Aberrant Methylation of *GCNT2* Is Tightly Related to Lymph Node Metastasis of Primary CRC

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Abstract. *Background: Glycoprotein expression profile is dramatically altered in human cancers; however, specific glycogenes have not been fully identified. Materials and Methods: A comprehensive real-time polymerase chain reaction (PCR) system for glycogenes (CRPS-G) identified several outstanding glycogenes. GCNT2 was of particular interest after GCNT2 expression and epigenetics were rigorously investigated in primary colorectal cancer (CRC). Results: The highlights of this work can be summarized as follows: (i) Expression of GCNT2 was remarkably suppressed. (ii) Silenced expression of GCNT2 was reactivated by combined demethylating agents. (iii) Promoter DNA methylation of GCNT2 was silenced in CRC cell lines and tissues. Hypomethylation of GCNT2 variant 2 is tightly associated with lymph node metastasis in primary CRC. (iv) GCNT2 methylation level in the normal tissues also showed a close association with that in the tumor tissues and reflected lymph node metastasis. Conclusion: We identified aberrant expression of GCNT2, which can be explained by promoter DNA hypermethylation. Hypomethylation of the GCNT2 variant 2 reflected lymph node metastasis of CRC in the tumor and normal tissues.*

The details of the functions of most glycoproteins remain obscure; however, the current study clarified two important roles. The first is distinction of cells and the second is conveying the signal. Both are related with the communication pathways between cells and play an important role not only at fundamental functions, such as generating specialization in human immunity but also at mechanism of metastasis of cancer as cancerous cells interact with a variety of host cells during growth and metastasis (1-3)

Some glycoproteins have important roles in guiding cancer cells to organs (3). Sialyl Lewis x (sLe^x), a ligand of E-selectin, is one of the important glycoproteins since, in metastasis, E-selectin, which is expressed by activated endothelium, is involved in adhesion of sLe^x-bearing cancer cells. The expression of sLe^x in cancer cells is caused in an epigenetic manner by gene silencing of *DTDST*, a sulfate transferase, which impedes the progression of differentiation of normal cells that bear glycoproteins interfering with relevant to the above pathway(s) (4). On the other hand, the carbohydrate tumor marker Sialyl Lewis a (sLe^a), also known as CA19-9, is likely to exhibit the similar function that may be explained by *ST6GalNAc6* (5). To put it in another way, simplification of glycan structure, sometimes elicited by epigenetic alteration of glycogenes, is associated with metastatic tendency. It is quite astonishing that there is a close connection between epigenetics, methylation of CpG islands and defect of gene activity. As there is no comprehensive study regarding the relationships between glycogenes and cancer, we surmised that a number of glycogenes could participate in the mechanism of oncogenesis or metastasis through epigenetic regulation. We, thus, herein used comprehensive real-time PCR system for glycogenes (CRPS-G) to identify genes aberrantly expressed in colorectal cancer (CRC) compared to the corresponding normal mucosa tissues and *GCNT2*, a gene-encoding glucosaminyl (N-acetyl) transferase 2, I-branching enzyme, that is of particular interest among the identified genes in terms of epigenetic alteration in CRC.

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Materials and Methods

CRC cell lines. CRC cell lines (HCT116, DLD1 and LoVo) were kindly provided by the Cell Response Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Caco2 was obtained from RIKEN

BioResource Center (Tsukuba, Japan). LoVo and DLD1 were grown in RPMI-1640 medium (Gibco, Carlsbad, CA, USA), while Caco2 was grown in MEM (Gibco) supplemented with 20% fetal bovine serum (FBS) and 0.1 mM NEAA (Invitrogen Carlsbad, CA, USA). HCT116 was grown in McCoy 5A mediums (Gibco) supplemented with 10% FBS.

Human primary CRC tissues. Thirty pairs of frozen primary CRC tissues and corresponding normal mucosal tissues, obtained at least 5 cm from the tumor edge, were used for TaqMan-real time polymerase chan reaction (PCR) analysis for mRNA expression. Formalin-fixed paraffin-embedded primary CRC tissues and paired normal mucosa specimens were obtained from 59 patients diagnosed with CRC and used for the quantitative methylation-specific PCR (Q-MSP). This study was performed under the approval of the Ethics Committee of the Kitasato University School of Medicine.

Comprehensive real-time PCR system for glycogenes (CRPS-G). A custom quantitative PCR (qPCR) array was used for analyzing transcript amount of genes relating to glycan synthesis pathway (6). The pathway-focused qPCR array consists of 189 sets of gene-specific PCR primer pair and Taqman™ probe from UPL library (Roche, Basel, Schweiz): 186 genes are "glycogene" and the rest are conventional reference genes, *ACTB, GAPDH* and *HPRT1* of human origin. For this quantitative analysis, total RNA was extracted from the tissues from CRC patients using the RNeasy plus mini kit (Qiagen, Düsseldorf, Germany). RNA concentration was measured with absorbance at 260 nm wavelength. Four μg of total RNA was subjected to cDNA synthesis using the Quntitect RT kit (Qiagen). The reaction mixture of 7.5 μl, including cDNA equivalent to 7.5 ng of starting RNA amount, was dispensed to each well of a 384-well format plate, which was prospectively dispensed with specific PCR primer and probe sets. Sequences of the primer and probe for glycogenes are available upon request. Each assay on the array was calibrated at two points 10^5 and 10^3 copies of reference template, as double stranded plasmid DNA pool of 189 target sequences.

Quantitative TaqMan real time PCR for mRNA expression of GCNT2 variants. Total RNA from the homogenized tissues or harvested cells was extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed with the SuperScriptIII reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). Quantitive TaqMan PCR was carried out using iQ multi SuperMix (Bio-Rad Laboratories, Hercules, CA, USA) for 3 spliced variants of *GCNT2* on the iCycler iQ real-time PCR detection system. Relative quantitative analysis normalized to β-actin (ACTB) was carried out according to comparative cycle threshold method (7).

