Table 4. Clinical and biochemical characteristics of NAFLD subjects in the health check-up study.

Variables	NAFLD subjects
Number	870
Age (yr)	55.3±6.8
Gender (M/F)	659/211
BMI (kg/m^2)	26.2±3.6
SBP (mmHg)	120.8±15.6
DBP (mmHg)	73.0±10.7
AST (U/L)	31.6±17.6
ALT (U/L)	43.4±25.0
AST/ALT ratio	0.87±0.39
GGT (U/L)	64.5±71.5
Albumin (g/dL)	4.4±0.2
Total cholesterol (mg/dL)	211.0±35.0
Triglyceride (mg/dL)	152.0±93.2
HDL-C (mg/dL)	53.9±11.4
Glucose (mg/dL)	122.0±34.1
Creatinine (mg/dL)	0.83±0.36
Uric acid (mg/dL)	6.0±1.3
Iron (μg/dL)	114.7±38.8
Platelet count (x10^9/L)	222.4±53.0
FIB-4 index	1.32±0.72
Fuc-Hpt (U/ml)	190.6±235.7

Data are presented as the mean ± SD.
doi:10.1371/journal.pone.0066328.t004

after adjustment for age, BMI, serum levels of AST, GGT, total cholesterol, triglyceride, IRI, ferritin, platelet count, and caspase-cleaved cytokeratin-18 (M30 antigen). Moreover, measurement of serum Fuc-Hpt concentrations was superior compared to measurement of the M30 antigen in distinguishing NASH patients from non-NASH patients and predicting both the presence of ballooning hepatocytes and fibrosis severity in NAFLD patients. Serum Fuc-Hpt levels can serve as a novel diagnostic biomarker for NASH.

Very recently, we reported that serum Mac-2 binding protein (Mac-2bp) levels constitute a superior biomarker for distinguishing NASH from non-NASH patients (manuscript in press). Mac-2bp is a glycoprotein that has seven potential *N*-glycosylation sites [35,36], and the *N*-glycans in Mac-2bp are susceptible to fucosylation [37]. We also found that Mac-2bp levels had a positive correlation with hepatocyte ballooning scores and significantly increased with increasing hepatocyte ballooning scores. We have reported previously that many glycoproteins in bile were strongly fucosylated compared to serum glycoproteins, and suggested that fucosylation may be a possible signal for the polarized secretion of glycoproteins into bile in the liver [20].

Indeed, we recently showed that fucosylated alpha-fetoprotein is more selectively secreted into bile [38]. Therefore, we hypothesized the reason for the elevation of serum Fuc-Hpt levels in NASH patients as follows. An increase in ballooning hepatocytes, which lose polarized secretion of fucosylated glycoproteins, would induce disruption of the fucosylation-based machinery. As a consequence, the secretion of Fuc-Hpt into the serum would increase, and serum Fuc-Hpt levels would become elevated in NASH patients. On the other hand, M30, cytokeratin-18 fragment, is produced from apoptotic hepatocytes, and correlate with the magnitude of hepatocyte apoptosis [33]. The secretion mechanisms into sera are thought to be different between Fuc-Hpt and M30. We think that this is the reason why these two serum proteins did not correlate each other. In the present study, we found that serum Fuc-Hpt levels were increased in NASH patients and that Fuc-Hpt levels were elevated with increasing hepatocyte ballooning scores. In addition, ballooning hepatocytes are also a typical pathological characteristic in alcoholic liver diseases, and Chambers et al. previously reported that serum Fuc-Hpt levels assessed by Western blotting were elevated in patients with alcoholic liver diseases [25]. These results would enhance the significance of our hypothesis regarding the elevation of fucosylated glycoproteins in NASH patients. Haptoglobin is a glycoprotein produced mainly in the liver, and is abundant in serum. The use of serum Fuc-Hpt levels to predict hepatocyte ballooning scores in NAFLD patients is a novel type of biomarker in NASH diagnosis. As far as we know, no serum biomarkers can predict hepatocyte ballooning scores with sufficient accuracy comparable to Fuc-Hpt.

In our study, there were a number of patients with low serum Fuc-Hpt levels even in the patients with high hepatocyte ballooning score. Therefore, the complete selection of cutoff value as a biomarker of NASH, hepatocyte ballooning score, and liver fibrosis degree is somewhat difficult at this time. We think there were at least two mechanisms that would explain the wide range of serum Fuc-Hpt levels in the patients with high hepatocyte ballooning score. First, haptoglobin has four potential *N*-glycosylation sites [17], and it has been reported that an *N*-glycan at a specific site plays a pivotal role in apical sorting in a glycoprotein possessing multiple *N*-glycans [39]. Site-specific fucosylation in the haptoglobin *N*-glycan might correlate with the elevation of serum Fuc-Hpt levels in NASH patients. Site-directed oligosaccharide analysis with mass spectrometry should be conducted to elucidate the features of the fucosylation site in NASH patients. Second, normal hepatocytes do not express fucosylation regulatory genes, while their expression is increased in hepatocellular carcinoma cells [38]. Recently, it is reported that fucosylation on serum alpha 1-acid glycoprotein is associated with liver fibrosis, suggesting that non-cancerous hepatocytes with high levels of fucosylation regulatory genes might lead to the secretion of fucosylated proteins into serum [40]. The hepatocytes of the patients with low serum Fuc-Hpt levels and frequent ballooning hepatocyte would have low fucosylation regulatory genes, and produce small amount of Fuc-Hpt in their hepatocytes. To elucidate this issue, further investigations should be required in our future study. In our present study, we adopted several cutoff values to examine the availability of Fuc-Hpt. We found that setting cutoff value to the mean of normal control plus 1 SD (484.3 U/mL) was able to distinguish NASH patients from simple steatosis patients with sufficient specificity and that this cutoff value increased the number of false negative subjects. However, at this time, we think that the best cutoff value is 484.3 U/mL to rule out false positive patients for distinguishing NASH patients from simple steatosis patients.

This study has several limitations. First, the proportion of non-NASH patients (14.5%) was small compared with that of NASH patients (85.5%) in the biopsy-proven NAFLD patients study. Second, patients were recruited from hepatology centers in Japan with a particular interest in studying NAFLD, and the possibility of some referral bias could therefore not be ruled out. A patient selection bias might also have existed, because liver biopsy might have been considered for NAFLD patients who were likely to have NASH. The findings of the biopsy-proven NAFLD study may thus

Table 5. Clinical and serological characteristics of the NAFLD subjects in health check-up study classified by FIB-4 index categories.

	FIB-4 index (cutoff values proposed by Shah et al.)			
Variables	**Low cutoff point (<1.30)**	**Indeterminate (1.30–2.67)**	**High cutoff point (>2.67)**	***P* value**
Number	525	315	30	
Age	53.2±5.7	58.2±6.9	62.2±8.7	<0.01
Gender (M/F)	391/134	244/71	24/6	0.5283 (chi square test)
BMI	26.4±3.7	25.9±3.5	25.2±4.3	0.0585
SBP	119.7±15.7	122.3±15.5	122±15.0	<0.05
DBP	73.1±11.0	73.2±10.2	70.1±10.3	0.201
AST	26.9±9.6	36.3±16.7	64.9±53.2	<0.01
ALT	41.5±22.4	45.9±27.8	52.0±32.1	0.0946
AST/ALT ratio	0.73±0.20	0.91±0.32	1.51±1.25	<0.01
GGT	57.8±53.2	65.6±61.5	172.6±219.4	<0.01
Albumin	4.4±0.2	4.4±0.2	4.3±0.3	0.1697
Total cholesterol	213.8±35.0	208.4±34.4	187.8±32.5	<0.01
Triglyceride	150.6±86.5	152.5±99.0	172.7±136.4	0.9267
HDL-C	53.2±10.8	54.8±11.9	57.7±15.0	0.1211
Glucose	122.9±35.5	120.7±31.9	121.6±32.9	0.5514
Creatinine	0.82±0.42	0.84±0.22	0.88±0.23	0.0824
Uric acid	6.0±1.3	6.0±1.3	5.7±1.2	0.364
Iron	111.5±36.5	116.3±37.8	147.5±63.3	<0.01
Platelet count	246.2±47.8	190.0±35.8	144.8±35.7	<0.01
FIB-4 index	0.95±0.21	1.69±0.34	3.92±1.61	<0.01
Fuc-Hpt	168.3±194.0	200.2±231.7	447.7±556.5	<0.01

Data are presented as the mean ± SD.
Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol.
**P* values correspond to the comparison among groups. Kruskal-Wallis test for continuous factors and Pearson's Chi-square test for categorical factors were used.
doi:10.1371/journal.pone.0066328.t005

not represent NAFLD patients in the general population. However, the increase in serum Fuc-Hpt levels with the FIB-4 index in the health checkup study would enhance the significance of serum Fuc-Hpt level measurement in the general population. Third, there were 15 differences among 126 liver specimens in scoring for hepatocyte ballooning between two hepatic pathologists at first in our present study. Interobserver variation in defining ballooning hepatocytes is one of the most important concerning in the NASH diagnosis [31,41–43]. Recently, Lackner et al. proposed more stringent definition of hepatocyte ballooning using keratin 8/18 immunohistochemistry, and loss of keratin 8/18 immunostaining can serve as an objective marker of a specific type of ballooning hepatocytes [32,44]. In this study, we diagnosed hepatocyte ballooning score using HE stained liver specimens. Therefore, the diagnosis of these samples was carefully discussed and made by consensus between two hepatic pathologists (YK and HF).

We conclude, despite these limitations, that serum Fuc-Hpt levels can distinguish NASH from non-NASH patients and estimate the increase in hepatocyte ballooning scores of NAFLD patients with an accuracy superior to that of the M30 antigen in our patients. Further investigation is needed using a larger number of biopsy-proven NAFLD patients.

Supporting Information

Figure S1.
(TIF)

Table S1 Distribution of parameters according to Matteoni's classification in the biopsy-proven NAFLD patients.
(DOCX)

Table S2 Histological Characteristics of the biopsy-proven NAFLD patients.
(DOCX)

Table S3 Correlation coefficients of relationships between serum Fuc-Hpt levels and various parameters in the biopsy-proven NAFLD patients.
(DOCX)

Table S4 Various Fuc-Hpt cutoff values for the detection of NASH, the presence of ballooning hepatocyte, and advanced fibrosis.
(DOCX)

Table S5 Multiple logistic regression analysis of factors associated with F3 (diagnosed by FIB-4 index).
(DOCX)

Author Contributions

Conceived and designed the experiments: TT EM. Performed the experiments: MA YT KN Hironobu Fujii. Analyzed the data: YK HA KM YY SK. Contributed reagents/materials/analysis tools: SY Hideki Fujii YS YD TY MM HE NH YI NK. Wrote the paper: YK.

