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DNA methylation and histone modifications modulate the β1,3 galactosyltransferase β3Gal-T5 native promoter in cancer cells

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ABSTRACT

The native promoter of β1,3 galactosyltransferase β3Gal-T5 contributes to the expression of the enzyme and its oligosaccharide products, such as Lewis antigens, in many tissues. It is mainly sensitive to nuclear factor NF-Y and located nearby two CpG islands. To elucidate the regulation of the native promoter, we analyzed NF-Y protein and *β3Gal-T5* mRNA, and found that NF-Y is scarcely modulated among various cell lines and biopsies from normal or cancerous colon. Conversely, *β3Gal-T5* expression levels vary in the cell lines and are strongly down-regulated in colon cancer. We also performed quantitative methylation analysis of *β3Gal-T5* CpG islands and found an inverse correlation between mRNA expression and DNA methylation. In particular, the methylation levels of both islands are always increased in cancer, with respect to the corresponding normal counterpart, in matched normal and tumor samples of colon and breast origin. Moreover, treatment with chromatin remodeling agents 5-aza-2′deoxycytidine and trichostatin A does not restore transcription in completely negative cells, but only increases expression in basally positive cells. However, methylation analysis after 5-aza-2′deoxycytidine treatment revealed partial demethylation of both islands in all treated cells. Finally, chromatin immunoprecipitation assays on *β3Gal-T5* promoter showed that histone H3K4 trymethylation, H3K79 dimethylation, and H3K9-14 acetylation are high in cells expressing the transcript, and very low in those negative, while H4K20 trimethylation and H3K27 dimethylation are the opposite. We conclude that complex epigenetic modulation underlies the regulation of *β3Gal-T5* native promoter.

1. Introduction

β1,3 galactosyltransferase β3Gal-T5 is responsible for type 1 chain oligosaccharide synthesis, including the selectin ligand sialyl-Lewis a (NeuAcα2,3Galβ1,3[Fucα1,4]GlcNAc), epitope of tumor marker CA19.9, and other Lewis antigens as Lewis a (Galβ1,3[Fucα1,4]GlcNAc) and Lewis b (Fucα1,2Galβ1,3[Fucα1,4]GlcNAc) (Isshiki et al., 1999). The role of type 1 chain oligosaccharide is not known in details. On one hand, sialyl-Lewis a is a selectin ligand potentially involved in cancer metastasis (Kannagi, 2007) and considered a marker of malignancy (Yamashita and Watanabe, 2009). On the other hand, the synthesis of type 1 chain oligosaccharides was reported to counteract the synthesis of polylactosamine chains and sialyl-Lewis x (NeuAcα2,3Galβ1,4[Fucα1,3]GlcNAc) (Salvini et al., 2001; Isshiki et al., 2003; Mare and Trinchera, 2004), another selectin ligand, both involved in tumor progression and metastasis. β3Gal-T5 is active in epithelia of various organs. In mammary gland, thymus, and trachea, as well as in some human cancer cell lines, transcription is mainly driven by a native promoter that was found sensitive to nuclear factor NF-Y, also in mice (Mare and Trinchera, 2007). In the organs of the gastrointestinal tract (as the colon, stomach and pancreas) another promoter is active and stronger than the native promoter (Isshiki et al., 2003; Mare and Trinchera, 2007; Dunn et al., 2003). This alternative promoter has a retroviral origin (named LTR), has been probably acquired about 10–15 millions years ago (Dunn et al., 2005), and is regulated through homeoproteins such as hepatocyte nuclear factor HNF1, and caudal-related homeobox Cdx (Isshiki et al., 2003; Dunn et al., 2003). In various cell lines of different tissue origin, but even among those derived from the same tissue, β3Gal-T5 transcript,

Abbreviations: Gal-T, galactosyltransferase; NF-Y, CAAT binding factor (also named CBF); LTR, long terminal repeat; ChIP, chromatin immunoprecipitation; 5AZA, 5-aza-2′deoxycytidine; TSA, trichostatin A; H3, histone 3; RT, reverse transcription.

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as a whole, is widely modulated (Isshiki et al., 1999; Mare and Trinchera, 2004). Moreover, it is strongly down-regulated in colon cancer with respect to the normal mucosa (Salvini et al., 2001; Isshiki et al., 2003): the mechanisms responsible for differential expression among cell lines and down-regulation in colon cancer are not known at present.

Since NF-Y is rather ubiquitous (Caretti et al., 2003) and the native promoter is located in between two CpG islands (supplemental Fig. S1), we wanted to elucidate whether modulation of NF-Y accounts for differential expression of the transcript, or whether epigenetic mechanisms, such as DNA methylation and histone modifications, are involved. To this aim we first compared the expression levels of NF-Y in various cell lines and tissues expressing different amounts of the native transcript. We then performed quantitative bisulfite DNA sequencing starting form the same sources, and treated cell lines with drugs affecting DNA methylation and histone deacethylation to rescue expression. Moreover, we performed ChIP analysis to asses the chromatin conformation in some cell lines expressing different levels of such transcript.