5-Aza-dC and trichostatin A (TSA) treatment. Cells were split to low density (1×10^6/T-75 flask) 12 to 24 h before treatments. Cells were then treated every 24 h for 5 days with either 1 or 5 μM 5-aza-2'deoxycytidine (5-Aza-dC; Sigma- Aldrich, St Louis, MO, USA) dissolved in 50% acetic acid or were mock-treated with PBS including the same amount of acetic acid. As indicated, 300 nM of TSA (Sigma-Aldrich) was added to the medium for the final 24 h (7).

Bisulfite treatment of CRC cell line DNA and sequencing analysis. Genomic DNA from CRC cell lines was extracted using the QIAamp DNA Mini Kit (Qiagen). Bisulfite treatment was carried out with the EZ DNA Methylation-Gold kit™ (Zymo research, lrvine, CA, USA) and the mixture was subsequently amplified by PCR for direct sequence or cloned sequence. Primer sequences for the *GCNT2* promoter regions were designed to recognize DNA alterations caused by bisulfite treatment. The PCR products were inserted into a pCR4-TOPO vector using the TOPO TA Cloning Kit for cloned sequence (Invitrogen). Ten or 5 selected clones for each sample and then sequenced.

Quantitative methylation-specific PCR. Tissue sections from tumor and corresponding normal mucosa were stained with hematoxylin and eosin and dissected under a microscope. Genomic DNA from the tissues was subsequently extracted using the QIAamp DNA FFPE Kit (Qiagen). For quantitative methylation analysis of *GCNT2* variant 2, TaqMan Q-MSP was carried out using iQ multi SuperMix (Bio-Rad Laboratories). All reactions were performed in triplicates. Relative quantitative analysis normalized to β-actin was carried out according to comparative cycle threshold method.

GCNT2 variant 2 expression vector and transfection. For transfection experiments, the *GCNT2* variant 2 plasmid (Origene technologies, Rockville, MD, USA) was used. The *GCNT2* variant 2 plasmid vectors were transfected into 2 CRC cancer cell lines (DLD1 and HCT116) using Lipofectamine 2000 reagent (Invitrogen). Stable clones with *GCNT2* or mock were established by G418 (GIBCO) selection (DLD1 and HCT116 1500 μg/ml).

Results

Identification of glycogenes aberrantly expressed in primary CRC by CRPS-G. In the 10 CRC tumor tissues and the corresponding normal tissues, CRPS-G for glycogenes (186 genes) was performed in order to identify glycogenes aberrantly expressed in primary CRC. The CRPS-G had been developed and granted by the New Energy and Industrial Technology Development Organization (NEDO) Medical Glycomics (MG) project (6). Identified glycogenes with outstanding features were differentially expressed in primary CRC tumor tissues as compared with the corresponding normal tissues (Figure 1a and 1b). Four specific genes, *GCNT2, B3GALT1, B3GALT4* and *GALNT6*, showed remarkable difference between primary CRC and the corresponding normal mucosa (Figure 1c). The first 3 genes were suppressed in expression in primary CRC as compared with that in the corresponding normal mucosa. On the other hand, GALNT6 (polypeptide N-acetylgalactosaminyltransferase 6), which is up-regulated in various cancers (8-10), showed a robust increase of its expression in primary cancer. *B3GALT1* and *B3GALT4* (beta-3-galactosyltransferase 1 and 4) coded beta-3-galactosyltransferases (11), which add galactose to core-2 protein and they are very important enzymes to consist sLe^a. These findings suggested that the tabulated genes from the CRPS-G experimental approach actually included onco-related glycogenes, which play a crucial role in cancer progression. However, there was no information of *GCNT2* expression in primary CRC.

Genomic structure of GCNT2 gene. *GCNT2*, also designated as IGNT (I-beta-1, 6-N-acetylglucosaminyl transferase) that actually codes 1.6-N-acetylgulcosaminyltransferase (12-14), is known as a causative gene for congenital cataract (15). *GCNT2* has 3 transcript variants (variant 1 NM_145649 (GenBank accession number), variant 2 NM_001491 and variant 3 NM_145655), which have different promoter regions (12). We, thus, designed specific primers and probes for expression analysis, as well as MSP primers and probes for bisulfite-treated DNA sequences, both of which are shown in Figure 2a.

GCNT2 expression in primary CRC tumor tissues and the corresponding normal colonic mucosa tissues. We first examined 4 CRC cell lines (DLD1, HCT116, Caco2 and LoVo) for expression of the 3 variants of *GCNT2* at the mRNA level (Figure 2b). In CRC cell lines, DLD1 cell is defective in expression of any *GCNT2* variants, while LoVo cell is abundantly expressed for all variants. In both HCT116 and Caco2, each variant showed unique expression. We then investigated 30 pairs of CRC tumor and corresponding normal mucosa tissues at the mRNA level, as depicted in Figure 2c and found an abundant expression of *GCNT2* in about half the normal mucosa tissues. All of the 3 isoforms of *GCNT2* genes showed reduced expression in primary CRC tumor tissues compared to the corresponding normal mucosa. This result was consistent with the screening process, except for the defective expression of the corresponding normal tissues in half of the cases. More surprisingly, expression of each isoform was strongly associated with other 2 isoforms in primary cancer or the corresponding normal mucosa tissues (R=0.99-995, $p<0.0001$; see Figure 2d).

Epigenetic silencing of GCNT2 variant 2 in CRC cell lines. As we postulated that reduced expression of *GCNT2* is mediated in an epigenetic manner, we performed demethylating treatments (5 Aza-dC and HDAC inhibitor: TSA) in the 4 CRC cell lines (Figure 3a). Among the 4 CRC cell lines, 2 (DLD1 and HCT116), in which the expression of all 3 *GCNT2* variants, exhibited robust re-activation of all variant was silenced after the aforementioned treatments. The re-activation was potent after combination treatment of both 5 Aza-dC and TSA but not induced by either one alone, suggesting that silenced expression of the all *GCNT2* variants was mainly regulated by hypermethylation of cytosine DNA of the promoter regions (16). On the other hand, *GCNT2* variants were constitutively expressed in both LoVo and Caco2, where the *GCNT2* variant 2 is exceptional; expression of the variant 2 is silenced in Caco2 and robustly reactivated by combination of 5 Aza-dC and TSA. The re-activation of the variant 2 was accompanied by augmented expression of other variants (variant 1 and variant 3).