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PLOS ONE

Mutation of GDP-Mannose-4,6-Dehydratase in Colorectal Cancer Metastasis

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Abstract

Fucosylation is a crucial oligosaccharide modification in cancer. The known function of fucosylation in cancer is to mediate metastasis through selectin ligand-dependent processes. Previously, we found complete loss of fucosylation in the colon cancer cell line HCT116 due to a mutation in the GDP-fucose synthetic enzyme, GDP-mannose-4,6-dehydratase (GMDS). Loss of fucosylation led to escape of cancer cells from tumor immune surveillance followed by tumor progression and metastasis, suggesting a novel function of fucosylation in tumor progression pathway. In the present study, we investigated the frequency of GMDS mutation in a number of clinical colorectal cancer tissue samples: 81 samples of primary colorectal cancer tissue and 39 samples of metastatic lesion including liver and lymph node. Four types of deletion mutation in GMDS were identified in original cancer tissues as well as metastatic lesions. The frequency of GMDS mutation was slightly higher in metastatic lesions (12.8%, 5/39 samples) than in original cancer tissues (8.6%, 7/81 samples). No mutation of the GMDS gene was observed in normal colon tissues surrounding cancer tissues, suggesting that the mutation is somatic rather than in the germline. Immunohistochemical analysis revealed complete loss of fucosylation in three cases of cancer tissue. All three cases had GMDS mutation. In one of three cases, loss of fucosylation was observed in only metastatic lesion, but not its original colon cancer tissue. These data demonstrate involvement of GMDS mutation in the progression of colorectal cancer.

Citation: Nakayama K, Moriwaki K, Imai T, Shinzaki S, Kamada Y, et al. (2013) Mutation of GDP-Mannose-4,6-Dehydratase in Colorectal Cancer Metastasis. PLoS ONE 8(7): e70298. doi:10.1371/journal.pone.0070298

Editor: Alfons Navarro, University of Barcelona, Spain

Received January 25, 2013; **Accepted** June 18, 2013; **Published** July 29, 2013

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Fucosylation is one of the most important oligosaccharide modifications in cancer and inflammation [1]. Fucosylation is regulated by various fucosyltransferases, guanosine 5′-diphosphate (GDP)-fucose synthetic enzymes, and GDP-fucose transporters. Most GDP-fucose is synthesized by the de novo pathway in which GDP-mannose is transformed into GDP-fucose by GDP-mannose-4,6-dehydratase (GMDS) and GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (FX) [2–4]. Several antibodies that recognize fucosylated glycoproteins or glycolipids in sera of patients with cancer have long been used as tumor markers [5]. The alpha-fetoprotein (AFP)-L3 fraction, which is fucosylated AFP, has also been used clinically as a tumor marker for hepatocellular carcinoma since 1996 in Japan and since 2005 in the United States [6]. In general, fucosylation levels are increased during carcinogenesis of several kinds of cancer [7,8]. Previously, however, we found that complete loss of fucosylation due to deletion mutation of GMDS gene allowed colon cancer cells to escape from natural killer cell-mediated tumor surveillance through modulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signaling [9], suggesting that a novel metastatic pathway dependent on loss of fucosylation. GMDS mutation has been observed in colon (HCT116, LS174T, NCI-H716) and gastric (SCH) cancer cell lines as well as in human colon and ovarian cancer tissues [9]. Interestingly, GMDS mutation was not found in any adjacent normal tissues, suggesting that GMDS mutation was somatic. If loss of fucosylation is critical for tumor metastasis during colorectal cancer progression, the frequency of GMDS mutation would likely be increased in metastatic lesions. In this study, we investigated the frequency of GMDS mutation in metastatic colorectal cancer tissues such as liver and lymph node.

Materials and Methods

Ethics Statement

The protocol and informed consent were approved by institutional review boards at Osaka University Graduate School of Medicine. Written informed consent was obtained from all patients, and the study was conducted in accordance with the Helsinki Declaration.

Tissue Samples

Thirty-one samples of metastatic liver cancer, 2 samples of metastatic other cancers (gastric cancer, thyroid cancer) and 81 samples of the original colon cancer tissues derived from patients with colorectal cancer who underwent primary resection at the Department of Surgery at Osaka Medical Center for Cancer and Cardiovascular Diseases from 1995 to 2005 were stored at −80°C

until used. Six samples from metastatic lymph nodes from patients with colorectal cancer who underwent primary resection at the Department of Surgery at Suita Municipal Hospital from 2010 to 2012 were also used in this study. Clinical parameters of patients in this study are summarized in Table 1. Some of the cancer tissues were embedded in paraffin and used for immunohistochemical analysis. These studies were approved by the institutional ethics committee of the Osaka University Hospital.

Screening of GMDS Mutation with Reverse Transcription-polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from frozen tissues according to a standard protocol using TRIzol (Invitrogen, Carlsbad, CA). The extracted RNA was reverse-transcribed using Super Script™ III reverse transcriptase and the Oligo dT primer (Invitrogen). Using synthesized cDNA, PCR was performed with KOD-Plus-DNA polymerase (TOYOBO, Osaka, Japan). The PCR primers for GMDS were as follows: F, 5′-GCAAGCTTAAAATGGCA-CACGCACCGGCAC-3′ and R, 5′-GCGGATCCTCAGG-CATTGGGGTTTGTC-3′. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as an internal control, and the following PCR primers were used to amplify GAPDH: F, 5′-AACGGGAAGCTTGTCATCAAT-3′ and R, 5′-GCCAGT-GAGCTTCCCGTTCA-3′. Sequence analysis was performed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Immunohistochemical Studies

Cancer and normal colon tissues were fixed with 10% formalin/phosphate-buffered saline (PBS) and stored as paraffin-embedded samples. The 4-μm tissue sections were de-waxed, and endogenous peroxidase activity was blocked by treatment with 0.3% hydrogen peroxide in methanol for 10 min. After washing twice with PBS, the samples were incubated with Tris buffered saline and Tween 20 containing 5% bovine serum albumin overnight at 4°C. The samples were incubated with biotinylated *Aleuria aurantia* lectin (AAL; 2.0 μg/ml) or rabbit anti-GMDS antibody (0.3 μg/ml) for 1 hour at room temperature. Samples were washed three times with PBS and subsequently incubated with the ABC kit (Vector Labs, Burlingame, CA) for AAL staining or with Dako Cytomation Envision+ System- HRP Labeled Polymer Anti-Rabbit antibody (Dako, Glostrup, Denmark) for GMDS staining at room temperature for 30 min. After samples were washed three times with PBS, positive staining was visualized using diaminobenzidine (Dako).

Table 1. Clinical parameters of patients in this study.

	Cases (n = 117)
Sex (men/women)	69/48
Age (MEAN ± SD)	66.2±11.5
Clinical stage	
0	4 (3%)
I	16 (14%)
II	41 (35%)
IIIa	12 (10%)
IIIb	6 (5%)
IV	38 (32%)
Primary tumor site	
Colon	106 (91%)
Rectum	11 (9%)

doi:10.1371/journal.pone.0070298.t001

Results

GMDS Mutation in Colorectal Cancer

To examine the frequency of GMDS mutation in original and metastatic colorectal cancers, total RNA was extracted from 81 samples of human original colorectal cancer tissues, 39 samples of metastatic cancer tissues, and adjacent normal colon tissues and was subjected to RT-PCR analysis. Four shorter PCR products were found in several original and metastatic cancer tissues (Fig. 1). Detailed sequence analysis revealed different types of deletion of GMDS exons: exons 2–4, 5–7, 2–7, and 3–7. Two of these mutations, deletion of exons 5–7 and exons 2–4, were identical to those in the HCT116 and SCH cell lines, respectively. Deletions of exons 2–7 and 3–7 of the GMDS gene represent novel mutations identified in this study. The GMDS mutations in the metastatic lesions were consistent with those from the original colorectal cancer tissues. Interestingly, the homozygote of GMDS mutation without normal type of GMDS transcript was found in one metastatic liver cancer tissue (case 1 in Fig. 1). The frequency of GMDS mutation in metastatic lesions was 12.8% (5/39 samples): 12.9% (4/31 samples) in liver, 16.7% (1/6 samples) in lymph node, and 0% (0/2 samples) in other organs (Table 2). A slightly lower frequency 8.6% (7/81 samples) of GMDS mutation was observed in the original cancer tissues compared to their metastatic lesions even though the difference was not statistically significant ($p<0.10$, by χ^2 test). No GMDS mutation was observed in 24 samples of adjacent normal colon tissues.

Immunohistochemistry

To examine cellular fucosylation level in these cancer tissues, 33 cases of original colorectal cancer tissues and four cases of their metastatic lymph nodes were stained with anti-GMDS antibody and a fucosylated glycan-binding lectin, AAL. RT-PCR analyses showed heterozygous GMDS mutation in five of the 33 cases. Twenty-eight colorectal cancer tissues without GMDS mutation showed positive staining for both GMDS and AAL. Representative pictures are shown in Fig. 2A (case-N). In contrast, two of five cases of the original cancer with GMDS mutation showed negative staining for both GMDS and AAL (case 4 and 6 in Fig. 2A). Interestingly, one of five cases with GMDS mutation showed negative staining for both GMDS and AAL in the metastatic lymph node in spite of positive staining in its original colon cancer tissue (case L-6 in Fig. 2B and C).

