An update on the I blood group system

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This update of the I blood group system (Cooling L. Polylactosamines, there's more than meets the "Ii": a review of the I system. Immunohematology 2010;26:133–55) continues to show the Ii antigens to be increasingly recognized as important posttranslational modifiers regulating cell adhesion, signaling, differentiation, and cancer. Ii antigens can modulate the immune response through the galectin lattice, as well as influence specific protein–protein interactions. Changes in *GCNT2* and I expression accompany stem cell differentiation and are associated with tumor progression in melanoma and breast and colon cancer. Regulation of *GCNT2* expression varies between cell types and differentiation. In red blood cell differentiation, *GCNT2* is regulated by methylation, microRNAs, and mitogen-activated protein kinase signaling pathways. Methylation and microRNAs also play a prominent role in altering *GCNT2* expression in several epithelial cancers. In congenital cataracts, *GCNT2* mutations may account for 4–6 percent of all cases. *GCNT2* may be particularly susceptible to gene deletion and rearrangements due to the density of Alu-repeat elements. ***Immunohematology* 2019;35:85–90.**

Key Words: I system, *GCNT2,* cancer, galectin

The I Gene

The I gene was originally named *IGnT* but has been renamed *GCNT2* (glucosaminyl *N*-acetyl transferase 2), with three tissue-specific mRNA isoforms: GCNT2A, GCNT2B, and GCNT2C (IGnTC).[1] In addition to the five coding exons, two noncoding exons have been identified upstream of exon 1A (E1A), resulting in a total of seven exons. Although most publications still only consider the five coding exons (E1A, E1B, E1C, E2, E3), a few recent publications have included the noncoding exons when discussing *GCNT2* structure and transcription (for example, exon E1A is exon 3).[2–4]

GCNT2 Mutations

Several new mutant *GCNT2* alleles have been identified, including two new missense mutations: one in E2 (G312D) and the other in E3 (Y349C) associated with congenital cataracts (Table 1).[1–3,5–7] In China, *GCNT2* mutations have been identified in 4–6 percent of children with cataracts.[8] Three previously identified i_{adult} alleles have been reclassified as I^{weak} (Table 1).[9]

Table 1. *GCNT2* mutations associated with I^{w+}, i_{adult}, and cataract phenotypes

Mutation			RBC phenotype		Congenital cataracts
Exon	Nucleotide*	Amino acid*	I+ RBC	i_{adult} RBC	
E1C	243T>A	N81L	I^{w}+	(↑i)	–
E1C	505G>A	A169T	I^{w}+	(↑i)	–
E1C	683G>A	R228Q	I^{w}+	(↑i)	–
E1C	651delA	V244X	–	+	–
E2	935>G>A	G312D	–	+	+
E2	984G>A	W328X	–	+	+
E2	1006G>A	G336R	–	+	+
E3	1046A>G	Y349C	–	(+)	+
E3	1049G>A	G350E	–	+	+
E3	1154G>A	R385H	–	+	+
Deletion	Δ98 kb	ΔE1A,E1B	(I+)		+
Deletion	Δ70 kb	ΔE1B,E3	–	+	+
Deletion	Δ93 kb	ΔE1B,E3	–	+	+
Deletion	Δ189 kb	ΔE1A,E3	–	(+)	+

*Nucleotide and amino acids are based on *GCNT2C,* which is 402 amino acids long. Please note that many publications have listed mutation nucleotide and amino acids associated with congenital cataracts based on *GCNT2A,* which is 400 amino acids long.

(+) Presumed I+ RBC phenotype based on genetic sequence.

RBC = red blood cell.

GCNT2 is located in an Alu-rich region. Alu repeats are short repetitive transposable elements that can facilitate mutation through deletion, translocation, and gene duplication.[2,9] This finding was inferred in one kindred, where Alu repeats flanked the deleted gene segment.[2] Currently, four deletion alleles involving most or all of the *GCNT2* gene have been identified. Most *GCNT2* deletion alleles are associated with the i_{adult} phenotype and congenital cataracts due to a profound loss of GCNT2 in all tissues.[2,3,7,9] One exception is a Pakistani kindred in which the deletion spanned the first two noncoding exons, exon E1A and exon E1B, but spared exons E1C/exon 5, E2/exon 6, and E3/exon 7.[2] As a result, the patients lack the GCNT2A and GCNT2B mRNA isoforms but should still be able to express and synthesize the GCNT2C isoform found in red blood cells (RBCs). Although not tested, it is probable that RBCs of these patients express a normal adult I phenotype.