Close association of DNA methylation of CpG islands of the promoter and expression of GCNT2 variant 2. To confirm actual epigenetic alterations of each GCNT2 variant, we further investigated the existence of CpG islands around the transcription start site (TSS) that seems to play a critical role in the transcriptional activity of genes (17). Although CpG islands were far from the TSS in both the variant 1 and the variant 3, we could find the CpG island near the TSS (within exon 4) only in the variant 2. We then examined the nearest to each promoter CpG islands and sequence cloning was performed after TA cloning of PCR products (Figure 3b). Interestingly, the methylation status is very consistent with the expression status only in the variant 2, but inconsistent in the variants 1/3, suggesting that the promoter region of the CpG islands close to exon 4 (near the TSS of the variant 2) plays a crucial role in the expression of variant 2 in CRC cell lines. Therefore, we examined the methylation status for variant 2 promoter regions in primary CRC, as well as the corresponding normal mucosa tissues.

Quantitative methylation-specific PCR (Q-MSP) for the DNA promoter of GCNT2 varitant 2 in primary CRC and the corresponding normal tissues. We performed quantitative methylation-specific PCR (Q-MSP) for 59 pairs of CRC and the corresponding normal mucosa tissues (Figure 4a). Unexpectedly, there was no statistical difference between the primary CRC and the corresponding normal mucosa tissues, while methylation levels of both tissues were mutually reflected (Figure 4a). Both CRC tumor and normal tissues have clear methylation in the promoter CpG islands of *GCNT2* v2. About half cases exhibited hypermethylation in both primary CRC and the corresponding normal tissues, with this result being consistent with that regarding half of the cases having silent *GCNT2* expression in both tissues. On the other hand, the remaining cases showed that the methylation level tended to be higher in the primary CRC than in the corresponding normal tissues (Figure 4b). In 21 cases, the methylation level of *GCNT2* was higher in the tumor tissues and weak in the corresponding normal tissues, although significance was statistically marginal ($p=0.064$). This methylation profile was consistent with the expression profiles of *GCNT2* (Figure 2c) suggesting that DNA methylation caused suppression of *GCNT2* expression in the colon tissues, including tumor tissues.

Promoter DNA hypomethylation of GCNT2 varitant 2 is tightly associated with lymph node metastasis in primary CRC, as well as in the corresponding normal tissues. We next examined the clinical relevance of *GCNT2* variant 2 methylation status in advanced colorectal cancer (stage II/III/IV). Analysis of variance or the Student's t test between each parameter elucidated that GCNT2 variant 2 methylation is the most highly associated with lymph node