Discussion

In previous our study, the GMDS mutation was identified by gDNA sequencing in two out of 100 cases of human colorectal cancer tissue and by RT-PCR analysis in five out of 10 cases of microdissected human ovarian cancer tissue. In this study, we further demonstrated the GMDS mutation in several human original and metastatic colorectal cancer tissues. The frequency of GMDS mutation was slightly higher in metastatic lesions (12.8%) than in the original cancer tissues (8.6%). Interestingly, in one case (L-6), loss of fucosylation was observed in the metastatic lymph node but not in its original cancer tissue (Fig. 2B and C). These results suggest that GMDS mutation is involved in the progression of colorectal cancer. The number of cases with GMDS mutation was not sufficient to examine the statistical correlation between GMDS mutation and disease activity with any certainty. Further

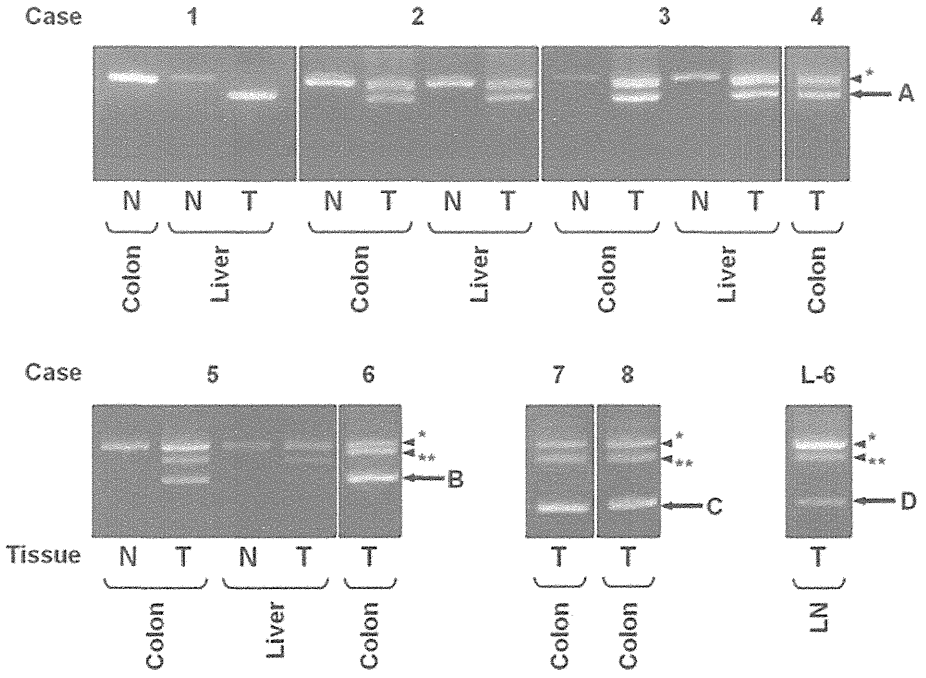

Figure 1. GMDS mutations in the original colorectal cancers and metastatic lesions. GMDS mutations were observed in seven cases of original colorectal cancer tissues and five cases of metastatic lesions. Arrows indicate bands representing GMDS mutation: deletion of exons 2–4 (A, 876 bp), 5–7 (B, 693 bp), 2–7 (C, 450 bp), and 3–7 (D, 495 bp). Arrowheads indicate wild type GMDS (*) and non-specific (**) bands. L-6 indicates one of the cases with metastatic lymph nodes. N, adjacent normal tissue; T, tumor; LN, lymph node.
doi:10.1371/journal.pone.0070298.g001

analysis with more number of samples will be required to determine the correlation between GMDS mutation and colon cancer progression. Four out of nine patients with GMDS mutation (case 1–8 and case L-6) were subjected to surgical operation within 3 years after diagnosis. Thus, a follow-up study is also required to investigate recurrence of metastasis.

Although most of the GMDS mutations observed in this study were heterozygous, homozygous deletion mutation was observed in one metastatic liver cancer tissue (case 1, Fig. 1). Since cancer tissues consist of a variety of cells, including not only cancer cells but also interstitial cells, it is difficult to demonstrate whether cancer cells harbor a heterozygous or homozygous type of mutation by RT-PCR analysis using whole cancer tissues. Thus, the possibility that cancer cells have a homozygous GMDS deletion mutation in tissues in which the heterozygous deletion mutation was observed remains. In fact, expression of GMDS and fucosylated glycans were barely detected by immunohistochemical analysis using anti-GMDS antibody and AAL in three of five cases with a heterozygous GMDS mutation, suggesting that cancer cells in these tissues may actually have homozygous GMDS mutation.

Table 2. Frequency of GMDS mutation in original and metastatic lesions of human colorectal cancer.

Tissue		Samples	Frequency (%)
Adjacent normal colon		0/24	0.0
Primary cancer		7/81	8.6
Metastasis		5/39	12.8
Breakdown of metastasis	Lymph node	1/6	16.7
	Liver	4/31	12.9
	Others	0/2	0.0

doi:10.1371/journal.pone.0070298.t002

Expression levels of the GMDS gene in cancer tissues are affected by not only gene mutation but also by transcriptional regulation. Although some of the glycosylation-related genes were reported to be epigenetically regulated [10,11], expression of GMDS was not altered by treatment with agents that modulating epigenetic DNA structure via DNA methylation or histone acetylation, suggesting that epigenetic effects do not play a major role in regulating GMDS gene expression [12] (data not shown). Further studies addressing gene expression regulation of the GMDS gene are warranted.

Among many fucosylation-related genes, mutation of the fucosyltransferases FUT1, 2, and 3, which catalyze α1–2 or 1–3/4 fucosylation, has been reported in patients with rare blood types [13,14]. In addition, GDP-fucose transporter has also been reported to be responsible for leukocyte adhesion deficiency type II which is a rare recessive syndrome characterized by growth and mental retardation and severe immunodeficiency [15–17]. No mutation of fucosylation-related genes has been reported in human cancer tissues before. GMDS is the first fucosylation-related gene that was found to be mutated in cancer tissues. Next-generation DNA sequence analysis may be a useful tool to determine why GMDS mutation occurs in several kinds of cancers.

High fucosylation level in cancer cells was demonstrated to increase metastasis through upregulating expression of sialyl Lewis A and X, selectin ligands, on cell surface [10]. In contrast, our results show that loss of fucosylation also increased metastasis even in the absence of selectin ligands. Cancer metastasis progresses through many steps: escape from immune cells, invasion, angiogenesis, intravasation, homing to metastatic tissues, extrav-

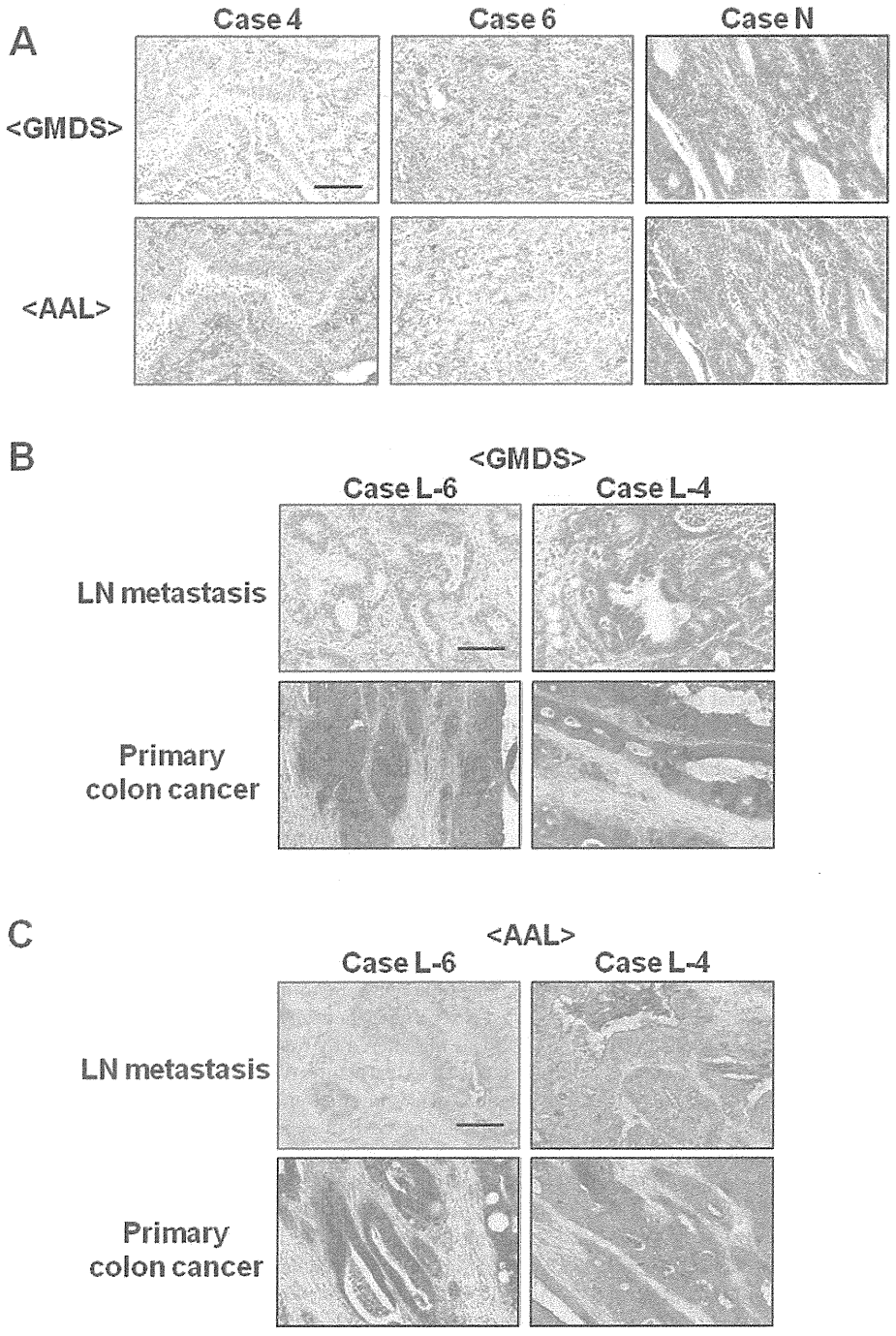

Figure 2. Immunohistochemical analyses of original colorectal cancers and their metastatic lymph nodes. Thirty-three cases of original colorectal cancer tissues and four cases of their metastatic lymph nodes were stained with anti-GMDS antibody and AAL. (A) Cases 4 and 6, which depict the original colorectal cancer with GMDS mutation, but not case N, which did not have GMDS mutation, showed negative staining for both GMDS and AAL. (B, C) In case L-6, which did harbor GMDS mutation, the metastatic lymph node was not stained for GMDS (B) and AAL (C) despite positive staining for both GMDS and AAL in the original colorectal cancer tissue sample. Case L-4 without GMDS mutation showed positive staining for GMDS and AAL in both the original and metastatic legions. Bar indicates 100 μm. LN, lymph node.
doi:10.1371/journal.pone.0070298.g002

asation, and colonization [18]. Fucosylation could have a different role in each step. Fucosylation in cancer cells needs to be tightly regulated and its dysregulaiton will cause further cancer progression and metastasis. In conclusion, the present study demonstrated that GMDS mutation should be involved in the progression of colorectal cancer. Next-generation DNA sequence analysis may give us more information about GMDS mutation in colorectal cancer.

