencing GnT-III expression. To address this question, we used MCF-7/AZ cell line that endogenously expresses E-cadherin and GnT-III, and verified that GnT-III knockdown did not affect E-cadherin transcriptional or transductional levels. However, when we compared cells silenced for GnT-III with cells treated with a scramble siRNA, there was a remarkable modification of the cellular phenotype. GnT-III knockdown MCF-7/AZ cells extensively lose their intercellular contacts, showing an increase of lamellipodia and filopodia extrusions, and E-cadherin membranar staining was persistant only in the focal points of intercellular contact. Moreover, there was an increase of cytoplasmic staining of E-cadherin comparing with control cells showing that GnT-III knockdown resulted in a membranar de-localization of E-cadherin with repercussions in the cellular phenotype. The cytoplasmic internalization of E-cadherin observed after GnT-III knockdown is in agreement with previous reports that described that the pool of E-cadherin undergoing endocytosis and recycling was markedly increased in cells without stable cell–cell contacts (29).

In response to GnT-III knockdown, we further evaluated the modifications in N-glycosylation profile of glycoproteins in general and on E-cadherin in particular. As expected, we
observed that in fact the GnT-III knockdown resulted in a decrease of bisecting GlcNAc structures on glycoproteins. In consequence, GnT-V has gained ability to synthesize β1,6 GlcNAc branched N-glycans, resulting in an increase of this product on glycoproteins. This remodeling of N-glycans was specifically observed in E-cadherin resulting in an increase of β1,6 branched structures, product of GnT-V, and a decrease of bisecting GlcNAc structures on E-cadherin molecule as presented in our model (Fig. 7C), leading to alterations of cell morphology and disruption of cell–cell contacts.

In conclusion, our \textit{in vitro} model shows a dynamic regulatory mechanism between E-cadherin and glycosyltransferases, GnT-III and GnT-V supporting the hypothesis that E-cadherin-mediated cell–cell adhesion may occur via remodeling of N-glycans controlled by glycosyltransferases (GnT-III and GnT-V) whose regulation and expression (GnT-III) can also be dependent of E-cadherin expression (see proposed model, Fig. 7).

This crosstalk between E-cadherin and GnT-III/GnT-V reported here \textit{in vitro}, was further confirmed for the first time, in an \textit{in vivo} model. We had chosen a model of Diffuse Gastric Carcinoma, based on the fact that E-cadherin deregulation is a common feature of these tumors (30). Mutations in the E-cadherin gene (CDH1) have been found only in Diffuse Gastric Cancer (31). The present study has demonstrated that in all cases of diffuse gastric carcinomas, a decrease or loss of E-cadherin expression was accompanied with a decrease of bisecting GlcNAc N-glycans, product of GnT-III. Conversely, GnT-V product remarkably increased with almost all isolated carcinoma cells labeled with L-PHA lectin (which looks like a tumor marker). Thus, the down-regulation of E-cadherin expression, observed in those carcinomas, suggest that, therefore, it was unable to regulate the transcription of GnT-III leading to a decrease of bisecting GlcNAc synthesis. Furthermore, the reduced enzymatic competition with GnT-V induced a significant increase of β1,6 GlcNAc branched synthesis.

In summary, our study provides strong evidences for a regulatory mechanism between E-cadherin, GnT-III and GnT-V (by enzymatic competition) which could underlie a possible novel signaling pathway in carcinogenesis. Moreover, our results open new insights into E-cadherin regulation, emphasizing its post-transcriptional modifications as another possible mechanism that could underlie E-cadherin dysfunction in cancer.

\section*{MATERIAL AND METHODS}

\subsection*{Cell culture}

Human MDA-MB435 cell line, which endogenously lacks E-cadherin expression at both the mRNA and protein level, and MDA-MB435 stably transfected with wild-type E-cadherin (MDA-MB435 + E-cadWT) cells (A.C.F. and J.C. Machado, manuscript in preparation) were maintained at
37°C in Dulbecco’s Modified Eagle’s Medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, under a humidified atmosphere containing 5% CO₂. Human MCF-7/AZ breast cancer cell line, which was kindly provided by Prof. Marc Mareel (Laboratory of Experimental Cancerology, Ghent University, Belgium), was maintained under the same culture conditions. MDA-MB435+E-cadWT cells were maintained under blasticidin selection (5 μg/ml; Invitrogen). GnT-III knockdown in MCF-7/AZ cells was generated through siRNA technique.