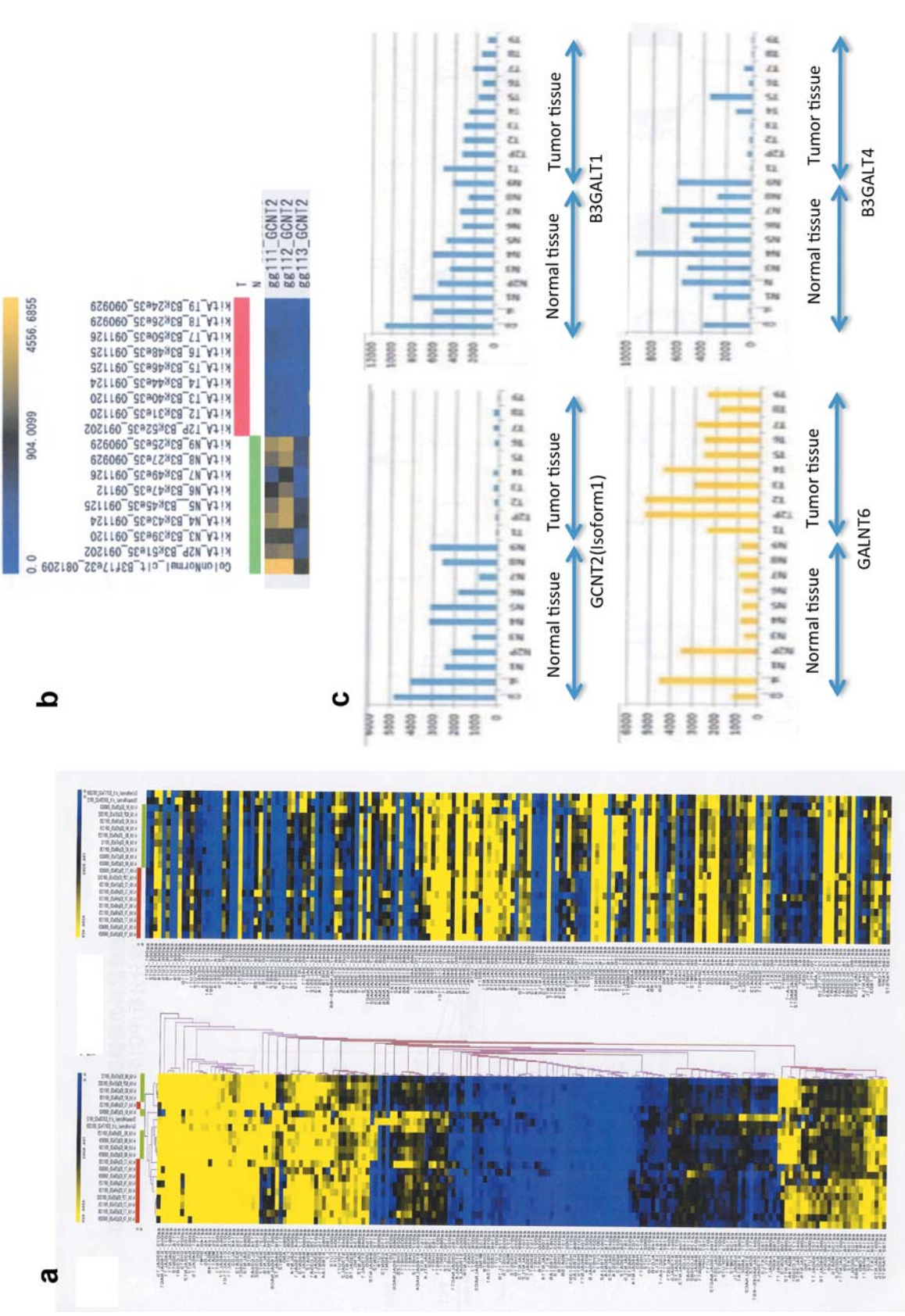

Figure 1. *(a) Comprehensive real-time PCR system for glycogenes (CRPS-G) for 186 glycogenes was applied to primary CRC tissues and the corresponding normal mucosa tissues of 10 CRC patients. This figure shows clustering of gene expression profiles of glycogenes in CRC. Yellow represents high expression, while blue shows low expression. The top red indicates tumor tissues (T), whereas the top green represents normal tissues (N). (b) This enlarged figure shows GCNT2 expression between T and N of CRC patients from Figure 1a. (c) GCNT2, B3GALT1, GALNT6 and B3GALT4 have differential expression in primary tumor and normal tissues of CRC patients. Note that GCNT2 expression is robustly suppressed in primary CRC tissues compared to that in the corresponding normal tissues.*

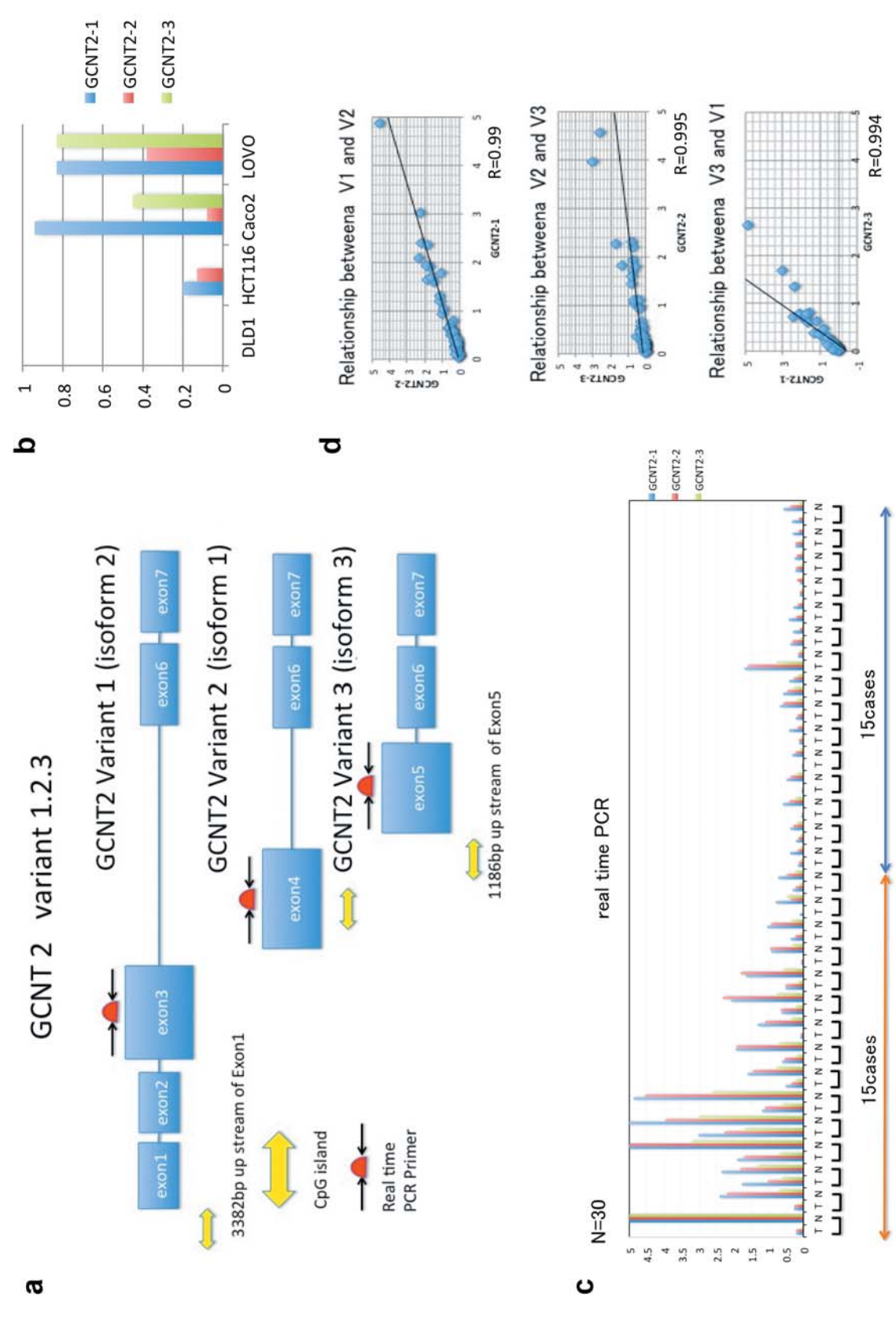

Figure 2. *(a) GCNT2 genomic structures were shown for variant 1, 2 and 3. The 3 different variants (variant 1/2/3) have different transcript start site (TSS) in exon 1/4/5, respectively, while all variants have common structure (exon 6 exon7). Positions of both PCR primers and CpG islands near the promoter region are shown. (b) Expression of each GCNT2 variant was analyzed by real time PCR in 4 CRC cell lines. DLD1 and HCT116 show low expression for all variants. Caco2 and LoVo show high expression of GCNT2. GCNT2 variant 2 is low in Caco2. (c) Expression of each variant of GCNT2 analyzed by real time PCR in primary CRC tumors and the corresponding normal mucosa tissues of the 30 CRC patients. (d) Correlative expression of each variant recognized in the primary CRC tumors and normal mucosa tissues.*