Acknowledgments

This study was performed as a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct),

Ministry of Education, Culture, Sports, Science and Technology of Japan and was partially supported by the New Energy and Industrial Technology Development Organization (NEDO) as a part of the Developing Technology Project for Implementing Sugar Chain Functions in Japan. This work was also supported in part by the Global COE Program of Osaka University funded by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Author Contributions

Conceived and designed the experiments: EM. Performed the experiments: KN TI. Analyzed the data: KN KM YK SS. Contributed reagents/materials/analysis tools: KM. Wrote the paper: KN KM YK.

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Twin studies on the effect of genetic factors on serum agalactosyl immunoglobulin G levels

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Received November 5, 2013; Accepted December 20, 2013

DOI: 10.3892/br.2014.216

Abstract. The level of immunoglobulin G (IgG) lacking the terminal galactose, referred to as agalactosyl IgG, was found to be increased in chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease (IBD), particularly in Crohn's disease, which is suggested to have a genetic component. This oligosaccharide modification of IgG is mainly regulated by the expression of glyco-genes; however, the association between genetic factors and changes in the IgG glycosylation has not been fully elucidated. The aim of the present study was to assess the role of genetics in this process by comparing the serum agalactosyl IgG levels between members of monozygotic and dizygotic twin pairs who underwent medical check-ups at the same time. The serum agalactosyl IgG level was assayed using high-performance liquid chromatography. Hematological and biochemical markers, including γ-glutamyltranspeptidase (γGTP), alanine aminotransferase (ALT) and white blood cell (WBC) count, were also measured. Although the serum γGTP levels (and, to a lesser extent, ALT and WBC levels) exhibited a correlation within monozygotic twin pairs, agalactosyl IgG levels were not found to be correlated between members of either type of twin pairs. Thus, the role of genetic factors in determining serum agalactosyl IgG levels may be less significant compared to the effect of environmental factors or the onset of inflammatory disease.

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Abbreviations: IgG, immunoglobulin G; IBD, inflammatory bowel disease

Key words: agalactosyl immunoglobulin G, environmental factors, genetic factors, monozygotic twin pairs, dizygotic twin pairs

Introduction

Immunoglobulin G (IgG) possesses complex-type biantennary *N*-linked oligosaccharides at asparagine 297 of the Cγ2 domain of the Fc fragment (1). Some of these oligosaccharides have bisecting *N*-acetylglucosamine (GlcNAc), core-fucose, galactose and sialic acid residues (2,3). Patients with rheumatoid arthritis (4) and other chronic inflammatory diseases, such as systemic lupus erythematosus, Sjogren's syndrome and tuberculosis (5,6), exhibit elevated serum levels of agalactosyl IgG, an IgG oligosaccharide that lacks the terminal galactose. We recently reported that serum agalactosyl IgG levels may be a novel diagnostic marker for the activity and clinical course of inflammatory bowel disease (IBD) (7) and developed a method to determine agalactosyl IgG using a lectin-antibody ELISA (8). Furthermore, we demonstrated the pathophysiological role of agalactosyl IgG in IBD using a mouse model of experimental colitis that is deficient in β-1,4-galactosyltransferase (9). Those experiments indicated that the increase in agalactosyl IgG levels in patients with IBD may be associated with the host's defense against inflammation, rather than the etiology of IBD.

We previously evaluated the levels of agalactosyl IgG by measuring the ratio of agalactosylated to fucosylated IgG oligosaccharides (G0F/G2F) (7) and demonstrated that G0F/G2F is a marker of IBD clinical activity and prognosis of recurrence. However, some patients with Crohn's disease do not exhibit elevated agalactosyl IgG levels, despite severe disease activity, suggesting that genetic factors may dictate IgG galactosylation. Furthermore, the level of IgG agalactosylation was shown to increase with age (10) and may be regulated by a variety of environmental factors, including food and infection; therefore, the relative effect of genetic and environmental factors has not been clearly determined. To determine the effect of genetic factors on the agalactosylation of IgG, we investigated the correlations of G0F/G2F and other biochemical data within pairs of monozygotic and dizygotic twins who underwent simultaneous medical check-ups.

Materials and methods

Subjects. The characteristics of the participants are summarized in Table I. Sixteen monozygotic twin pairs (14 males and 18 females, aged 40.8±19.3 years) and 13 dizygotic twin pairs

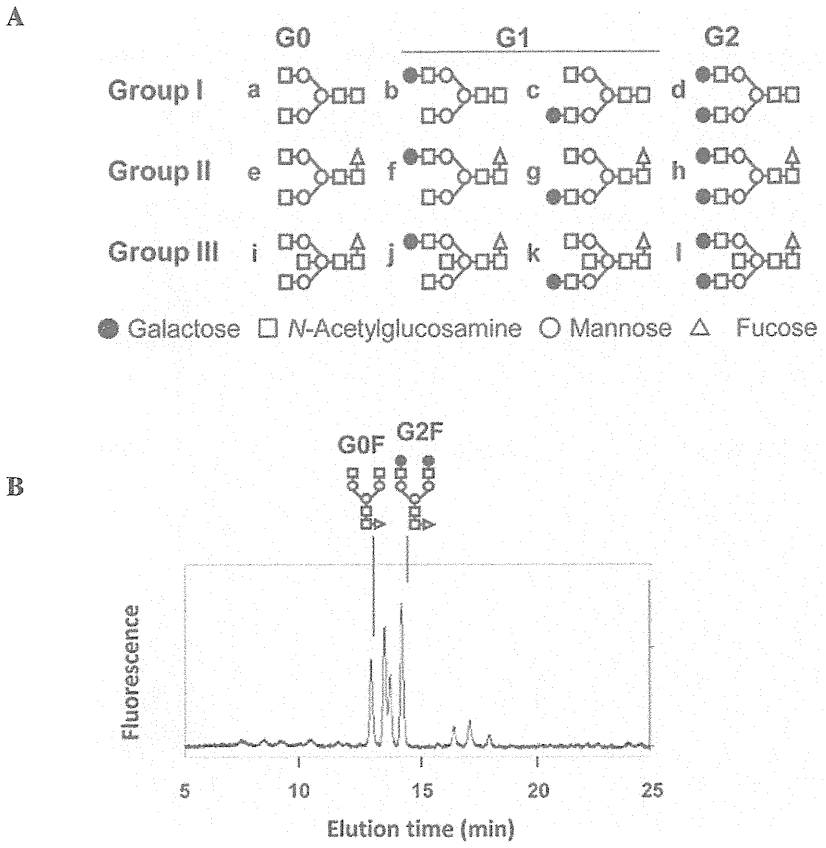

Figure 1. Analysis of 2pyridylamino (PA)-labeled IgG oligosaccharides with high-performance liquid chromatography. (A) Structural patterns of N-linked neutral oligosaccharides on IgG. (B) Representative profiles of 2PA-labeled oligosaccharides derived from IgG under neutral conditions.

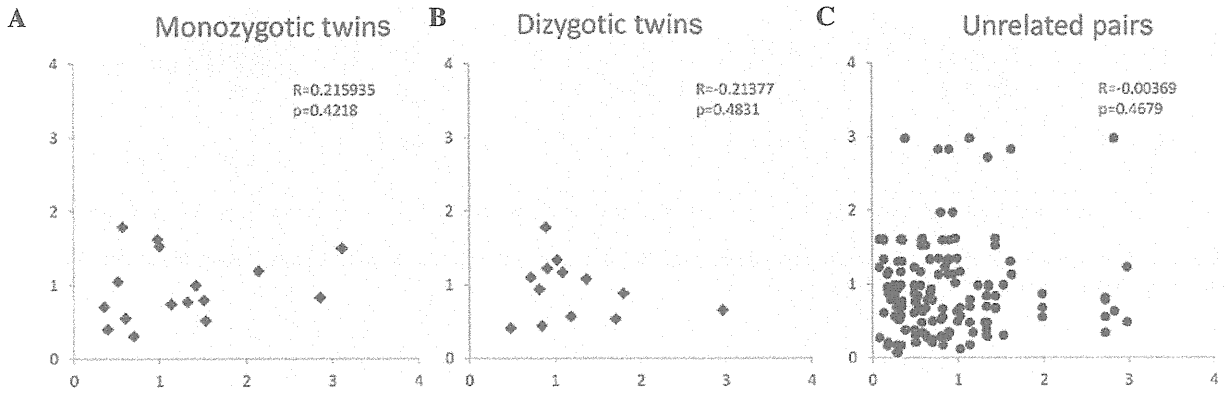

Figure 2. Scatterplots of G0F/G2F ratios for (A) monozygotic twins; (B) dizygotic twins; and (C) unrelated pairs. In (A) and (B), the higher G0F/G2F ratio within the pair was plotted on the horizontal axis.

Table I. Subject participant characteristics (means ± SD).

Characteristics	Monozygotic twins	Dizygotic twins
Pairs (n)	16	13
Male/female	14/18	10/16
Age (years)	40.8±19.3	42.5±16.9
γ-glutamyltranspeptidase (IU/l)	25.4±25.7	22.8±35.4
Alanine aminotransferase (IU/l)	16.8±9.01	14.8±9.30
White blood cells/μl	5,909±1,819	5,276±1,505
G0F/G2F ratio	1.10±0.68	1.07±0.55

(10 males and 16 females, aged 42.5±16.9 years) who underwent simultaneous medical check-ups as pairs between 1984 and 1994 were enrolled in this study. All the participants were healthy. Written informed consent was obtained from each subject and the study protocol was approved by the Ethics Committee of Osaka University. We also randomly selected unrelated pairs from this pool of participants and a total of 145 unrelated pairs were analyzed to serve as controls for genetic association.