GCNT2 Transcription

RBCs

The laboratory of Twu and colleagues has pursued additional work in deciphering *GCNT2/IGnT* regulation during hematopoiesis and RBC maturation.[10,11] To review, a RBC-specific promoter site was identified upstream of exon 1C (E1C/exon 5), which regulates expression of the GCNT2C mRNA isoform in RBCs. This promoter region contains consensus sequences for three transcription factors: Oct-2, Sp1, and c/EBPα. Previous work showed that although all three transcription factors can bind the *GCNT2C* promoter, c/EBPα alone is critical for *GCNT2* transcription in RBCs.[10,12] Furthermore, *GCNT2* transcription is regulated based on the presence or absence of serine phosphorylation on the c/EBPα (Ser-21). The dephosphorylated c/EBPα avidly binds to the *GCNT2* promoter, whereas phosphorylation (c/EBPα-p21) suppresses c/EBPα binding and decreases *GCNT2* expression.[12]

The role of serine phosphorylation indicated the involvement of mitogen-activated protein kinase (MAPK) signaling pathways. MAPK pathways are integral in transmitting signals from the extracellular membrane to the nucleus to effect changes in transcription, cell proliferation, and differentiation.[13] MAPK enzymes transfer phosphate groups to specific serine, threonine, or tyrosine residues on target proteins. This phosphorylation effectively acts as a molecular switch to turn proteins "on" or "off" within minutes of an extracellular signal or stimuli. MAPK phosphorylation is balanced by phosphoprotein phosphatases, which act to remove phosphate groups.[14] SHP2 (Src homology region 2 domain–containing tyrosine phosphatase) is a major phosphatase expressed in hematopoietic cells and is a positive regulator in early erythroid differentiation.[13] Among the three major MAPK families, JNK and p38MAPK are required for normal erythroid development.[13] The role of ERK is less clear although ERK may permit naive stem cells to escape self-renewal, an early step toward lineage differentiation. In mice, ERK promotes survival of early CD34+ erythroid progenitors, but does not contribute to later erythroid differentiation.[13] In fact, ERK acts to degrade GATA-1 binding, a transcription factor important for erythroid differentiation, while promoting myeloid differentiation.

Using the erythroleukemia line K562, Twu and colleagues were able to map upstream regulation of *GCNT2C* by ERK2 and SHP2.[15] Treatment of K562 cells with sodium butyrate to induce erythroid differentiation resulted in increased GCNT2C mRNA and I antigen expression, with equivalent decreases in phosphorylated ERK2 (pERK2) and c/EBPα-p21. These results were recapitulated by transfecting cells with mutant ERK2 that lack phosphorylated tyrosine residues (dominant-negative mutants due to Tyr185Ala and Tyr187Ala substitutions). In the absence of pERK2, there was a 50 percent decrease in c/EBPα-p21 and a converse sixfold increase in GCNT2C mRNA. Moreover, only GCNT2C was upregulated by blocking ERK2 activity. There was no effect on the expression of GCNT2A and GCNT2B isoforms. Similar results were observed with SHP2 dominant-negative mutants. In their model, erythroid differentiation leads to hypophosphorylation of SHP2 with reduction in ERK2 activity and c/EBPα-p21. As a result, the non-phosphorylated c/EBPα binds the *GCNT2C* promoter with *GCNT2C* transcription and causes I antigen expression.

Methylation

CpG islands, potential sites for cytosine methylation, are present in the majority of genes (60–70%).[16] Methylation of CpG islands in the promoter region can suppress transcription, whereas hypomethylation can increase gene expression. Epigenetic changes in promoter methylation, as well as other regions of the genome (CpG shores, intragenic CpG), are common in cellular differentiation and in malignancy.[16]

Studies have shown widespread hypomethylation during erythroid differentiation, with upregulation of several genes and transcription factors important to RBC development.[17,18] Lessard et al.[18] showed an inverse correlation between GCNT2 mRNA and methylation of CpG (cg14322298) within the *GCNT2* promoter, close to the transcription start site. *GCNT2* was hypermethylated in the earliest erythroblasts (days 0–3).

Changes in *GCNT2* methylation and expression are also described in colon and adrenal cancers. *GCNT2* was among 34 genes found to be hypomethylated, with increased GCNT2 mRNA, in aldosterone-secreting adrenal adenomas.[19] In colorectal cancer cell lines, all three GCNT2 mRNA isoforms could be upregulated by treating cells with demethylating agents. Methylation of CpG islands upstream of E1A/exon 3 (3382 bp upstream), EIB/exon 4 (within exon E1B), and E1C/exon 5 (1186 bp upstream) was demonstrated; however, only methylation of the CpG island located within the E1B exon was associated with *GCNT2* suppression.[4] Clinically, *GCNT2B* hypomethylation was a marker of tumor aggressiveness, with tumors showing deeper invasion and higher incidence

of lymph node metastasis.[4,20] Methylation of *GCNT2* is also inferred in gastric carcinoma.[21]