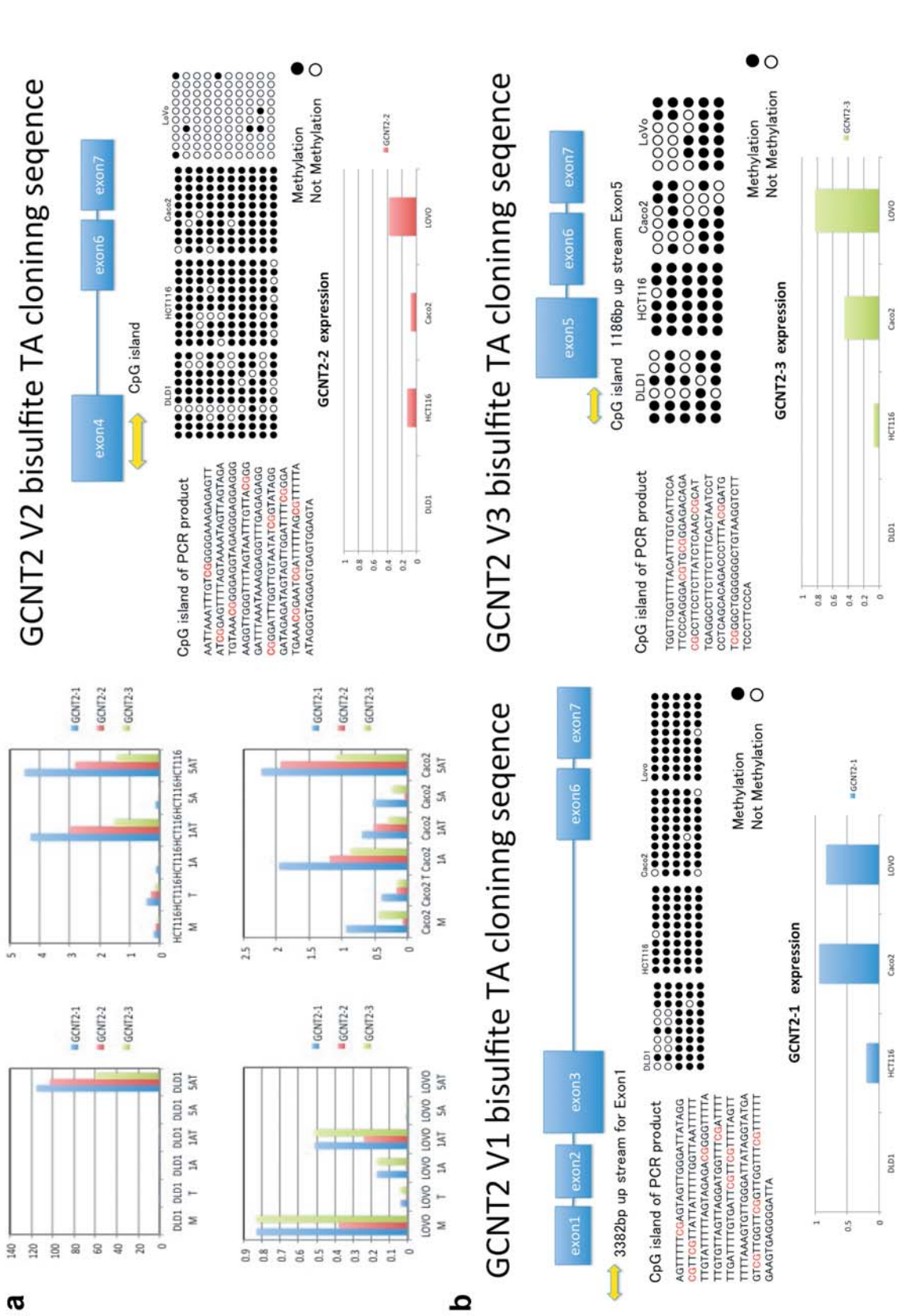

Figure 3. *(a) The 4 CRC cell lines were treated with demethylating agents variously combined; 5 Aza-dC (A) alone, TSA (T) alone or the combination (AT). The numerical indication shows the concentration of 5 Aza-dC (1 μM or 5 μM). Among the 4 CRC cell lines, 3 cell lines, which had silenced variant expression, are robustly reactivated by combination of demethylating agents. (b) The methylation and expression levels are consistent in GCNT2 variant 2. Cloned sequences demonstrate the methylation level after bisulfite treatment of the DNA after TA cloning. Black dots indicate methylation, white dots signify unmethylation in the cloned sequence analysis.*