IgG purification. Serum IgG was purified using protein G sepharose (Amersham Pharmacia Biotech, Buckinghamshire,

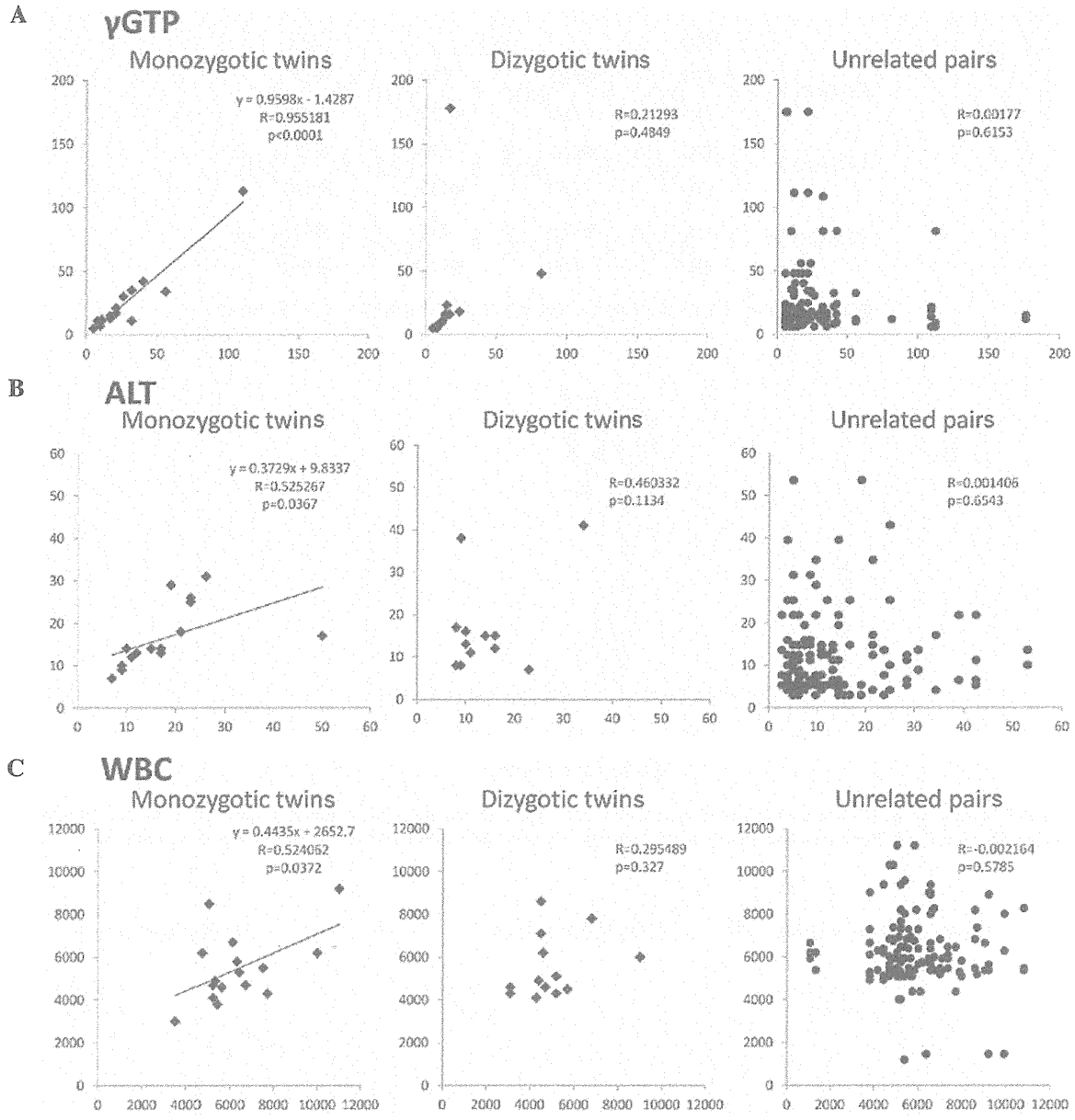

Figure 3. Scatterplots of serum levels of (A) γ-glutamyltranspeptidase (γGTP); (B) alanine aminotransferase (ALT); and (C) white blood cell (WBC) count for monozygotic twins, dizygotic twins and unrelated pairs.

UK). Briefly, serum diluted 1:1 with phosphate-buffered saline (PBS) was loaded onto a protein G sepharose column. The column was subsequently washed with a minimum of 10 column volumes of PBS, followed by the same volume of 10 mM ammonium bicarbonate. Column-bound IgG was eluted using 0.1% trifluoroacetic acid.

Analysis of IgG oligosaccharides. The pyridylaminated N-linked oligosaccharide of IgG was analyzed using reverse-phase high-performance liquid chromatography (HPLC). *N*-linked oligosaccharides were released from serum IgG and labeled with 2-aminopyridine as previously described (7). Briefly, *N*-linked oligosaccharides were released from purified IgG samples following overnight incubation with 0.5 mU glycopeptidase F (Takara Bio, Inc., Sigma, Japan) at 37˚C. The oligosaccharides were then incubated with 0.5 mM ammonium acetate (pH 4.0) for 30 min, lyophilized and labeled with 2-aminopyridine using GlycoTag (Takara Bio, Inc.) according to the manufacturer's instructions. Excess reagent was removed with a cellulose cartridge glycan preparation kit (Takara Bio, Inc.) and the oligosaccharides were incubated with 2 M acetic acid at 80˚C for 2 h to remove sialic acids. The pyridylamino (PA)-oligosaccharides from IgG were analyzed with reverse-phase HPLC (Hitachi High-Technologies Corporation, Tokyo, Japan) using a LaChrom Ultra C18 (2-μm) column (Hitachi High-Technologies Corporation) with 10 mM sodium phosphate (pH 4.4, solvent A) and 10 mM sodium phosphate plus 0.5% 1-butanol (solvent B) at a flow rate of 0.5 ml/min at 40˚C. The glycans were separated with a gradient of 0-50% solvent B for 30 min, followed by 50% solvent B for 10 min. The PA-oligosaccharides were detected using a fluorescence detector (LaChrom Elite, Hitachi) at wavelengths of 320 nm for excitation and 400 nm for emission.

Statistical analysis. The patient characteristics are presented as mean ± SD. The Spearman's rank correlation coefficient was used to assess the correlation of continuous variables within each pair. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IgG oligosaccharide profiles. The normal oligosaccharide structures of neutral human IgG contain 12 major structural variants (Fig. 1A). We analyzed the profiles of IgG neutral oligosaccharides using HPLC in combination with fluorescent labeling of oligosaccharides. In our previous study (7), the G0F/G2F ratio was described as the ratio of the peak height of G0 (agalactosylated IgG) to G2 (fucosylated IgG oligosaccharide group II) (Fig. 1B). Since the majority of IgG oligosaccharides belong to group II, the G0F/G2F ratio represents the total agalactosylation of IgG.

G0F/G2F ratio. We measured the G0F/G2F ratio of IgG oligosaccharides in 32 monozygotic and 26 dizygotic twin pairs. The G0F/G2F ratio was not found to be significantly correlated within monozygotic twin (R=0.215935), dizygotic twin (R=-0.21377), or unrelated pairs (R=-0.0369) (Fig. 2A-C).

Correlations of different markers within pairs. The correlations in serum γ-glutamyltranspeptidase (γGTP) levels were higher within monozygotic twin (R=0.955181) compared to those within dizygotic twin (R=0.21293) and unrelated pairs (R=0.00177) (Fig. 3A). Alanine aminotransferase levels (R=0.525267 for monozygotic, R=0.460332 for dizygotic and R=0.001406 for unrelated pairs) and white blood cell (WBC) count (R=0.524062 for monozygotic, R=0.295489 for dizygotic and R=-0.002164 for unrelated pairs) did not exhibit a strong correlation within twin pairs, although both were found to be significant in monozygotic twin pairs (P=0.0367 and P=0.0372, respectively) (Fig. 3B-C).

Discussion

The agalactosylation of IgG increases with age and is associated with a number of inflammatory diseases. Although the present study included a limited number of twin pairs, the results clearly demonstrated that IgG agalactosylation was not significantly affected by genetics. Of note, γGTP levels were found to be significantly correlated in the 16 pairs of monozygotic twins investigated. Since γGTP levels are often associated with alcohol consumption, this finding suggests that taste and metabolism of alcohol are associated with genetic factors. Although the WBC count is known to vary under different conditions, it was similar between the monozygotic twins in this study. Therefore, compared to WBC, the agalactosylation of IgG appears to be less affected by genetic and more by environmental factors. Furthermore, our studies indicated that twin studies may not a suitable approach to glycobiology investigations.

As the HPLC analysis of IgG oligosaccharides is costly and time-consuming, high-throughput systems, such as ELISA, are required to investigate large numbers of monozygotic/dizygotic twins. Although the lectin-antibody ELISA that we recently developed (8) may be a suitable tool for large-scale analysis of IgG oligosaccharides, it is difficult to evaluate the normal levels of IgG agalactosylation using this method.

To summarize, although the ABO blood type is completely regulated by genetic factors, our results indicated that IgG oligosaccharides are more closely associated with environmental factors and genetic factors do not play a significant role. There are several reports available on the epigenetic regulation of glycosyltransferase genes (8,11) and further studies are required to investigate the epigenetic and environmental factors affecting the agalactosylation of IgG.

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Negative expression of N-acetylglucosaminyltransferase V in oral squamous cell carcinoma correlates with poor prognosis

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Abstract

N-acetylglucosaminyltransferase V (GnT-V), an enzyme with a key role in the branching of asparagine-linked oligosaccharides, is strongly linked to tumor invasion and metastasis of many solid tumors. Here we searched for correlations between the clinical features of patients with oral squamous cell carcinoma (OSCC) and GnT-V expression in the tumor, and we studied the feasibility of using GnT-V as a marker for oral cancer prognosis. Samples from 68 patients with OSCC were examined by immunohistochemistry using antibodies against GnT-V. Correlations between the expression level of GnT-V in the tumor and patient clinical features were statistically analyzed. Positive GnT-V expression was found in 48 cases (70.6%), and negative GnT-V expression was found in 20 cases (29.4%). Negative GnT-V expression was associated with mode of invasion by multiple logistic regression analysis (OR: 3.605; P = 0.048). Biological characteristics of tumors and the Ki-67 labeling index were higher in tumors with negative GnT-V expression than in those with positive GnT-V expression, although the difference was not significant (P = 0.176). Patients with negative GnT-V expression had significantly shorter survival than those with tumors having positive GnT-V expression (5-year survival rate, 58.2% and 86.5%, respectively; P = 0.025). Negative GnT-V expression was a significant unfavorable prognostic factor for OSCC (hazard ratio, 4.246; P = 0.045). The loss of GnT-V expression is a likely indicator of tumors with high potential of tumor invasion and poor prognosis in OSCC patients.

