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ORIGINAL ARTICLE

Glycoproteomic analysis reveals the effects of bisecting GlcNAc in intrahepatic cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinoma (ICC) is the second major subtype of primary liver cancer and has caused more and more attention with increasing incidence and mortality worldwide. Our previous study found that bisecting *N*-glycans are commonly increased in ICC, while the effects and potential functions of bisecting GlcNAc in ICC are still largely unclear. In this study, we further confirmed that the structures of bisecting GlcNAc were significantly up-regulated in ICC compared with paracancer tissues by glycoproteomic data and lectin histochemistry. The expression of its glycosyltransferase MGAT3 was also up-regulated in ICC tissues at both mRNA and protein levels, and expression of *MGAT3* is negatively correlated with overall survival explored by bioinformatic analyses and published datasets from 255 patients. Next, the silencing of *MGAT3* could inhibit the growth and invasion of ICC cells, and overexpressing of *MGAT3* only promoted ICC cell invasion. Further glycoproteomic analysis showed that the commonly glycoproteins modified by bisecting GlcNAc after *MGAT3*-overexpression in two ICC cell lines were mainly involved in cell movement-related biological processes, such as cell adhesion, integrin-related and ECM-receptor interaction. This study sheds light on the potential effects of bisecting GlcNAc in ICC cells and suggests that *MGAT3* might be used as a potential target in the therapy of ICC.

Keywords Intrahepatic cholangiocarcinoma · Bisecting GlcNAc · *MGAT3* · Glycoproteomics

Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver malignancy, which arises from bile duct epithelium within the liver [1]. The incidence and subsequent mortality of ICC has been growing around the world [2]. Currently, surgical excision remains the most effective treatment for ICC, but most patients are diagnosed at an advanced stage due to atypical symptoms. After surgery, the 5-year overall survival rate is only 30–35%, and the 5-year survival rate for patients who are unable to undergo surgical resection is less than 10% [3].This fact emphasizes the importance of developing new therapeutic strategies for ICC.

Glycosylation is one of the most important post-translational modifications of proteins and plays a role in a variety of biological activities, including cell communication, adhesion, immunological defense, and cell growth [4]. Glycosylation abnormalities also play a vital role in tumor formation and progression [5]. We recently revealed that partial bisecting glycans were commonly increased in ICC and hepatocellular carcinoma (HCC) tumors by comparing differences in aberrant site-specific *N*-glycosylation between ICC and HCC tumors using a newly developed method (StrucGP) for *N*-glycoproteomics combined with stable isotope labeling-based quantification [6, 7]. The bisecting GlcNAc structure, a modification mostly found in complex and hybrid *N*-glycans, is the attachment of an *N*-acetylglucosamine (GlcNAc) to the core mannose of the *N*-glycan via a MGAT3-catalyzed β1,4 linkage [8]. Bisecting GlcNAc has been demonstrated to influence cancer development in the past, although whether it acts as an inhibitor or a promoter is

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still up for debate. In ovarian cancer, hepatocarcinoma, leukaemia and in some cells, including K562 cells, Hela cells and B16-hm cells, it had been demonstrated that *MGAT3* had a promoting effect on cancer [9–14]. In metastatic ovarian cancer, mammary tumor, and MKN45 cells, *MGAT3* had the effect of inhibiting cancer [15–17]. All these results illustrate the heterogeneity of *MGAT3* on cancer and cancer cells. Bisecting GlcNAc effects and molecular mechanisms in the development of intrahepatic cholangiocarcinoma also remain unclear.

In this study, we comparatively analyzed the bisecting GlcNAc changes between ICC and paracancer tissues using glycoproteomic data and lectin histochemistry. The expression and clinical relevance of *MGAT3* were explored based on TCGA and recently published RNA-seq data from 255 ICC tumor and related tissues [18]. Molecular biology experiments were used to examine the effects of *MGAT3* on ICC cells. In addition, a comparative glycoproteomic analysis were performed on ICC cells with and without *MGAT3* overexpression. Overall, our data preliminarily elucidate the possible mechanisms of bisecting GlcNAc and *MGAT3* in the progression of ICC, and provide new ideas for the discovery of possible therapeutic targets for ICC.

Materials and methods

Cell culture

RBE cell line was purchased from Chinese Academy of Sciences Cell Bank. HUCCT1 cell line was purchased from the cell bank of Japanese Collection of Research Bioresource (JCRB, Shinjuku, Japan). RBE and HUCCT1 cells were cultured in RPMI-1640 (VivaCell, Shanghai, China) with 10% fetal bovine serum (VivaCell, Shanghai, China) at 37 °C with 5% CO_2.

Fluorescence-based lectin histochemistry

Tissue samples were prospectively collected from patients undergoing ICC resection at the First Affiliated Hospital of Xi'an Jiaotong University, China. The study was approved by Human Ethics Committee at the First Affiliated Hospital of Xi'an Jiaotong University, and written informed consents were obtained from all participants. Formalin-fixed paraffin-embedded (FFPE) tissue sections from the paired tumor and paracancer tissue of ICC were dewaxed and hydrated with xylene and gradient alcohol, respectively. For lectin histochemistry, Cy3-labeled PHA-E was applied to detect the Bisecting GlcNAc structures. Briefly, the sections were washed, blocked with 5% (w/v) BSA and 0.02% Triton for 1 h and incubated with 1 $\mu g \cdot \mu L^{-1}$ Cy3-labeled PHA-E overnight at room temperature in the dark. Then nuclei were stained with DAPI (Solarbio, Beijing, China) for 10 min [19]. The fluorescence images were acquired by a fluorescence microscope, and the relative intensity of fluorescence was quantified by imagej software (http://imagej.net).