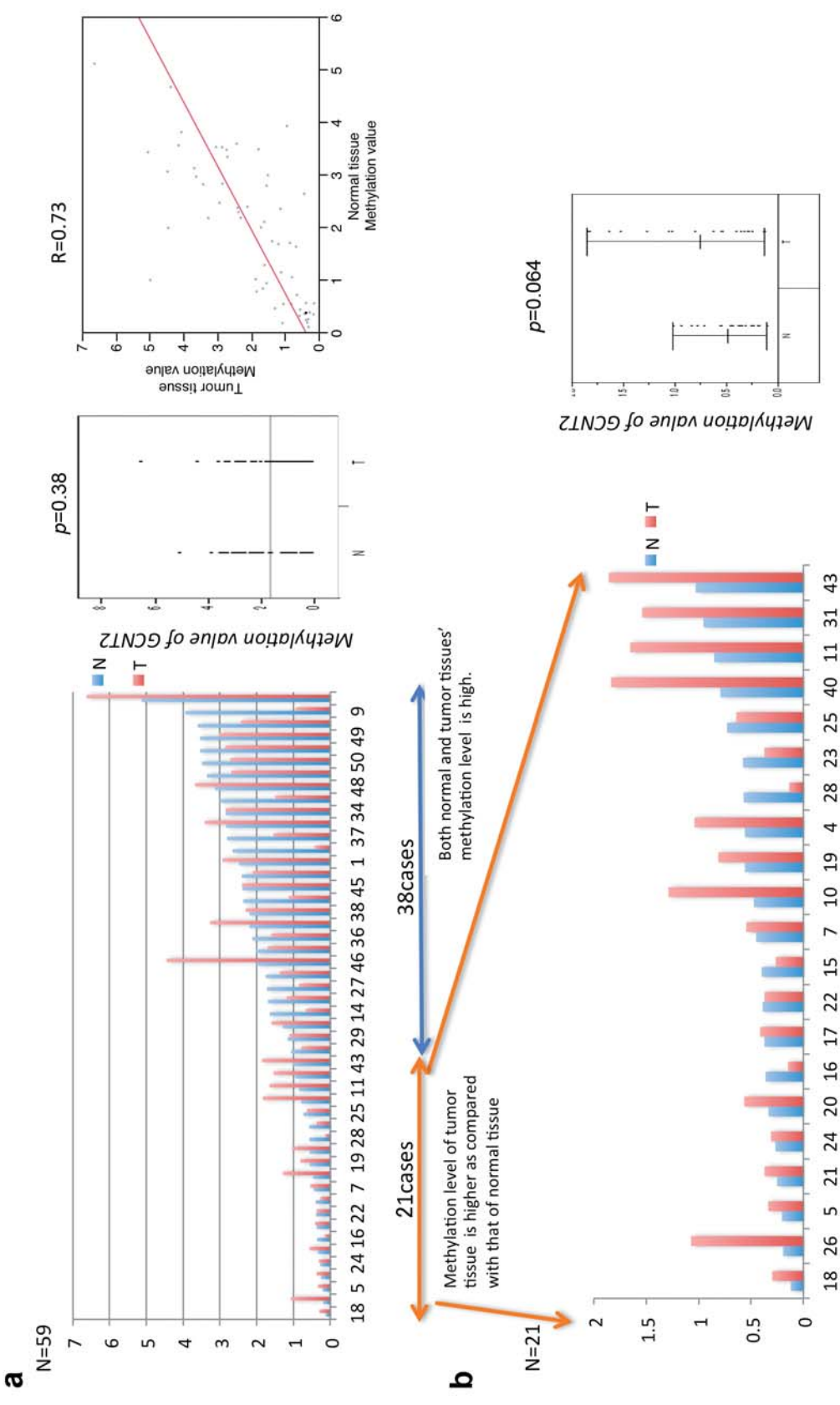

Figure 4. *(a) Quantitative analysis of the methylation level of the promoter CpG islands of the GCNT2 variant 2 for 59 tumors and the corresponding normal mucosa tissues. There is no significant difference for the total number of cases. The methylation value of both tumor tissue (T) and normal tissue (N) are related (R=0.73). (b) Quantitative analysis of the methylation level of the promoter CpG islands of the GCNT2 variant 2 for the 21 cases with weak methylation of the corresponding normal mucosa exhibit higher methylation in primary CRC tissues (T) than the corresponding normal tissues (N). This difference is statistically marginal (p=0.064).*

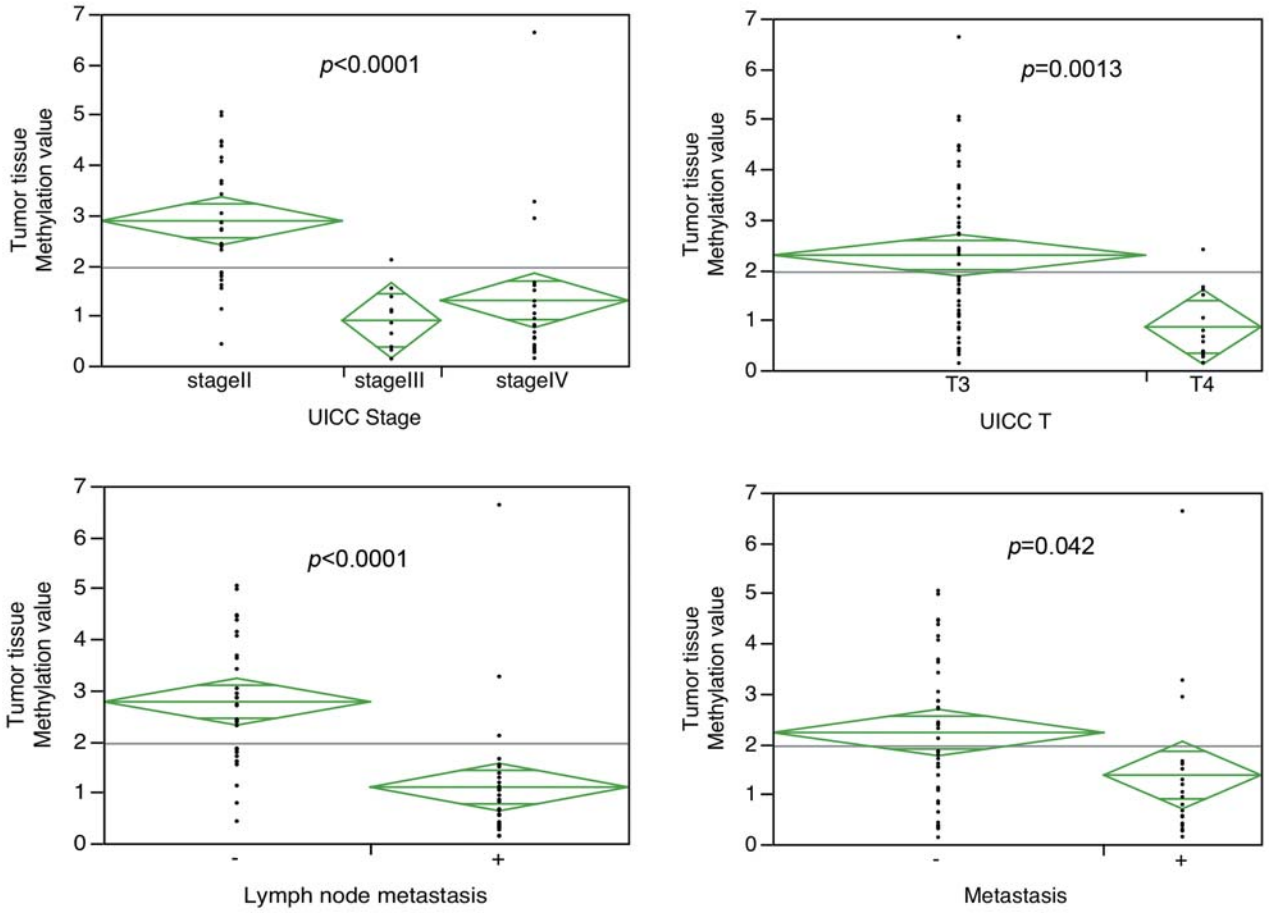

Figure 5. *These graphs show the relationship between methylation value of the promoter region of the GCNT2 variant 2 in the tumor tissues and several critical prognostic parameters such as stage according to the 6thUICC classification, T factor, lymph node metastasis and distant metastasis. Every graph shows that hypomethylation represents high tumor stage.*