MicroRNA

MicroRNA (miRNA) are small, evolutionarily conserved, 18- to 22-nucleotide oligomers that are abundant in the human genome (~1%).[22] miRNA act posttranscriptionally to suppress protein expression by binding the 3′UTR of target mRNA to either promote mRNA degradation or directly interfere with mRNA translation. It is estimated that 30 percent of all proteins are potentially regulated by miRNAs.[22]

The let-7 miRNA family is expressed in human blood cells, with the highest levels observed in reticulocytes (let-7a/let-7b).[23] Inhibition of let-7 miRNA suppresses many aspects of erythroid differentiation, including globin-chain switching and enucleation.[23] Likewise, loss of let-7 miRNA function led to a decrease in GCNT2 mRNA, suggesting an indirect role for let-7 miRNA in GCNT2 expression.

The miRNA-199a is associated with GCNT2 regulation in stem cells and non-erythroid tissues. *GCNT2* is expressed in embryonic stem cells; however, *GCNT2* is suppressed by rising miRNA-199a during pancreatic islet cell differentiation.[24] The miRNA-199a/b family also regulates GCNT2 protein expression in colon epithelial cells.[25] Chao et al.[25] identified two miRNA-199 binding sites (ACACUGG) in the GCNT2-3′UTR: the miRNA site proximal to the poly-A tail was critical for miRNA-199 binding and GCNT2 suppression.

Polylactosamines and Galectins

Galectins are highly conserved galactose binding proteins that recognize lactosamine via a 130-amino-acid-long carbohydrate binding domain.[26,27] To date, 15 galectin families have been identified in mammals.[27] Some galectins bind terminal lactosamine on extended type 2 chain structures (Gal-1, -2, -3), whereas other galectins preferentially bind internal lactosamine motifs (Gal-9).[26] Galectin families differ in the size of the epitope (disaccharide or repeating lactosamine tetrasaccharide) and their ability to tolerate branching, sialylation, and fucosylation. Some galectins (Gal-1, Gal-3) are able to polymerize to form oligomers.[26,27]

In general, glycoproteins bearing several large, complex, branched N-glycans (or O-glycans) with long extended polylactosamines are the preferred receptors for galectins. Galectin binding to N-glycans is believed to create an extracellular lattice structure several angstroms above the lipid membrane.[26] This galectin lattice clusters and restricts glycoprotein diffusion, while promoting protein retention in the membrane. As such, galectins are integral in determining the composition and functioning of membrane microdomains, particularly with regard to protein signaling, and protein–protein interactions.[26,27] Cellular changes that increase N-glycan branching and elongation promote glycoprotein recruitment into the lattice.[26] Glycoproteins with five or more N-glycan residues are usually associated with the lattice.[26]

Galectins are modulators of adaptive immunity, including T-cells, B-cells, NK cells, macrophages, and dendritic cells.[27] Galectins can also affect neutrophil, mast cell, and platelet activation and tissue repair of endothelial, epithelial, and muscle cells. Aberrant galectin expression is described in cancer, which acts to suppress the immune response.

GCNT2 and Galectin-9 in B-Cell Receptor Signaling

Galectin-9 (Gal-9) recognizes internal lactosamine residues.[26] Interestingly, Gal-9 binds to naive and memory B-cells, but not to germinal center B-cells.[28] A comparison of glycan structures shows that naive and memory B-cells express predominantly (>90%) linear, i-type N-glycans, whereas germinal center B-cells express GCNT2 with a mix of linear (58%) and branched, I-type N-glycans (42%).[28] This finding can be recapitulated in Ramos, a Burkitt lymphoma B-cell line that strongly binds Gal-9: transfection of Ramos cells with *GCNT2* blocks Gal-9 binding.

It appears that GCNT2 helps regulate B-cell receptor (BCR) signaling by modulating CD45–CD22 binding. CD22 is a negative regulator of BCR signaling and B-cell activation.[29] CD45 acts to enhance BCR signaling by binding and sequestering CD22, making CD22 unavailable to interact with the BCR complex. It is hypothesized that Gal-9 binding to linear, i-type N-glycans on CD45 interferes with CD45–CD22 interaction, leaving CD22 free and available to associate with the BCR.[28] The expression of I antigen on CD45, however, blocks Gal-9 binding, thereby favoring CD45–CD22 binding.

GCNT2 and Leukocytes

The I antigen may enhance NK cell killing of leukemia cells.[30] Leukemia cell lines that strongly express I antigen were more sensitive to NK cell cytotoxicity. Moreover, transfecting cells with *GCNT2* can increase NK cell cytotoxicity nearly twofold. It appears that I expression enhances NK cell interaction with leukemia cells, leading to NK cell activation and degranulation. Incubating leukemia cells with monoclonal anti-I to block I antigen led to a 30 percent decrease in

NK-leukemia cell aggregation and a nearly 50 percent decrease in NK cell activation. Conversely, GCNT2 overexpression increased NK-leukemia cell interaction. The nature of NK cell-I antigen interaction is not known at this time.