Stable knockdown and overexpressed cell lines were generated by using the lentiviral system

To generate *MGAT3* knockdown variants, lentiviral shRNA was constructed with the pGreenPuro vector (System Biosciences, CA). The target sequences (5'-GAGTCCAACTTCACGGCTTAT-3') of the MAGT3 were used as sh-*MGAT3*, and a scrambled shRNA was used as a control. To generate *MGAT3* overexpression cell variants, full length *MGAT3* cDNA from humans was amplified using PCR SuperMix High Fidelity (Takara, Japan) and ligated into the eukaryotic expression vector pCDH-CMV-MCS-EF1-Puro plasmid (System Biosciences, CA). An empty vector was used as a control. Lentiviral supernatants were generated by co-transfection of helper plasmids (System Biosciences, CA) into the packaging cell line (HEK293TN). Viral supernatants were harvested at 48–72 h after transfection. RBE and HUCCT1 cells were infected with filtered retroviral supernatant and selected with 2 μg/mL puromycin (Solarbio, China).

Western blot analysis

Proteins were extracted with RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Total protein was separated by 10% SDS-PAGE and then transferred to 0.45 μm PVDF membranes (Millipore, USA). Membranes were blocked with 5% non-fat milk at room temperature for 1 h, followed by incubation with primary and secondary antibodies. Blots were developed with ECL reagent and analyzed on a chemoluminiscence instrument (ChemiScope 6200, CLINX, China).

Clone formation assay

The stable cells (1×10^3) were plated in a six-well plate. Media were changed every 3–4 days. After 10–14 days, surviving colonies were fixed with methanol for 15 min, and then stained by 0.1% crystal violet. The colony formation ability was presented as percentage compared with control group, and three independent experiments were performed.

Transwell assay

Cell invasion was assessed by transwell chambers with 8.0 μm pore membranes (Corning, USA). About 1×10^5 cells

in precooled serum-free 1640 were seeded into the upper chamber coated with Matrigel (R&D, USA), and medium supplemented with 10% FBS was added to the lower chamber as a chemoattractant. Cells were then incubated at 37 °C with 5% CO_2 for 24 h. The invaded cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet. Finally, different fields of cells were then randomly selected and photographed. The average of cell counts in four microscope fields per condition was used for plotting results.

Gene expression analyses in public datasets

The transcriptome profiling profiles of ICC were downloaded from The Cancer Genome Atlas (TCGA) Data Portal [20] (https://www.cancer.gov), including 33 tumors and 8 normal tissues. Statistical significance of the expression differences between groups was determined using Wilcox's test.

Glycoproteomic analyses

The analytical methods of glycoproteomics have been described in detail in previous studies [21, 22]. In a nutshell, the details of sample treatment including cell lysis, protein extraction, protein digestion, peptide desalting, and enrichment of intact glycopeptides using MAX columns. LC-MS/MS analysis, database search for intact glycopeptide identification were performed based on StrucGP [22].

Bioinformatic analyses

Protein-protein interaction network was analyzed and performed by the STRING (http://string-db.org/) [23]. Gene Ontology (GO) and KEGG pathway was performed by Database for Annotation, Visualization, and Integrated Discovery (DAVID) V6.8 (https://david.ncifcrf.gov/home.jsp) [24]. Whole genome as background and *P*-value less than 0.05 was regarded as the significant pathway.

Results

Bisecting GlcNAc is upregulated in ICC tumors

Our existed LC-MS/MS data on ICC tumor (n = 6), HCC (n = 6) and their paired paracancer tissue (n = 12) were re-analyzed in this study [19]. After filtering results by ≥ 5 glycopeptide-spectrum matches (GPSMs), a total of 56 glycopeptides modified by bisecting GlcNAc were identified from ICC tumors and paracancer tissues, which accounted for 5.17% of all intact glycopeptides (1084) (Fig. 1 A, **Sup Table S1**). The identification of glycopeptide with bisecting GlcNAc was determined by a series of feature Y ions (such as peptide + HexNAc3Hex1 at *m/z* 1429.69 (z = 2), peptides + HexNAc3Hex1Fuc1 at *m/z* 1501.71 (z = 2)) **(Fig. S1)**.

The TMT-labeled quantitation results showed that 47 of 56 glycopeptides (95.7%) modified with bisecting GlcNAc were up-regulated, and 2 (3.6%) of them were down-regulated in ICC tumors with 1.7-fold as a cutoff (Fig. 1B), which was determined based on the LC-MS analysis of ICC and HCC paracancers tissues. (95.2% of glycopeptides were within 1.7-fold changes, **Fig. S2)**. These differently expressed glycopeptides were comprised of 21 *N*-glycan structures and 22 glycosites from 20 glycoproteins (Fig. 1 C-D).

To further confirm the increase of bisecting GlcNAc in ICC, we conducted lectin histochemistry analysis on three ICC tumors and their paired paracancer tissues using Cy3-labeled PHA-E for the detection of bisecting GlcNAc. The histochemical results demonstrated that the binding of PHA-E was significantly stronger ($P < 0.001$) in ICC tumors than in their adjacent normal tissues (Fig. 1E).