Keywords: N-acetylglucosaminyltransferase V; GnT-V; Oral squamous cell carcinoma; OSCC; Biomarker

Introduction

Head and neck carcinoma, which includes cancers of the oral cavity, oropharynx, larynx, and hypopharynx, is the sixth most common cancer worldwide and has an incidence of around 600,000 cases per year (Kamangar et al. 2006). Oral cancer, the largest subset of head and neck cancer, has become one of the most lethal malignancies (Chen et al. 2013), of which oral squamous cell carcinoma (OSCC) is the most frequent histological type (Parkin et al. 2005). The current management and treatment of OSCC involves multimodal approaches comprising surgery, chemotherapy, and radiotherapy (Seiwert and Cohen 2005). Despite recent advances in early detection, diagnosis, and treatment, the 5-year survival for patients with OSCC has remained at 50% for the past 30 years (Forastiere et al. 2003). Because of the high prevalence and mortality rate of oral cancers, prevention and early intervention are important strategies for managing the disease.

Glycosylation of cell-surface glycoproteins is widely accepted to play a key role in various specific biological interactions. The glycosyltransferase plays a crucial role on the protein glycosylation. Glycosyltransferase, located in the Golgi apparatus, includes at least six N-acetylglucosaminyltransferase (GnT) defined as GnT-I-VI (Taniguchi et al. 1999). GnT-V, a glycosyltransferase encoded by the *Mgat5* gene that catalyzes the formation

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of β1,6GlcNAc (N-acetylglucosamine) branches on N-glycans, is believed to be associated with cancer growth and metastasis (Taniguchi et al. 1999; Lau and Dennis 2008). Moreover GnT-V protein could results in tumor angiogenesis, and its mechanism as an inducer of angiogenesis was different from original function as a glycosyltransferase (Saito et al. 2002).

Numerous studies have shown that GnT-V is positively correlated with malignancy in many types of tumor, including breast, colon, endometrial, and ovarian mucinous tumors (Fernandes et al. 1991; Murata et al. 2000; Yamamoto et al. 2007; Takahashi et al. 2009). In contrast, the opposite results have been found for lung, thyroid, and liver tumors. As such, GnT-V expression and its functional and prognostic significance in human cancer remain controversial. The relationship between GnT-V expression and malignancy has been studied in many types of tumor, but not in human oral SCC. In vitro analysis, it was reported that the decrease inβ1, 6GlcNAc branching on cisplatin-resistant human SCC cell line, so the GnT-V expression in SCC may be inversely corelated with prognosis (Nakahara et al. 2003).

In this study, we examined GnT-V expression by immunohistochemistry for surgically resected OSCC and analyzed the correlation with clinical features of OSCC.

Materials and methods

Patients and tissue specimens

Tumor specimens were obtained from 68 patients with OSCC seen at the Department of Oral and Maxillofacial Surgery, University of Tsukuba Hospital, Ibaraki, Japan during the period 1994–2004. Patients were followed for more than 60 months. Tumors were staged according to the International Union Against Cancer scheme (Sobin and Wittekind 1997), and clinical data were obtained from patient medical records. Specimens were obtained after patients gave informed consent, and the study protocol was reviewed and approved by the Research Ethics Committee of the University of Tsukuba (H25-43).

Immunohistochemistry

For immunostaining of GnT-V, 2-μm thick sections from patient samples were stained using the Vecta staining kit according to the manufacturer's instructions with anti-GnT-V antibody obtained from Dr. Eiji Miyoshi (Osaka University, Osaka, Japan). GnT-V expression levels were classified into two groups according to the percentage of positively stained cells in the cancerous area: ≥ 30% (positive) and < 30% (negative) (Takahashi

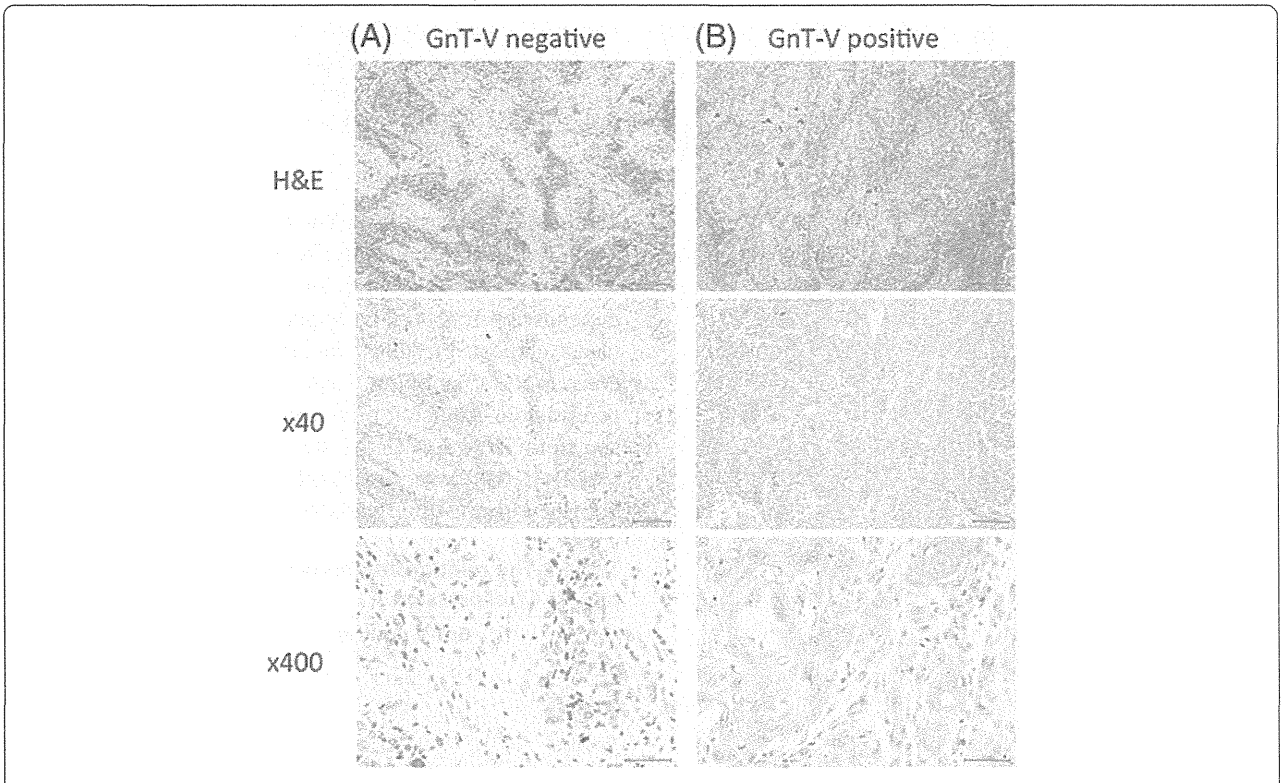

Figure 1 Representative photomicrographs of immunohistochemical staining with N-acetylglucosaminyltransferase (GnT-V) antibodies Negative staining (total absence of GnT-V immunostaining) (A), and positive staining (B) (original magnification, ×40 and × 400).

et al. 2009) (Figure 1). The scoring procedure was carried out twice by two independent observers who were blinded to the clinical data.

Table 1 Relationship between GnT-V expression and clinical and clinicopathological characteristics in all 68 patients

	GnT-V positive	GnT-V negative	P
Age			
Average	68.88	59.15	0.006
Gender			
Male	22	15	
Female	26	5	0.028
Alcohol			
no	33	8	
yes	15	12	0.027
Smoking			
no	32	9	
yes	16	11	0.096
T-Category			
1, 2	30	13	
3, 4	18	7	0.845
Clinical Stage			
I, II	27	9	
III, IV	21	11	0.397
Differentiation			
Well	33	9	
Moderate, poor	15	11	0.066
Mode of invasion			
1 to 3	32	7	
4	16	13	0.016
pN			
Negative	42	15	
Positive	6	5	0.202
Recurrence			
Negative	38	15	
Positive	10	5	0.178
Metastasis			
Negative	38	14	
Positive	10	6	0.416
Survival			
Alive	43	13	
Dead	5	7	0.015
theraphy			
Operation	34	11	
Chemoradiotheraphy	14	9	0.208

Statistical analysis

To simplify the correlation analysis of GnT-V expression with clinical features, tumors were divided into the T-category groups T1 + T2 or T3 + T4. Clinical stage was classified as I + II or III + IV, and differentiation as well-/moderately or poorly differentiated. Anneroth grade to denote tumor invasion was assigned as 1–3 or 4. For univariate analysis, we used the Chi-squared test, Student's test, or Welch's *t*-test. For multivariate analysis, we used multiple logistic regression analysis. All analysis was performed using the statistical software package SPSS.

Results

Univariate analysis of GnT-V expression

Positive GnT-V expression was observed in 48 specimens (70.6%) and negative GnT-V expression in 20 specimens (29.4%). Table 1 shows the correlation between GnT-V expression and clinicopathological features. The GnT-V-negative group included significantly more young patients (P = 0.006), more males than females (P = 0.028), alcohol consumption (P = 0.027), more invasive tumors (P = 0.016), and a higher 5-year survival rate (P = 0.015). No significant difference in GnT-V expression was observed with respect to other factors, including, smoking, T-category, clinical stage, cellular differentiation, pN positive or negative, local recurrence, lymph node metastasis, and treatment type. No difference was observed between GnT-V expression and p53 expression. Ki-67 labeling index values were higher in tumors with negative GnT-V expression than in those with positive GnT-V expression, but not significantly (P = 0.176) (Table 2).

Multivariate analysis of GnT-V expression

The predictor variables in the 68 patients were used in a logistic regression model with GnT-V expression as the dependent variable. The logistic model was constructed using clinical variables, including age, gender, alcohol consumption, smoking, stage, differentiation, and Mode of invasion. Adjusted odds ratios (OR) and P values are shown in Table 3. Negative staining for GnT-V (OR = 3.605 and P = 0.048) was significantly associated with invasion but not with the other variables.