metastasis ($p<0.0001$), as well as depth invasion ($p=0.0013$), distant metastasis ($p=0.042$) and stage ($p<0.0001$) according to the 6th UICC classification (Figure 5). This result is recapitulated by the GCNT2 variant 2 methylation level even in the corresponding normal tissue (lymph node metastasis ($p<0.0001$), depth invasion ($p=0.001$), distant metastasis ($p=0.038$) and the 6th UICC stage ($p<0.0001$)), putatively because the methylation level in the normal tissues largely reflects that of the tumor tissues (Figure 4a, right panel). The optimal cut-off level of GCNT2 methylation was selected as 1.54 by the receiver operating characteristic curve to predict lymph node metastasis (Figure 6a). Using this cut-off level, 29 patients with a high methylation level included 25 patients with no lymph node metastasis (86.2%), while 30 patients with low methylation level included 26 patients with lymph node metastasis (86.7%) (diagnostic accuracy=86.4%, $p<0.0001$). This result also reflects the data obtained from the corresponding normal mucosa (data not shown).

Log rank plot of methylation values of the tumor tissues determined the best cut-off value (1.70) for prognostication, which was almost the same with the value determined by lymph node prediction. On the other hand, the log rank plot of the corresponding normal tissues determined the best cut-off value (0.37), where low methylation represents dismal prognosis. This finding suggested that the methylation level of GCNT2 in the corresponding normal mucosa may especially affect dismal prognosis of advanced colorectal cancer during the early term of colorectal carcinogenesis.

Lastly, prognosis according to GCNT2 methylation level was examined for stage II/III CRC. The GCNT2 methylation in CRC tumor tissues showed an excellent prognostic stratification ($p=0.0082$), while that in the corresponding normal tissues did not ($p=0.076$) (the cut-off line of each analysis was based on lymph node metastasis study). This finding suggested that the GCNT2 methylation level could be promising for prediction of prognosis, as well as lymph node metastasis, even preoperatively by biopsy samples in curative CRC.

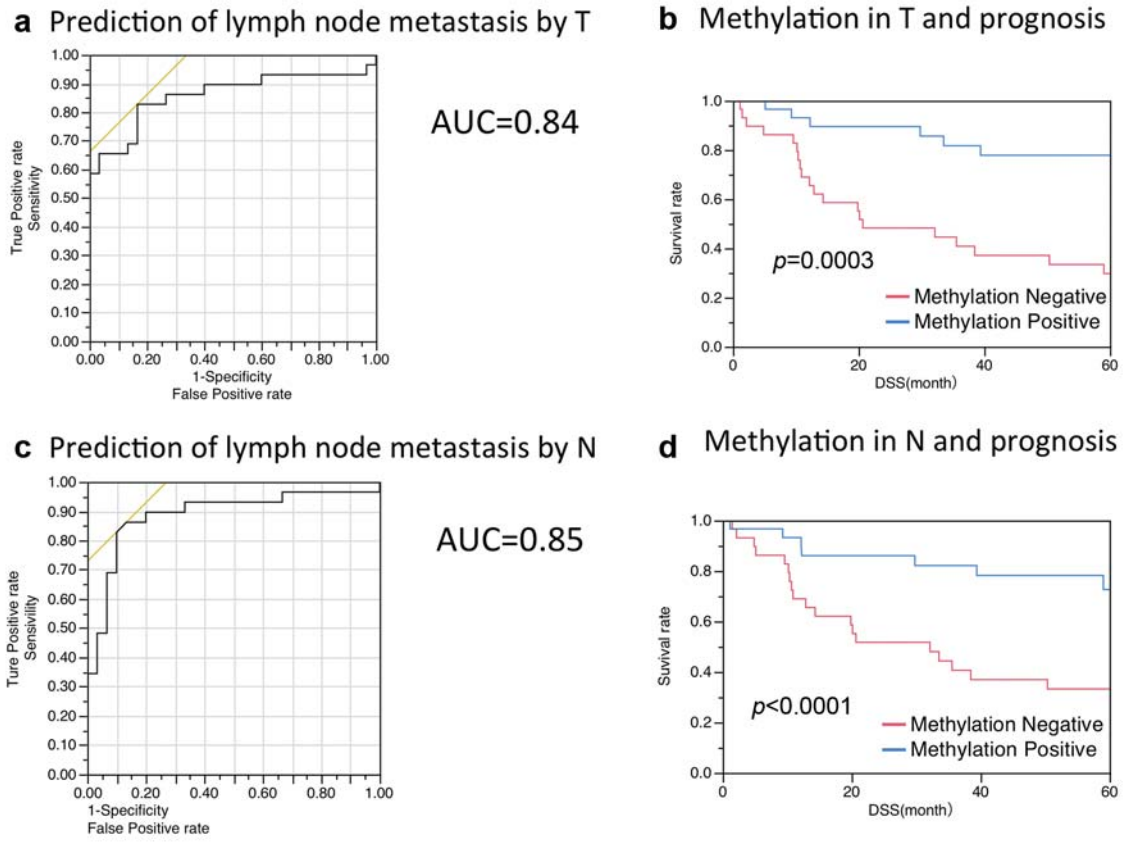

Figure 6. *(a) This is a ROC curve to predict lymph node metastasis according to methylation of the GCNT2 variant 2 promoter CpG islands of tumor tissues (T). The area under the curve (AUC) shows 0.84. We established cut-off level as 1.54. (c) This graph is a ROC curve to predict lymph node metastasis according to methylation of the GCNT2 variant 2 promoter CpG islands of the corresponding normal tissues (N). AUC shows 0.85. We established cut-off level as 1.75.*

Discussion

In the present study, we demonstrated, for the first time, that a GCNT2 is aberrantly expressed in primary CRC. All 3 variants of GCNT2 were substantially suppressed in primary CRC, while abundant expression of GCNT2 was found in considerable numbers of the corresponding normal mucosa. Since previous studies have elucidated that down-regulation in primary cancer tissues represents a possession of tumor suppressive function (17-19), these findings suggested that *GCNT2* is a tumor suppressive molecule in primary CRC.