Upregulated*MGAT3*is negatively correlated with overall survival of ICC patients

To further confirm the increase of bisecting GlcNAc in ICC, the MGAT3 expression was profiled at both mRNA and protein levels in ICC (Fig. 2 A). Based on the transcriptome data in the TCGA database, the mRNA level of *MGAT3* was significantly elevated ($P < 0.0001$) in ICC tumors (n = 33) compared with normal tissues (n = 8) (Fig. 2B). According to the immunohistochemistry data in the Human Protein Atlas (HPA) database, the staining positivity of GnT-III increased in ICC tissue, which was consistent with the mRNA upregulation in ICC patients identified in TCGA data (Fig. 2 C).

To elucidate the relevance of *MGAT3* with tumor progression, we further investigated the relationship between *MGAT3* expression and clinical features of ICC based on a recently published RNA-seq dataset from 255 ICC tumor tissues [18]. Based on the median *MGAT3* mRNA expression, we divided the patients into high and low *MGAT3* mRNA-expressing groups. For TNM stage analyses, the transcription levels of *MGAT3* were significantly higher in the III-IV than in the I- II (Fig. 2D), while tumor size showed no significant relevance with *MGAT3* expression (Fig. 2E). *MGAT3* appeared to be highly expressed in patients with other clinicopathological features, including vascular invasion, regional lymph node metastasis, and perineural invasion (Fig. 2 F). Furthermore, survival statistics indicated a significant reduction of overall survival in ICC patients with high *MGAT3* expression compared with

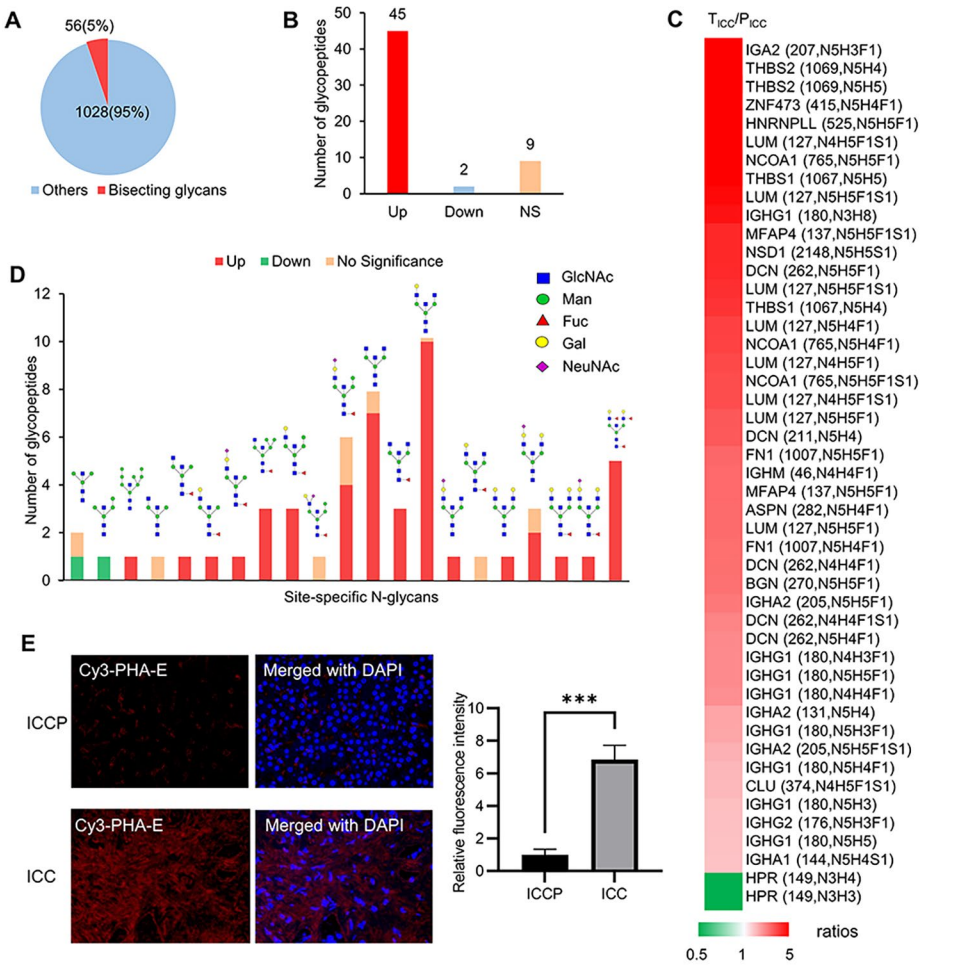

Fig. 1 Bisecting GlcNAc altered in ICC tumors compared with paracancer tissues. A, Percentage of unique glycopeptides with bisecting GlcNAc in all glycopeptides. B, Changes of bisecting *N*-glycopeptides in ICC compared with paired paracancer samples. "Up" and "Down" indicate fold change of glycopeptides ≥ 1.7 and ≤ 0.588 in ICC, respectively. "NS" (no significant change) stands for fold change < 1.7 or > 0.588 in ICC tumors compared with paracancerous tissues. C, Heatmap of differentially expressed bisecting *N*-glycopeptides. TICC, tumors of ICC; PICC, paracancers of ICC; Gene name (glycosite, glycan composition). D, Frequencies of changed site-specific bisecting GlcNAc in ICC (n = 6). E, Lectin histochemistry analysis of ICC tumors and paired paracancer tissues (n = 3, ±SD, P < 0.001 by paired t-test) based on the WFA binding (40×magnification). A-D were derived from reanalysis of the data from previous research [19]

low *MGAT3* expression (Fig. 2G). These results suggested that the *MGAT3* expression differed significantly among ICC tumors, and was associated with ICC progression.