RNA extraction and analysis of mRNA expression by RT–PCR and real-time PCR
Total RNA from MDA-MB435 mock, MDA-MB435+E-cadWT and MCF-7/AZ cells were reverse transcribed using Tri-Reagent (Sigma) according to the manufacturer’s protocol. RNA yield and quality were determined spectrophotometrically, and 5.0 μg of total RNA was reverse transcribed using the Superscript III RNase H Reverse Transcriptase kit (Invitrogen) according to the manufacturer’s instructions. Primers used in PCR analysis for GnT-III and hypoxanthine phosphoribosyltransferase (HPRT) mRNA expressions were the following: human GnT-III forward (5’-GCCGCTATCAACGCCATCAA-3’) and human GnT-III reverse (5’-CAGGTAGTCTGCGGCCGATCCA-3’); human HPRT forward (5’-TTCCTCCTGAGCAGTCAG-3’), and human HPRT reverse (5’-GTCGGCGATCCA-3’). The product sizes obtained by the PCR were 260 bp for human GnT-III and 756 bp for human HPRT. The PCR conditions used for both gene analyses were 35 cycles of 15 s at 94°C, 30 s at 65°C and 2 min at 70°C. Reaction products obtained were submitted to electrophoresis in 1.6% agarose gels containing ethidium bromide, and bands were quantified using Quantity One software (BioRad). Quantitative real-time PCR analysis of GnT-III mRNA expression was performed using SYBRgreen reagent (Applied Biosystem). Each sample was amplified in triplicate in an ABI Prism 7000 (Applied Biosystem). Expression of 18S was also measured in triplicate for each sample and used for normalization of target gene abundance. Specificity of amplification was confirmed by melt curve analysis. Primers used in the quantitative real-time PCR analysis were as follows: human GnT-III forward (5’-GCCGCTATCAACGCCATCAA-3’) and human GnT-III reverse (5’-CAGGTAGTCTGCGGCCGATCCA-3’); human HPRT forward (5’-TTCCTCCTGAGCAGTCAG-3’), and human HPRT reverse (5’-GTCGGCGATCCA-3’). The PCR conditions were 45 cycles at 95°C for 5 s and 60°C for 30 s. Results of GnT-III mRNA expression levels are presented as the fold increase of E-cadWT cells, a Student’s t-test, with Microsoft Excel, was performed in the triplicates.

Immunoprecipitation, western blot and lectin blot analysis
Cell cultures were washed with phosphate-buffered saline (PBS) and then lysed in cold PBS containing 1% Triton X-100, 1% NP40, protease inhibitor cocktail (Roche 1 tablet/50 ml buffer) and phosphatase inhibitor cocktail (Sigma, 1:100 dilution). Total protein was quantified using a BCA protein assay kit (Pierce). Equal amounts of total protein (750 μg) from each cell lysate were precleared with 25 μl of protein G-sepharose beads (Sigma) for 1–2 h. After centrifugation, the supernatant was incubated overnight with 5 μg of monoclonal antibody against E-cadherin (BD Biosciences). After that, incubation with protein G-sepharose for 2 h was performed. Next, the beads were washed three times with immunoprecipitation buffer. The immune complexes were released by boiling for 5 min at 95°C in Laemmli sampling buffer and the immunoprecipitates were subjected to 7.5% SDS–PAGE and the separated proteins were transferred to a nitrocellulose membrane. The blots were then probed with anti-E-cadherin antibody or biotinylated Phaseolus vulgaris erythroagglutinin (E-PHA, which binds to bisecting GlcNAc structures) or biotinylated P. vulgaris leucoagglutinin (L-PHA, which recognizes β1,6 branched structures) lectins (Vector Laboratories). Immunoreactive bands from lectin blots were then visualized using the Vectorstain ABC kit (Vector Laboratories) and detection was performed by an ECL plus reagent. Equal amounts of total cell lysates were also subjected to SDS–PAGE electrophoresis and then transferred to nitrocellulose membranes. The membranes were incubated with L-PHA and E-PHA lectin for lectin blot analysis or with anti-E-cadherin antibody for immunoblot analysis. Blots probed with anti-actin antibody (Santa Cruz Biotechnology) were performed for loading control analysis.

siRNA transfection
A set of four different siRNAs targeting GnT-III mRNA were purchased from Dharmacon (IL, USA), and prepared according to manufacturer’s instructions. In parallel, non-silencing siRNA duplexes were used as a negative control. Prior to transfection, 60% confluent monolayers of MCF-7/AZ cells plated onto six-well plates were washed with PBS and incubated in serum and antibiotic-free medium. Cells were transiently transfected with 0–200 nM of siRNA, using the Lipofectamine 2000 transfection reagent (Invitrogen). At the end of each transfection, putative cytotoxic effects were evaluated, analyzing cell viability by trypan blue dye exclusion test. Efficiency of GnT-III knockdown was maximum with 150 nM of siRNA at 24 h.

E-cadherin staining
MCF-7/AZ cells were plated on six-well plates with coverslips on the bottom of each well. GnT-III siRNA transfection using the optimized conditions described earlier was performed. After the siRNA experiment, cells were fixed and incubated with anti-E-cadherin monoclonal antibody and with Alexa Fluor® 488 anti-mouse secondary antibody (Invitrogen). The nuclear staining was performed and images were then visualized in a fluorescence microscope (Zeiss).

Histology
Formalin-fixed tissues were used. For E-cadherin staining, heat-induced antigen retrieval was done by microwave in
citrate buffer (pH 6.0) for 10 min, after blocking the endogenous peroxidase. The sections were stained with anti-E-cadherin monoclonal antibody or with biotinylated lectins E-PHA or L-PHA (Vector Laboratories), according to manufacturer’s instructions. For E-cadherin staining, slides were incubated for 30 min with a 1:200 dilution of biotin-labeled rabbit anti-mouse secondary antibody (Dako). Incubation with avidin–biotin–peroxidase complex method using a commercial kit (Vectastain ABC Kit, Vector Laboratories) was then performed. 3,3'-diaminobenzidine (DAB) was used as chromogen and hematoxylin was used as a counterstain. Six human gastric adenocarcinoma, classified as diffuse type following the Lauren’s classification (32) or as terstain. Six human gastric adenocarcinoma classified as chromogen and hematoxylin was used as a counterstain. E-cadherin and anti-E-cadherin monoclonal antibody or with biotinylated lectins E-PHA or L-PHA (Vector Laboratories), according to

L-PHA lectins reactivity was evaluated by three independent observers and estimated visually for the entire lesion as follows: <5% of labeled cells; 5–25%; 25–50%; 50–75% and >75% of positive cells. The negative control sections were treated with PBS.

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Conflict of Interest statement. None declared.

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