Table 2 The relationship between GnT-V expression and p53, ki 67 expression

	GnT-V positive	GnT-V negative	P
p53			
Negative	33	16	
Positive	15	4	0.346
Ki67 mib index (Mean ± SD)			
	14.0 ± 1.9	19.8 ± 4.6	0.176

Table 3 Multiple logistic regression analysis for the correlation between GnT-V expression and clinical characteristics

Characteristics	Odds ratio	P
Age	1.048	0.052
Gender (male/female)	0.502	0.415
Alcohol (no alcohol/alcoho drinker)	1.750	0.464
Smoking (non smoker/smoker)	1.378	0.639
Stage (I + II/III + IV)	1.135	0.866
Differentiation (well/moderate + poor)	1.391	0.597
Mode of invasion (1-3/4)	3.605	0.048

GnT-V expression and prognosis of OSCC

We next analyzed the relationship between GnT-V expression and patient survival and the importance of GnT-V as a prognostic factor. Kaplan-Meier survival curves clearly demonstrated that patients with negative GnT-V expression had significantly shorter survival than patients with positive GnT-V expression (5-year survival rate, 58.2% and 86.5%, respectively; P = 0.025; Figure 2). Cox proportional-hazard analysis was performed to compare the impact of GnT-V expression on survival with currently used clinicopathological prognostic factors such as age, gender, alcohol consumption, smoking consumption, stage, differentiation, and GnT-V expression. Negative GnT-V expression was the only significant unfavorable prognostic factor in our analysis (hazard ratio, 4.246; P = 0.045) (Table 4).

Discussion

Glycosylation is one of the most common posttranslational protein modifications, and nearly half of all known proteins in eukaryotes are glycosylated (Saxon and Bertozzi 2001). Cell surface glycosylation not only regulates the stability and activity of structural proteins and receptors on the cell membrane, but also participates in the maintenance of cell morphology and cell-cell interactions (Hirai-Fujita et al. 2008; Krishnan et al. 2005; Rak et al. 1991). Changes in glycans are associated with many physiological and pathological events, including cell adhesion, migration, and invasion (Dennis et al. 1987).

The present report shows that GnT-V expression in OSCC is associated with age (P = 0.006), gender (P = 0.028), alcohol consumption (P = 0.027), mode of invasion (P = 0.016), and 5-year survival (P = 0.015). Although our results revealed that there were no significant differences between GnT-V expression and T or Clinical stages, it was reported that GnT-V expression is upregulated in the early stages of almost all cancers (Miyoshi et al. 2012). However Multiple logistic regression analysis to determine the correlation between GnT-V expression and clinical and clinicopathological characteristics showed that the cases of negative GnT-V expression tended to be more invasive as determined by Anneroth grade.

Kaplan-Meier survival curves clearly demonstrated that patients with negative GnT-V expression had significantly shorter survival than patients with positive GnT-V expression (5-year survival rate, 58.2% and 86.5%, respectively; P = 0.025; Figure 2). Historogy was significantly correlated with GnT-V expression and low GnT-V expression was more frequently found in squamous cell carcinomas than non-squamous cell carcinomas (Akita 2004). Our data strongly suggested that the relationship between GnT-V expression and the prognosis depends on the histrogical type, as well as the original organ of the cancer. When considering survival rate, the type of treatment (surgery or chemoradiotherapy) was taken into account, but we found no significant correlation between GnT-V expression and treatment type. Moreover, in patients with negative GnT-V expression that correlated with survival rate, we found no significant

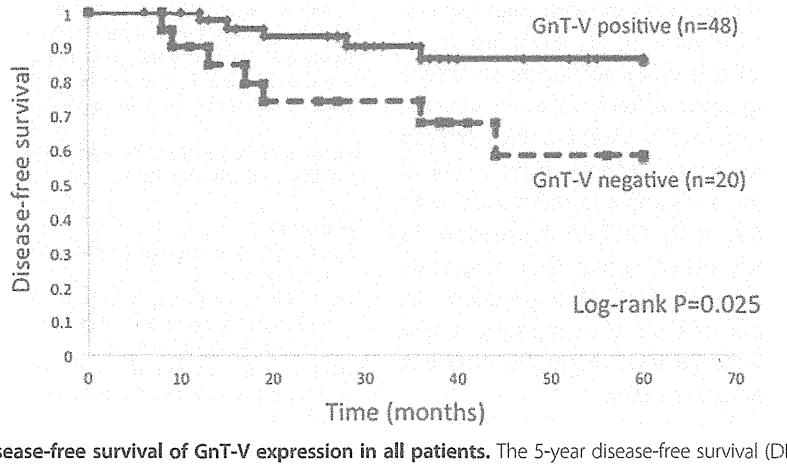

Figure 2 Kaplan Meier disease-free survival of GnT-V expression in all patients. The 5-year disease-free survival (DFS) rates of positive GnT-V expression and negative GnT-V expression were 86.5 % and 58.2%, respectively (P = 0.025).

Table 4 Cox proportional hazards model analysis of prognostic factors in patients

Characteristics	Hazard ratio	95% CI	P
Age	1.060	0.992-1.132	0.083
Gender (male/female)	0.488	0.093-2.565	0.397
Alcohol (no alcohol/alcoho drinkerl)	1.436	0.333-6.199	0.627
Smoking (non smoker/amoker)	1.814	0.444-7.408	0.407
Clinical stage (I, II / III, IV)	2.428	0.541-10.896	0.247
Differentiation (well/moderate + poor)	1.016	0.286-3.612	0.980
GnT-V expression	4.246	1.0320-17.586	0.045

correlation between GnT-V expression and local recurrence or node metastasis. This suggests that negative GnT-V expression reduces the efficacy of chemoradiotherapy as a second treatment. This implies that OSCC patients with negative GnT-V expression are more likely to have poor prognosis.

The relationship between cisplatin-resistance and α5β1 integrin with β1-6GlcNAc branching has been reported in an established cisplatin-resistant head and neck carcinoma cell line, but reasons for the relationship are unclear (Nakahara et al. 2003). Down-regulation of GnT-V enhances nasopharyngeal carcinoma cell radiosensitivity both in vitro and in vivo, and is linked to the G2-M cell cycle arrest and the reduction of the BcL-2/Bax ratio (Zhuo et al. 2012). Conversely, a correlation was found between the high expression levels of GnT-V in neuroblastoma patients with a favorable prognosis, suggesting that GnT-V can cause neuroblastomas to regress by increasing their susceptibility to apoptosis (Inamori et al. 2006).

Low expression of GnT-V may contribute to altered biological properties of bladder cancer as well as non-small cell lung cancer and hepatocellular carcinoma by decreasing the synthesis of β1-6 branching oligosaccharides of certain target glycoproteins, resulting in shorter survival in patients having tumors with low GnT-V expression compared with patients having tumors with high GnT-V expression (Akita 2004; Ishimura et al. 2006; Ito et al. 2001). The importance of this oligosaccharide structure as a precursor to malignancy differs between organs, and the target substrate of GnT-V might differ between oral cancer and other carcinomas. However, from a clinical background, there is not a significant difference with the tumor differentiation with GnT-V expression in OSCC as observed in the other cancer that prognosis was inversely correlated with GnT-V expression. In addition, since expression of GnT-V expression is low in young people, histologic pattern might be different in GnT-V positive and negative cases.

Taken together, immunohistochemistry of OSCC specimens can provide information that could help physicians make appropriate decisions for the treatment of cancer patients. For example, if GnT-V expression is absent, the tumor is more likely to have poor prognosis, and radical treatment in such a case would be a better choice. However the potential oncogenic role and underling mechanisms of GnT-V in OSCC have not been investigated. Clealy, further studies are needed to elucidate the mechanisms of GnT-V promoting the development and metastasis of OSCC in detail.

Competing interest

The authors declare that they have no competing interest.

Authors' contributions

HB, JS, KO and TY conceived the experiments. KS, FU, OB, MY and RK performed the experiments, and analysed the data together with EW, SS, SH, KY and EM provide valuable help on the optics. KS and FU co-wrote the paper. All authors read and approved the final manuscript.

Funding

This study was supported by Grants-in-Aid for Scientific Research (B) (grant no. 24390449 and grant no. 22390379) from the Japan Society for the Promotion of Science (JSPS).

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Received: 28 August 2013 Accepted: 26 November 2013
Published: 6 December 2013

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Cite this article as: Seto *et al.*: Negative expression of N-acetylglucosaminyltransferase V in oral squamous cell carcinoma correlates with poor prognosis. *SpringerPlus* 2013 **2**:657.

Cancer Science
The official journal of the Japanese Cancer Association

Review Article

Role of α-gal epitope/anti-Gal antibody reaction in immunotherapy and its clinical application in pancreatic cancer

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(Received September 27, 2012/Revised December 4, 2012/Accepted December 10, 2012/Accepted manuscript online December 15, 2012/Article first published online February 6, 2013)

Pancreatic cancer is one of the most common causes of death from cancer. Despite the availability of various treatment modalities, such as surgery, chemotherapy and radiotherapy, the 5-year survival remains poor. Although gemcitabine-based chemotherapy is typically offered as the standard care, most patients do not survive longer than 6 months. Therefore, new therapeutic approaches are needed. The α-gal epitope (Galα1-3Galβ1-4GlcNAc-R) is abundantly synthesized from glycoproteins and glycolipids in non-primate mammals and New World monkeys, but is absent in humans, apes and Old World monkeys. Instead, they produce anti-Gal antibody (Ab) (forming approximately 1% of circulating immunoglobulins), which specifically interacts with α-gal epitopes. Anti-Gal Ab can be exploited in cancer immunotherapy as vaccines that target antigen-presenting cells (APC) to increase their immunogenicity. Tumor cells or tumor cell membranes from pancreatic cancer are processed to express α-gal epitopes. Subsequent vaccination with such processed cell membranes results in *in vivo* opsonization by anti-Gal IgG in cancer patients. The interaction of the Fc portion of the vaccine-bound anti-Gal with Fcγ receptors of APC induces effective uptake of the vaccinating tumor cell membranes by the APC, followed by effective transport of the vaccinating tumor membranes to the regional lymph nodes, and processing and presentation of the tumor-associated antigens. Activation of tumor-specific B and T cells could elicit an immune response that in some patients is potent enough to eradicate the residual cancer cells that remain after completion of standard therapy. This review addresses these topics and new avenues of clinical importance related to this unique antigen/antibody system (α-gal epitope/anti-Gal Ab) and advances in immunotherapy in pancreatic cancer. (*Cancer Sci* 2013; 104: 282–290)

Pancreatic cancer, which is the fifth leading cause of cancer death worldwide, is a devastating disease associated with an extremely poor prognosis.[1] Immunotherapy designed to target tumor-associated antigens (TAA) is a promising treatment approach for pancreatic cancer. However, immunotherapy alone is limited by the number of cytotoxic T lymphocytes (CTL) that can penetrate large pancreatic tumors. Therefore, there is a need to find other modalities of immunotherapy for the treatment of this disease. In part, this is due to the nature of pancreatic cancer, which is often highly immunosuppressive, diagnosed late, and with a short time to death.[2] To develop efficient immunotherapy for pancreatic cancer, it is important to have an understanding of the following basic issues: (i) the nature of the immune network against autologous tumor cells; (ii) the identity of tumor antigens and means to evaluate the immune response in patients with pancreatic cancer; (iii) tumor escape mechanisms from the immune system and strategies to overcome them; and (iv) development of efficient immune interventions to eliminate pancreatic cancer cells. In particular, identification of appropriate tumor antigens is an essential step for the development of effective immunotherapy against pancreatic cancer.