Overexpression of bisecting GlcNAc promotes ICC cell invasion

To evaluate the effect of *MGAT3* expression on malignant biological behaviors, stable *MGAT3* overexpression (OE) and knockdown (KD) of RBE and HUCCT1 cell lines were established via lentiviral transfection. Colony formation and transwell assays were performed to determine the effects of *MGAT3* on colony formation and invasion of ICC cells. The colony formation analysis revealed that knockdown of *MGAT3* inhibited the clonal formation of RBE and HUCCT1 cells, while overexpression of *MGAT3* showed no significant effect on clonal formation (Fig. 3 A). Transwell invasion assays revealed that knockdown of *MGAT3* significantly inhibited cell invasion and overexpression of *MGAT3* significantly promoted cell invasion in RBE and HUCCT1 cells (Fig. 3B).

The detection of E-cadherin and vimentin protein expression was conducted by western blot analysis (Fig. 3 C). The results showed that E-cadherin expression was negatively correlated with *MGAT3*, while expression of *MGAT3* did not have a significant effect on vimentin expression. Taken together, these findings demonstrated that *MGAT3* promoted ICC cell invasion and was related to metastasis of ICC.

Functional investigation of glycoproteins with bisecting GlcNAc

To explore the biological relevance of bisecting GlcNAc in ICC cells, *N*-glycoproteome analyses were again performed on the control and overexpressed RBE and HUCCT1 cells. Glycoproteome analysis identified 1,994–2,404 glycopeptides from RBE and HUCCT1 cell lines with/without *MGAT3* overexpression **(Sup Table S2-S5)**. The number of glycopeptides modified by bisecting GlcNAc was significantly increased in *MGAT3* overexpressed RBE and HUCCT1 cell lines (Fig. 4 A). A total of 38 glycoproteins (204 glycopeptides) and 30 glycoproteins (135 glycopeptides) with bisecting GlcNAc were identified in *MGAT3* overexpressed RBE and HUCCT1 cell lines, respectively (PSMs ≥ 5), of which 17 glycoproteins are shared in both cell lines (Fig. 4B). The number of unique