GCNT2 may play a role in the malignant T-cell process. Transcriptome analysis identified *GCNT2* as one of 13 genes associated with mycosis fungoides and adult T-cell lymphoma leukemia/lymphoma.[31]

Stem Cells

GCNT2 is one of 90 proteins identified as a marker of human embryonic stem cells.[24,32] In murine hematopoietic stem cells, *GCNT2* is expressed by actively dividing stem cells but is decreased in quiescent stem cells.[33] In human hematopoiesis, GCNT2 mRNA is expressed by the earliest pluripotent progenitors but is progressively lost in early thymocyte progenitors and lymphoid lineage commitment due to *GCNT2* hypermethylation.[34]

The i antigen was identified as a marker of hematopoietic-derived mesenchymal stem cells but is lost with adipogenic and osteogenic differentiation, presumably because of terminal substitutions (e.g., sialylation) or branching.[35,36] Increased i expression on mesenchymal stem cells is linked to elevated β3GnT5 and β3GnT1.[36] Elevated i antigen on mesenchymal cells is accompanied by increased galactin-3 binding.[36]

Epithelial Cancers

Increased GCNT2 is a common feature in metastatic breast cancer and high-risk, hormone receptor–negative tumors.[37] Likewise, breast cancer patients frequently have elevated I antigen in sera.[38,39] In breast cancer cell lines, high GCNT2 mRNA expression enhanced the ability of cells to detach and migrate, with increased endothelial cell adhesion, invasion, and pulmonary metastasis.[37] *GCNT2* upregulation depends on the tumor growth factor β/Smad signaling pathway and may facilitate the epithelial-mesenchymal transition—an early critical step in epithelial cancers.[37] In genome-wide association studies, a specific single-nucleotide polymorphism (rs9348512, A allele) on 6p24 was linked to decreased GCNT2 mRNA expression and to a 15 percent decrease in breast cancer risk among carriers of the BRAC2 mutation.[40]

Similar findings are reported in prostate and colon cancer. In prostate cancer, high GCNT2 expression was linked to cell migration, invasion, and extracapsular extension.[41] In addition, the prostate cancer associated antigen F77 was identified as a H-active, branched type 2 chain antigen on glycolipids and glycoproteins.[42] In colon cancer, *GCNT2* is also upregulated during epithelial-mesenchymal transition.[25] As discussed earlier, the increase in GCNT2 expression is a result of decreasing miRNA-199a/b, which acts to suppress GCNT2 translation.[25]

Melanoma

In contrast to epithelial cancers, GCNT2 is inversely correlated to melanoma invasiveness and progression. Sweeney et al.[43] mined publicly available microarray data and identified GCNT2 as a biomarker for metastatic melanoma. GCNT2 protein and mRNA were markedly decreased in metastatic melanoma isolated from lymph nodes and viscera when compared with the primary lesions. This finding was confirmed by comparing N-glycan and GCNT2 mRNA expression in established melanoma cell lines and normal human epidermal melanocytes. Melanoma lines expressed predominantly simple, long, i-active polylactosamines, whereas normal melanocytes expressed complex, branched N-glycans. Forced overexpression of GCNT2 in melanoma cell lines decreased tumor growth in vitro and in vivo.

Subsequent studies showed that branched, I-active N-glycans modified cell signaling in melanocytes.[43] Specifically, branched N-glycans diminished ligand binding to insulin growth factor receptor and α/β-integrins, thereby diminishing overall cell activation. In melanoma, however, the loss of I-active N-glycans leads to enhanced cell signaling and a global increase in phosphorylation that favors both cell growth and survival. Altered glycosylation may also modify receptor dimerization and clustering, further enhancing cell signaling and adhesion.

The molecular basis for *GCNT2* downregulation in melanoma is not described. One study found rare examples of *GCNT2* copy number variation due to large chromosomal deletions in familial melanoma.[44] Unlike colon cancer, *GCNT2* downregulation in melanoma does not appear to be mediated by miRNA-199a: miRNA-199a is dramatically downregulated in metastatic melanoma, which should act to increase and prolong GCNT2 mRNA survival analogous to that seen in colon cancer.[25,45] Aberrant methylation is a common feature in melanoma and might account for *GCNT2* downregulation.[46]

Summary

The cloning of the I gene, *GCNT2,* has led to a significant understanding of the role of branched, I-active polylactosamines in cellular, cancer, and immune biology. *GCNT2* expression is highly regulated through a complex array of tissue-specific promoters, methylation, and miRNA.

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