Anti-Gal is an IgG antibody (Ab) present in large amounts in normal subjects and patients with malignancies. Galili[3] and Macher and Galili[4] estimated that approximately 1% of B cells in humans can produce anti-Gal. Most of these B cells (designated here as "anti-Gal B cells") are in a quiescent state within the lymph nodes and spleen. Natural anti-Gal Ab is produced primarily by anti-Gal B cells present along the gastrointestinal tract, because of continuous stimulation by bacteria of the natural flora.[3,4] Anti-Gal Ab specifically interacts with α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R) on cell surface glycolipids and glycoproteins. Once anti-Gal Ab binds to α-gal epitopes on the cells, its Fc portion readily binds to Fcγ receptor (FcγR) III on dendritic cells and macrophages. This interaction induces effective phagocytosis of the anti-Gal-Ab-opsonized cells by antigen-presenting cells (APC).[5–7] Pig xenografts transplanted into humans release into the circulation α-gal epitopes on xenoglycoproteins that reach the quiescent anti-Gal B cells, activate them and induce extensive production of high affinity anti-Gal IgG molecules.[3,4] Indeed, anti-Gal Ab can bind extensively to α-gal epitopes on xenograft cells and exacerbate xenograft destruction, primarily through antibody-dependent cell-mediated cytotoxicity (ADCC).[3,4] Whereas anti-Gal Ab has a detrimental effect in xenotransplantation, its strong immunological potential might be exploited for immunotherapy in patients with pancreatic cancer. In previous studies, Rossi *et al.* exploited this unique immune mechanism to create a whole cell cancer vaccine to treat melanoma tumors.[8,9] B16 melanoma vaccines genetically modified to express α-gal epitopes effectively targeted pre-existing subcutaneous and pulmonary α-gal-negative melanoma tumors in α1,3-galactosyltransferase (α1,3 GT) knockout mice.[8,9]

In this article, we address the basic problem of cancer immunotherapy and detail our recent work in tumor antigen vaccination using α-gal epitopes/anti-Gal interaction. A series of preclinical experiments demonstrated that tumor-associated

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doi: 10.1111/cas.12084

antigens engineered to express α-gal epitopes elicit enhanced immunogenicity and an effective antitumor immune response. We also discuss the novel approach of immunotherapy that targets pancreatic cancer stem cells using stem cell markers, remodeled to express α-gal epitopes.

Distribution of α1,3 GT, α-Gal Epitopes and Anti-Gal Antibody in Mammals

The α-gal epitope (Galα1-3Galβ1-4GlcNAc-R) with a unique carbohydrate structure is absent in humans but naturally expressed on glycolipids and glycoproteins in non-primate mammals, prosimans and New World monkeys.[3,4] In 1968, Yamakawa and colleagues were the first to isolate ceramidepentahexoside (CPH), a glycolipid, from rabbit red blood cells, which contained the non-reducing terminal sequence, Galα1-3Galβ1-4GlcNAc-R.[10] Subsequently, the structure of the rabbit red blood cell CPH was further characterized by Hakomori and colleagues in 1973.[11] The expression of α-gal epitopes was assessed by measuring the binding of anti-Gal Ab, a natural antibody, to this epitope in humans and to the α-gal epitope-specific lectin, *Bandeiaea (Giffonia) simplicifolia* IB4[12] in various cells. Although this epitope is not expressed on the cells of non-mammalian vertebrates, it is abundantly expressed on cells of non-primate mammals, including marsupials.[3,4] Among primates, the α-gal epitope is found on red blood cells and nuclear cells of prosimans (e.g. lemura) and New World monkeys (e.g. monkeys of South and Central America). In contrast, Old World monkeys (e.g. monkeys of Asia and Africa), apes and humans lack α-gal epitopes and instead produce large amounts of anti-Gal Ab (Fig. 1).[3,4]

The reason for this unique pattern of distribution is the differential activity of α1,3 GT, a glycosyltransferase enzyme.[3,4] This enzyme catalyzes within the Golgi apparatus the synthesis of α-gal epitopes on the carbohydrate chains of glycolipids and glycoproteins. α1,3 GT appeared early in mammalian evolution, prior to the divergence of marsupials and placental mammals (>125 million years ago), and has been active in non-primate mammals as well as in ancestral primates (Fig. 1).[3,4] However, after the geographic separation of South America from the African continent, ancestral pressure resulted in inactivation of the α1,3 GT gene. Cloning of the α1,3 GT gene in bovine and mouse cells enabled the identification of α1,3 GT pseudo-genes in humans, Old World monkeys and apes.[3,4,13,14] Based on the sequences of pseudo α1,3 GT genes in various primates, this evolutionary inactivation is estimated to have occurred approximately 20–25 million years ago.[3,4,13] A possible evolutionary scenario that could have resulted in inactivation of the α1,3 GT gene is the appearance of an infection agent that expressed α-gal epitopes, which was detrimental to monkeys and apes, and endemic only in the Old World (i.e. it did not reach the South American continent due to geographic barriers). Such an infectious agent could have induced selective pressure for the evolutionary story of primates suppressing α-gal epitope expression (i.e. inactivation of the α1,3 GT gene) and producing anti-Gal Ab as a means of defense.[3,4]

In 1984, Galili[3] and Macher and Galili[4] reported that 1% of circulating IgG in human sera showed specificity for α-linked galactose. This antibody, anti-Gal Ab, is found in high titer in sera of non-immunocompromised humans. It is produced throughout life as a result of continuous antigen stimulation by carbohydrate antigens of the normal flora of the gastrointestinal tract, including *Klebsiella pneumonia*, Escherichia coli and *Serratiamarcecens*.[3,4] Characterization of the immunoglobulin genes encoding the anti-Gal heavy chain indicates that a number of closely related genes encode this chain, implying that anti-Gal Ab is a polyclonal antibody.[3,4] The polyclonality of anti-Gal Ab was subsequently confirmed by isoelectric focusing analysis.[3,4] In spite of its polyclonality, anti-Gal

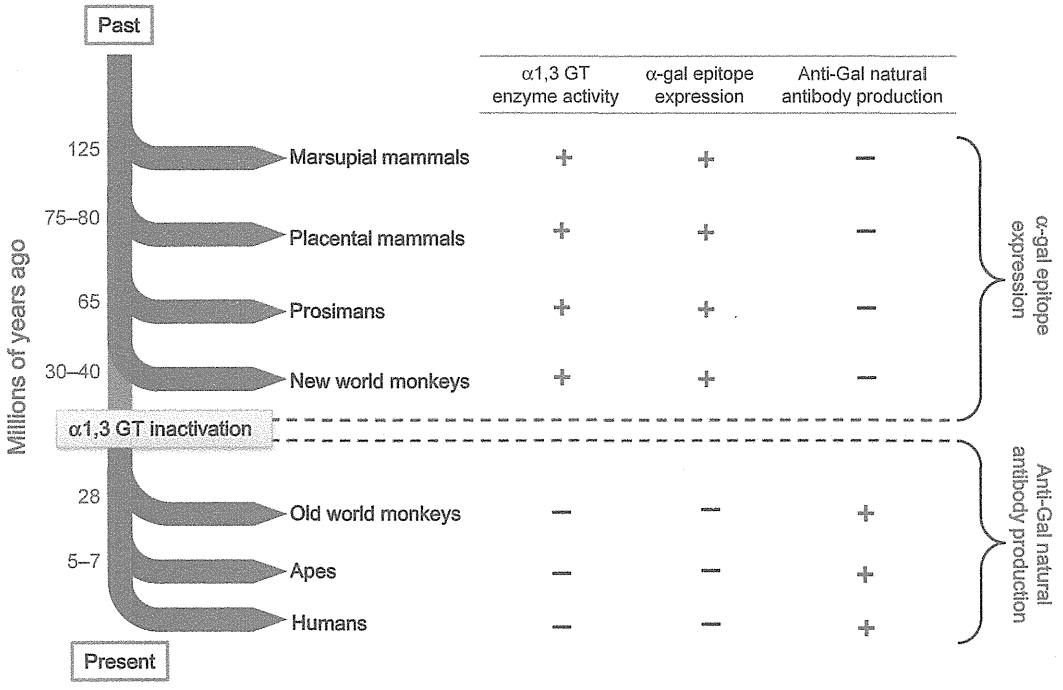

Fig. 1. Reciprocal evolution of α1,3-galactosyltransferase (α1,3 GT) enzyme activity, α-gal epitopes and anti-Gal antibody in mammals. α-gal epitopes have been synthesized in mammals by α1,3 GT for more than 125 million years, since before the divergence of placental mammals and marsupials. All non-mammalian vertebrates lack α1,3 GT and do not express α-gal epitopes. Expression of this epitope was suppressed in ancestral Old World primates after they diverged from New World monkeys, and probably after apes and monkeys diverged from each other. Suppression of α-gal epitopes was followed by production of anti-Gal natural antibody, which is absent in non-primate mammals, prosimians and New World monkeys.