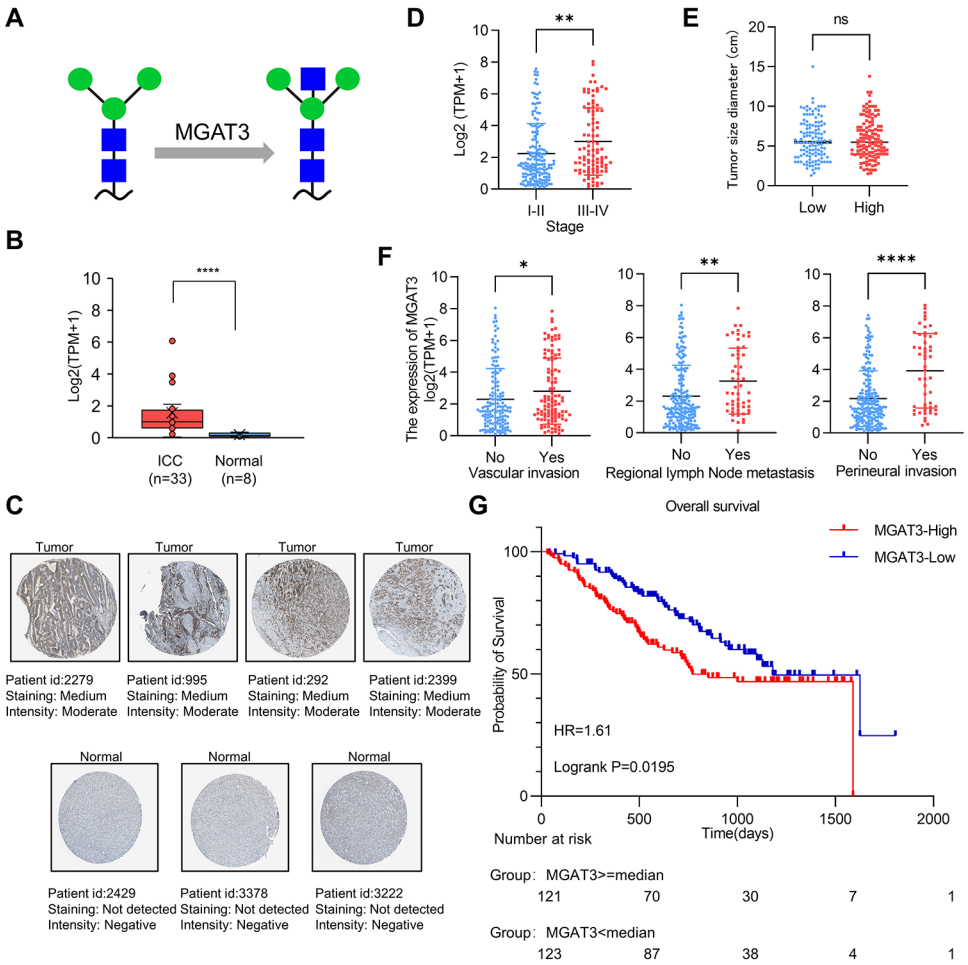

Fig. 2 Expression levels of*MGAT3*and survival analysis of ICC patients. A, *MGAT3* encodes for the glycosyltransferase GnT-III that catalyzes the transfer of *N*-acetylglucosamine (blue box) to the core mannose of the *N*-glycan in β1,4 linkage to form the bisecting *N*-linked glycan structure. B, Transcript levels of *MGAT3* in ICC tumors (n = 33) and normal tissues (n = 8) by TCGA database (https://www.cancer.gov), (****P < 0.0001, Wilcoxon's test). C, Validation of *MGAT3* in the translational level. Immunohistochemistry (IHC) staining data were obtained from the Human Protein Atlas (HPA) database. D-F, Comparison of *MGAT3* expression in TNM stage (D), tumor size (E), Vascular invasion, regional lymph node metastasis and perineural invasion (F) based on a recently published RNA-seq dataset from 255 ICC tumor tissues [18]. Different groups were separated by the median expression. G, Expression of *MGAT3* correlates with survival in 255 ICC tumor tissues (Only 244 patient samples had overall survival information) [18]. The median value of expression was set as the cut-off point. The number of individuals at risk is depicted at the bottom. Significance was determined with log-rank test. P = 0.0195; HR = 1.61. Data are presented as mean ± SEM; P-values were determined by unpaired two-tailed (B-F). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, no significant difference

glycopeptides from those glycoproteins are increased to varying degrees (**Sup Table S6**).

To further explore the biological functions of 17 common glycoproteins with increased bisecting GlcNAc in *MGAT3* overexpressed RBE and HUCCT1 cells, GO and KEGG pathway analyses were performed. These glycoproteins were mainly involved in cell adhesion, viral entry into host cell, cell-matrix adhesion, integrin-mediated signaling pathway and cell adhesion mediated by integrin; and were mainly localized in extracellular exosome, cell surface, external side of plasma membrane, integrin complex and azurophil granule membrane. In addition, integrin binding, virus receptor activity, laminin binding, fibronectin binding, and protease binding were the most enriched in terms of molecular function (MF) enrichment (Fig. 4 C). KEGG pathway analysis showed the significant enrichment of ECM-receptor interaction, phagosome, focal adhesion, proteoglycans in cancer, and PI3K-Akt signaling pathway and lysosome of these glycoproteins (Fig. 4D). To obtain a better idea of the potential relationships between those 17 glycoproteins, protein–protein interaction network analysis was also performed by the STRING database. Many glycoproteins have interactions, and 10 proteins are related to cell adhesion (GO:0007155). All these data indicated that bisecting GlcNAC mainly affects cell behavior by biological processes

related to cell movement, such as cell adhesion, integrin-related and ECM-receptor interaction in RBE and HUCCT1 cells.

Discussion

In recent years, the incidence and subsequent mortality of intrahepatic cholangiocarcinoma (ICC) have been increasing worldwide despite recent advances in therapeutic approaches [25]. Therefore, an in-depth understanding of its mechanism and a more effective treatment strategy are urgently needed. Aberrant glycosylation has been recognized as a fundamental feature of many cancers and plays an important role in key pathological steps in tumor development and progression [5]. We previously performed precision *N*-glycoproteomic analysis on both ICC and HCC tumors as well as paracancer tissues to investigate their aberrant site-specific *N*-glycosylation [26], and found that partial bisecting GlcNAc structures were commonly elevated in ICC and HCC. However, the overall changes of bisecting GlcNAc in ICC and the effects are still unclear.

In this study, we found that the bisecting GlcNAc and its glycosyltransferase MGAT3 were significantly up-regulated in ICC. By silencing or overexpression of *MGAT3* in RBE and HUCCT1 cell lines, and combined with phenotypic experiments and glycoproteomic analysis, the effects and possible mechanisms of bisecting glycopeptides on ICC cells were systematically analyzed. In addition, our new method StrucGP can provide overall distributions of bisecting GlcNAc, the modified glycoproteins, glycosites, and possible *N*-glycan structures [7, 27]. The application of StrucGP in this study greatly facilitates *N*-glycoproteomic analysis [7].

We identified 47 bisecting *N*-glycopeptides from 20 glycoproteins that were altered in ICC, with 95.7% of altered glycopeptide were up-regulated. Among the 20 glycoproteins, the highest number of bisecting GlcNAc were identified from IGHG1 and lumican, with nine and eight bisected glycans,