2. Materials and methods

2.1. Cell line, tissues, and cell treatments

Human breast cancer cell lines MCF-7 and MDA-MB-231, human gastric cell line MKN-45, human bile duct carcinoma cell line HuCC-T1, and human colon cancer cell lines HT-29, HCT-15, COLO-205 and SW1116 were cultured as reported (Mare and Trinchera, 2004, 2007). Human breast cancer cell line BT-474, a gift of Dr. G. Fontana (University of Milan), was cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 1 mg/ml streptomycin, and 2 mM L-glutamine. Human breast samples were collected at surgery and kindly donated by Dr. Filippo Mare (Hospital di Circolo, Varese, Italy); human colon samples were as reported (Salvini et al., 2001; Trinchera et al., 2011). For treating cells with drugs affecting DNA methylation and histone deacethylation, HCT-15, MDA-MB-231, MCF-7, and MKN-45 ($1–4 \times 10^5$ cells) were plated in 6-well plates, incubated 24 h with regular medium that was replaced with medium containing different amount of 5AZA (Sigma, dissolved in DMSO as 10 mM stock solution) and/or TSA (Sigma, dissolved in ethanol as 1 mg/ml stock solution). Media were replaced every 24 h with media containing freshly diluted drugs. At the end of treatment cells were harvested by trypsinization and processed for DNA or RNA preparation.

2.2. Western blot

Freshly collected cell pellets, upon trypsinization and washing with PBS, were processed to obtain nuclear extracts using a commercially available kit (NEPER, Pierce) as reported (6). Frozen biopsies from colon and breast were dounce homogenized and submitted to nuclear extraction as for cell pellets. Protein concentration was determined by the Coomassie Plus Protein Assay (Pierce). Aliquots of nuclear extracts (5–10 μg of protein) were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane (Trans-Blot SD Semi Dry Transfer Cell, BIORAD) and blotted with rabbit polyclonal anti NF-YA (Santa Cruz sc-10779, 1:200) or rabbit polyclonal anti H3 (Cell Signaling, 1:500), according to our published protocol (Caretti et al., 2010).

2.3. Bisulfite sequencing

Genomic DNA was extracted from human tissues and cell lines using a commercially available kit (QIAamp DNA, Qiagen) and 0.1–1.5 μg were submitted to bisulfite treatment and purification using the Epitect bisulfite Kit (Qiagen), or the MethylCode bisulfite conversion Kit (Invitrogen). For cloning, the obtained material was amplified by PCR as follows. Amplifications (35 cycles) were performed in 25 μl using an hot start Taq (Promega) according to the manufacturer's recommendations in the presence of an enhancer (PCRx Enhancer system, Invitrogen) with 2 μl of bisulfite converted DNA as template and first reaction primers (see supplemental Table S1). Amplification program included a single treatment at 94 °C for 3 min followed by cycles consisting of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C, and a final extension step at 72 °C for 8 min. Nested PCRs (25 cycles) were performed in a final volume of 50 μl using 1–2.0 μl of the PCR products and inner primers designed to contain restriction sites (see supplemental Table S1). Reaction mixtures and amplification programs were as above but annealing temperature was 62 °C. Amplified fragments were column-cleaned, digested with appropriate restriction enzymes, column cleaned again, and cloned into pGl3 vector for sequencing (at Eurofins sequencing service).

Pyrosequencing experiments were aimed to quantitatively evaluate the methylation levels of two islands, CpG 1 and CpG 2, allowing us to investigate 4 CpG sites on each island (for details on PCR and sequencing primers see supplemental Table S1 and supplemental Fig. S1). PCR were carried out starting from 20 ng of bisulfite-converted DNA, in a final volume of 50 μl. PCR conditions were the same for both regions and included: 48 cycles consisting of 95 °C for 30 s, 56.4 °C for 30 s and 72 °C for 20 s; 30 μl of PCR products were loaded on PyroMArk ID instrument (Biotage AB, Uppsala, Sweden) and quantitative DNA methylation analyses were performed in the PSQ HS 96 System (Biotage), with the PyroGold SQA reagent kit (Biotage AB, Uppsala, Sweden) according to the manufacturer's instructions. Raw data were analyzed using the Q-CpG software v1.0.9 (Biotage AB, Uppsala, Sweden), that calculates the ratio of converted C's (T's) to unconverted C's at each CpG, giving the percentage of methylation.

For each sample, the methylation value represents the mean between at least two independent PCR and Pyrosequencing experiments.

2.4. RT-PCR

Quantification of β3Gal-T5 native transcripts was performed by competitive RT-PCR as previously described (Mare and Trinchera, 2004; Trinchera et al., 2011). Briefly, total RNA, prepared and DNase-treated using a commercially available kit (SV RNA, Promega) was quantitated by fluorometry with Qubit (Invitrogen). First strand cDNA was synthesized in a 20 μl volume by Moloney Murine Leukemia virus reverse transcriptase (2500 U/ml, USB-Affymetrix) in the presence of 1.0–2.0 μg RNA, 0.4 μM oligo-$(d)T_{12-18}$ primer, the supplied buffer, and 1000 U/ml human placental RNase inhibitor. Reactions were kept at 37 °C for 45 min and then at 42 °C for 45 min. Control reactions were prepared by omitting the reverse transcriptase. cDNAs were amplified in a volume of 25 μl in the presence of the indicated amounts of competitor, for 35 cycles (β3Gal-T5) or 5 pg competitor for 25 cycles (β-actin), using 2.5 U/ml of GoTaq Hot Start polymerase (Promega) in a mixture containing the supplied buffer, 2.0 mM $MgCl_2$, 0.1 mM dNTPs, 250 pmol/ml of each primer, and different amounts of cDNAs. Amplification program included a single treatment at 94 °C for 3 min followed by cycles consisting of 1 min at 94 °C (melting) and 3.5 min at 72 °C (annealing plus extension) and a final extension step at 72 °C for 8 min. No amplification was detected with control reactions. Competitor cDNAs were as reported (Salvini et al., 2001; Mare and Trinchera, 2007). Parallel PCR amplifications were performed on known amounts of standard cDNAs premixed with the competitors. Standard cDNAs were the original cloned sequences quantitated and diluted as for the competitors. Aliquots of PCR reactions were analyzed on 1% agarose gels stained with ethidium