On the other hand, *GCNT2* has been reported to be over-expressed in highly metastatic breast cancer cell lines of human and mouse origin and basal-like breast tumor samples (20). Moreover, functional studies showed that ectopic expression of *GCNT2* enhances cell detachment, adhesion to endothelial cells, cell migration and invasion *in vitro*, as well as lung metastasis of breast cancer cells *in vivo*. These findings suggested that *GCNT2* is an oncogenic molecule in breast cancer. Concerning its expression profile, *GCNT2* shows a reduced expression in almost all primary CRC, which is totally different from the results obtained in breast cancer cases.

Thus far, there have been few glycogenes reported that are regulated by promoter DNA methylation in primary CRC. Specifically and among glycogenes, only *B4GALT1* was reported to be hypermethylated compared to the normal mucosa (8, 21). The CRPS-G experimental approach identified *GCNT2* as a candidate methylation gene that supported our hypothesis. In CRC, epigenetically-regulated glycogenes have been reported as *DTDST* (4), a sulfate transferase, and *ST6GalNAc6* (22) to regulate production of sLe^X and sLe^a (CA19-9), respectively, which were both clinically available tumor markers in the serum; however, direct demonstration has been inconclusive for DNA methylation of the promoter CpG islands.

Our data propose that the *GCNT2* gene is epigenetically-regulated in CRC and normal colonic mucosa and variant 2

plays a central role in its expression. As opposed to other variants (variant 1 and variant 3), the promoter CpG islands of only the *GCNT2* variant 2 is close to TSS and the CpG island methylation status of the promoter region is consistent with the expression status of *GCNT2* variant 2 (Figure 3b); CRC cell lines with hypermethylation, such as DLD1, HCT116 and Caco2, are defective in the variant 2 expression, while the hypomethylated LoVo was the only cell line constitutively expressing *GCNT2* variant 2. Such consistent findings could not be demonstrated in the CpG islands of either variant 1 or variant 3 nearest to TSS. Combined treatment with demethylating agents robustly reactivated (hypermethylation) the expression of the *GCNT2* variant 2 in CRC cell lines (DLD1, HCT116 and Caco2).

To support the hypothesis that *GCNT2* variant 2 methylation is critical for its expression *in vivo*, we examined primary CRC cancer *versus* the corresponding normal mucosa tissues. Interestingly, about half the samples of both tumor and normal tissues showed remarkably high methylation, which is consistent with the finding that about half the cases exhibited silenced expression of *GCNT2* variant 2 in both tumor and normal tissues. On the other hand, primary CRC that showed less methylation in the corresponding normal tissues, tended to exhibit a higher *GCNT2* variant 2 methylation value (Figure 4b). Since hypomethylation is important for *GCNT2* variant 2 expression, these findings indicate that expression of the *GCNT2* variant 2 is epigenetically-regulated.

Moreover, variant 2 expression was accompanied by the expression of other variants in primary CRC supporting the notion that *GCNT2* variant 2 expression was epigenetically-regulated and playing a key role even in primary CRC tissues. In order to confirm whether either *GCNT2* variant 1 or variant 3 was induced by *GCNT2* variant 2, we created CRC cell lines (DLD1 and HCT116) stably expressing the *GCNT2* variant 2 gene. Quite interestingly, variant 1 and 3 expression was remarkably induced, while variant 3 expression patterns were all same after *GCNT2* variant 2 transfection in both DLD1 and HCT116 cells. This finding suggested that *GCNT2* variant 2 expression can activate variant 1 and 3 transcription and play a pivotal role in total GCNT2 expression.

Hypomethylation in the tumor tissues was herein assessed by Q-MSP quantification and a was proven to predict CRC progression status, especially for lymph node metastasis (Figure 5, 6a). Preoperative diagnosis of lymph node metastasis is quite important in advanced-stage CRC as it can even predict future prognosis (Figure 6b). Importantly, this is recapitulated even in cases without distant metastasis (stage II/III cases). Diagnostic accuracy of lymph node metastasis by *GCNT2* hypomethylation reached 86.2%, that was much higher than that by the present imaging system, such as computed tomography or magnetic resonance imaging, having a novel potential as a diagnostic tool to predict lymph node metastasis. Moreover, the methylation level of the tumor tissues was similar to that of the corresponding normal tissues (Figure 4a), and methylation level in the corresponding normal tissues can be used as well (Figure 6c, d). These findings suggested that lymph node metastasis was affected by the surrounding normal mucosa. Log rank plot analysis also elucidated that hypomethylation of the *GCNT2* variant 2 in the corresponding normal tissues predicts dismal prognosis of CRC. These clinical findings, that hypomethylation of *GCNT2* represents an aggressive phenotype of CRC, suggested that *GCNT2* is an oncogenic molecule in CRC, as well as in breast cancer. Interestingly, the CRC cell line LoVo, constitutively expressing *GCNT2* and derived from subclavian lymph node metastasis, exhibited highly invasive properties (23).

In conclusion, CRPS-G elucidated differential expression of glycogenes in primary CRC and identified *GCNT2* as an epigenetically-regulated gene candidate. The mechanism of *GCNT2* expression are very unique because variant 2 is closely associated with other variants; variant 2 induced and represented other variants. On the other hand, hypomethylation in the tumor tissues was closely associated with lymph node metastasis and prognosis, and the same phenomenon is caused in the background normal mucosa tissues. This is the first report showing that the methylation status of colorectal normal mucosa tissues predicted lymph node metastasis of primary advanced CRC. Such diagnostic potential needs to be further studied in the field of translational cancer research.

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