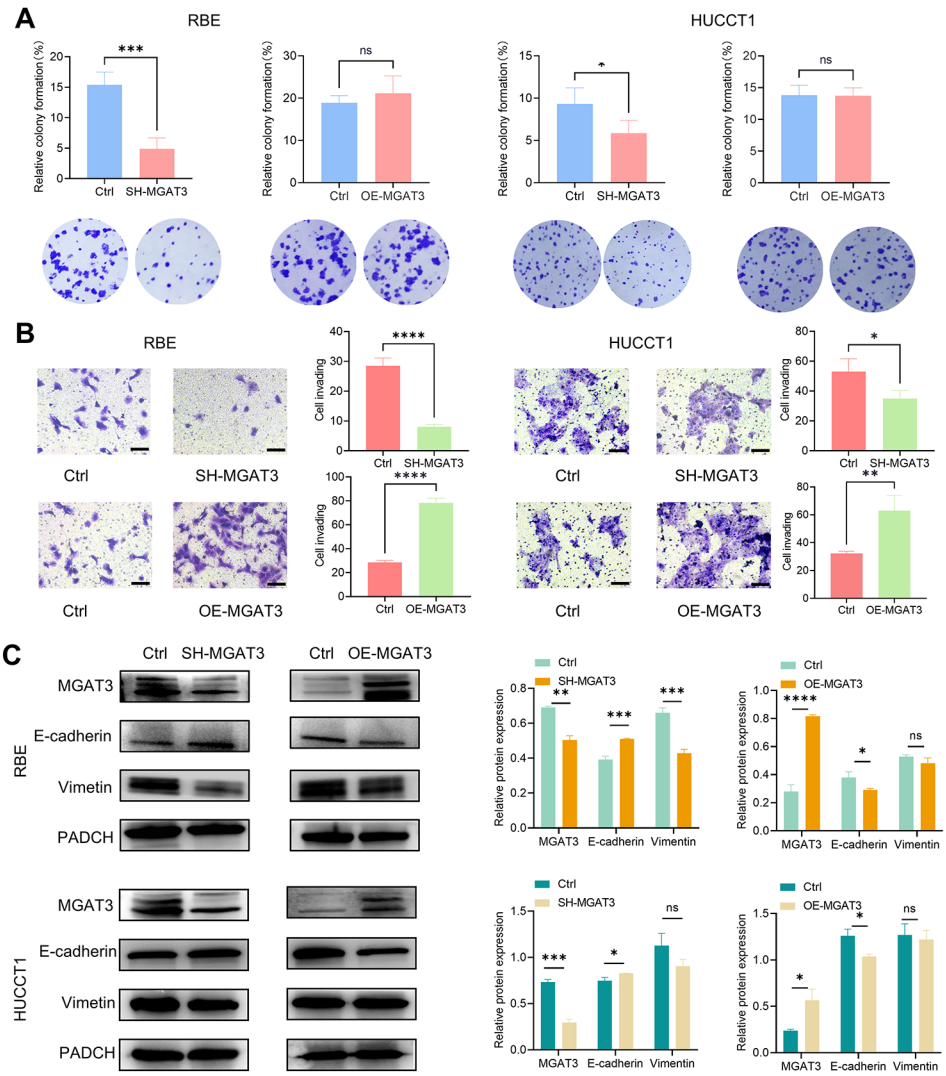

Fig. 3 Overexpression of *MGAT3* promotes invasion of ICC cells A, Representative microphotographs of clone formation experiments in stable ICC cell lines of *MGAT3* knockdown and overexpression. B, The Matrigel Trans-well invasion assay (Scale bar = 10 μm) in stable ICC cell lines of *MGAT3* knockdown and overexpression. C, Protein expression levels of *MGAT3*, E-cadherin and vimentin in RBE and HUCCT1 cell lines with *MGAT3* knockdown and overexpression (left). Quantifications analysis of the protein expression levels of *MGAT3*, E-cadherin and Vimentin (right). The grayscale values were measured from the western blotting data with Image J. Analyses were performed in triplicates for each condition. Data are presented as mean ± SEM; P-values were determined by unpaired two-tailed t-test (A and B) or One-way ANOVA (C). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, no significant difference

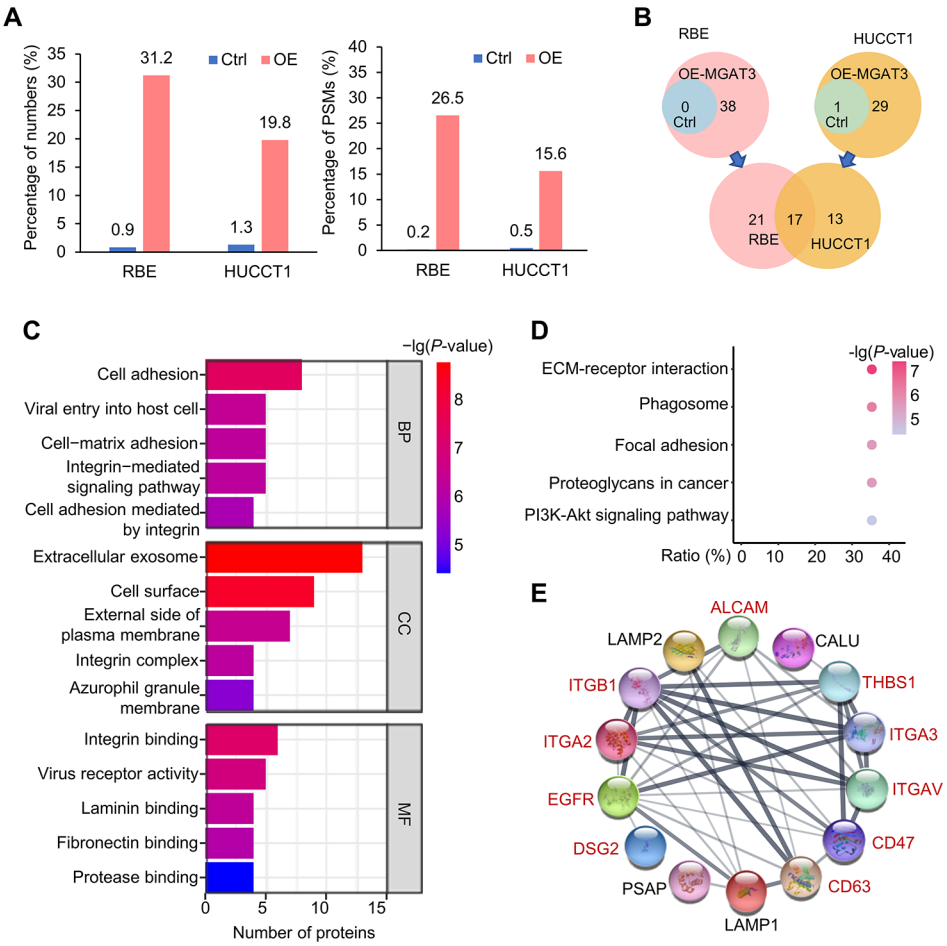

Fig. 4 Functional enrichment analysis of glycoproteins with bisecting GlcNAc in RBE and HUCCT1 cells. A, Percentages of glycopeptides with bisecting GlcNAc in RBE and HUCCT1 cell. Percentages were calculated based on the unique glycopeptides (left) and PSMs (right) B, Glycoproteins modified by bisecting GlcNAc that were commonly identified in two ICC cell lines (PSM ≥ 5). C and D, Gene Ontology (GO) (C) and KEGG pathway (D) analysis of common glycoproteins modified by increased bisecting GlcNAc between RBE and HUCCT1 cell lines ($P < 0.05$). Biological process (BP), cellular component (CC), and molecular function (MF). E, Protein-protein interaction network of common glycoproteins modified by bisecting GlcNAc identified in two ICC cell lines. The red lables mark the glycoproteins involved in cell adhesion (GO:0007155). Nodes represent proteins; edges, network connections; line thickness, strength (thicker = stronger). The minimum required interaction score selects medium confidence (0.400)

respectively. It has been reported that bisecting GlcNAc on IGHG1 were also highly expressed in cirrhosis (CIR) and HCC [28, 29]. Studies showed that bisecting GlcNAc could change the conformation of IGHG1 to influence the binding of IgG to its receptors, and affect the progression of liver diseases [30]. Glycosylation of lumican may protect collagen against MMP-14 proteolysis by decrease MMP-14 activity in tumor cells, and thus influences cell–matrix interaction in tumor progression [31]. Considering the regulatory roles of bisecting GlcNAc in N-glycan biosynthesis, it is possible that bisecting N-glycans on lumican could be involved in the interaction between lumican and MMP-14.

MGAT3 expression was also consistently up-regulated in ICC tissues at both the mRNA and protein levels. Using RNA-seq data from 255 ICC tumor tissues, we found that increased expression of *MGAT3* is associated with decreased overall survival. Some clinical events, including vascular invasion, regional lymph node metastasis, and perineural invasion, were shown to be related to MAGT3 expression. Vascular invasion and regional lymph node metastasis are associated with overall survival, and perineural invasion is associated with recurrence of cholangiocarcinoma [3, 32]. However, there was no significant correlation between *MGAT3* expression and tumor size, which is consistent with the conjecture that *MGAT3* is involved mostly in cell differentiation rather than cell proliferation during cell culture [33]. Similar with our results in ICC, the over-expression of *MGAT3* was also found to be associated with enhanced tumor progression in spleen, which was partially mediated by CD44-hyaluronan interactions enhanced by the bisecting GlcNAc on CD44 [13].

Our finding shows that *MGAT3* expression is positively correlated with cell invasion. Meanwhile, *MGAT3* expression levels were slightly negatively correlated with the expression levels of E-cadherin. According to previous studies, different glycan structures were described to regulate E-cadherin expression through interfering with its basic mechanisms including E-cadherin-mediated cell-cell adhesion, E-cadherin cellular trafficking, and E-cadherin-dependent signaling pathways [34]. As for bisecting GlcNAc, its over-expression induced by *MGAT3* might affect the expression of E-cadherin by regulating its turnover rate and its release from cell surface [35] [36]. In present study, glycosylated E-cadherin was not identified in RBE and HUCCT1 cell lines, which may be due to the low abundance of glycosylated E-cadherin or the expression of E-cadherin is

only slightly affected by other pathways without glycosylated. Altering the expression of glycosyltransferases has a significant impact on the glycosylation remodeling of various glycoproteins, thereby affecting various cellular behaviors such as cell invasion. The detailed mechanism behind this process is complex and worthy of further investigation. In previous studies, *MGAT3* also has been shown to promote the progression of malignant behavior in other cells and cancers. *MGAT3* overexpression in K562 cells promotes cell spleen colonization [9]. In HeLa cells, overexpression of *MGAT3* promotes cell metastasis by increasing EGFR signaling and decreasing cell adhesion [11]. In addition, it has also been observed that *MGAT3* is proportional to invasive ability in ovarian cancer [10, 37]. However, several other studies have demonstrated that *MGAT3* can also act as a tumor suppressor [17] [38]. All these results illustrate the heterogeneity of *MGAT3* on cancer cells, and the reason remains to be further investigated.

By using high-throughput glycoproteomics, we systematically charactered bisecting GlcNAc in RBE and HUCCT1 cells, including the corresponding glycoproteins information. We detected a serial of glycoproteins with increased bisecting GlcNAc in *MGAT3* overexpressed RBE and HUCCT1 cells. Evidences have shown that bisecting GlcNAc might modulate the biological activity of its carrier proteins. For example, overexpression of bisecting GlcNAc on CD44 enhances its adhesion to hyaluronate, and affects local tumor growth and metastatic growth in the spleen [13]. Bisecting GlcNAc on EGFR could regulate endocytosis of EGFR and further modulate downstream signaling [39]. So far, details of molecular function of bisecting GlcNAc in regard to the regulation of its carrier proteins associated with cellular invasiveness (especially promotion of invasive) are complex and still remain unclear due to the varied consequences and possibly mechanisms of bisecting GlcNAc expression among different cell types. Further studies are still required to fully explore molecular functions of bisecting GlcNAc associated with cellular invasiveness. ECM-receptor interaction is the most prominent pathway enriched in the KEGG pathway. ECM-receptor interaction mainly controls cell adhesion, migration, proliferation, and coagulation cascade activation [40]. Our research provides rich data for exploring mechanisms between bisecting GlcNAc and their carrier proteins.

Based on all the above analyses, *MGAT3* plays an important role in ICC carcinogenesis. Although the exact molecular mechanism of *MGAT3* on cell behavior is still unclear, the glycoproteomics data presented here provides useful insights for future researches. These achievements will be greatly beneficial for further in-depth functional investigation and specific regulatory mechanisms of bisecting GlcNAc and *MGAT3* in ICC.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10719-022-10085-5.

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Author contributions W.D. and S.S. designed the experiments; L.H. collected the tissue samples; W.D. and C.L. performed experiments with help from P.L., J.L, J.L.; W.D., C.L., Z.C and J.L., J.S., analyzed data; W.D., J.L., P.L., L.D., M.X., Z.Y., and S.S. wrote the paper with help from all authors.

Data Availability The mass spectrometry data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD035321.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Ethical statements All human studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. This study does not involve experiments of animal models.

Ethical approval Tissue samples were prospectively collected from patients undergoing ICC resection at the First Affiliated Hospital of Xi'an Jiaotong University, China. The study was approved by Human Ethics Committee at the First Affiliated Hospital of Xi'an Jiaotong University, and written informed consents were obtained from all participants.

Consent to participate All persons gave their informed consent prior to their inclusion in the study.

Consent to publish Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

References

1. Aljiffry, M., et al.: Evidence-based approach to cholangiocarcinoma: a systematic review of the current literature. J. Am. Coll. Surg. **208**(1), 134–147 (2009)
2. Gupta, A., Dixon, E.: Epidemiology and risk factors: intrahepatic cholangiocarcinoma. Hepatobiliary Surg. Nutr. **6**(2), 101–104 (2017)
3. de Jong, M.C., et al.: Intrahepatic Cholangiocarcinoma: An International Multi-Institutional Analysis of Prognostic Factors and Lymph Node Assessment. J. Clin. Oncol. **29**(23), 3140–3145 (2011)
4. Ohtsubo, K., Marth, J.D.: Glycosylation in cellular mechanisms of health and disease. Cell. **126**(5), 855–867 (2006)
5. Liu, H.M., et al.: Progress in research into the role of abnormal glycosylation modification in tumor immunity. Immunol. Lett. **229**, 8–17 (2021)

6. Li, J., et al.: Precision N-glycoproteomics reveals elevated LacdiNAc as a novel signature of intrahepatic cholangiocarcinoma. Mol. Oncol. **16**(11), 2135–2152 (2022)
7. Shen, J.C., et al.: StrucGP: de novo structural sequencing of site-specific N-glycan on glycoproteins using a modularization strategy. Nat. Methods. **18**(8), 921– (2021)
8. Nishikawa, A., et al.: Purification, cDNA cloning, and expression of UDP-N-acetylglucosamine: beta-D-mannoside beta-1,4 N-acetylglucosaminyltransferase III from rat kidney. J. Biol. Chem. **267**(25), 18199–18204 (1992)
9. Yoshimura, M., et al.: Bisecting N-acetylglucosamine on K562 cells suppresses natural killer cytotoxicity and promotes spleen colonization. Cancer Res. **56**(2), 412–418 (1996)
10. Allam, H., et al.: The glycosyltransferase GnT-III activates Notch signaling and drives stem cell expansion to promote the growth and invasion of ovarian cancer. J. Biol. Chem. **292**(39), 16351–16359 (2017)
11. Sato, Y., et al.: Overexpression of N-acetylglucosaminyltransferase III enhances the epidermal growth factor-induced phosphorylation of ERK in HeLaS3 cells by up-regulation of the internalization rate of the receptors. J. Biol. Chem. **276**(15), 11956–11962 (2001)
12. Yoshimura, M., Ihara, Y., Taniguchi, N.: CHANGES OF BETA-1,4-N-ACETYLGLUCOSAMINYLTRANSFERASE-III (GNT-III) IN PATIENTS WITH LEUKEMIA. Glycoconj. J. **12**(3), 234–240 (1995)
13. Sheng, Y., et al.: Remodeling of glycoconjugates on CD44 enhances cell adhesion to hyaluronate, tumor growth and metastasis in B16 melanoma cells expressing beta1,4-N-acetylglucosaminyltransferase III. Int. J. Cancer. **73**(6), 850–858 (1997)
14. Bhaumik, M., et al.: Progression of hepatic neoplasms is severely retarded in mice lacking the, bisecting N-acetylglucosamine on N-glycans: Evidence for a glycoprotein factor that facilitates hepatic tumor progression. Cancer Res. **58**(13), 2881–2887 (1998)
15. Li, J., et al.: MGAT3-mediated glycosylation of tetraspanin CD82 at asparagine 157 suppresses ovarian cancer metastasis by inhibiting the integrin signaling pathway. Theranostics. **10**(14), 6467–6482 (2020)
16. Song, Y.H., et al.: The Bisecting GlcNAc on N-Glycans Inhibits Growth Factor Signaling and Retards Mammary Tumor Progression. Cancer Res. **70**(8), 3361–3371 (2010)
17. Akama, R., et al.: N-acetylglucosaminyltransferase III expression is regulated by cell-cell adhesion via the E-cadherin-catenin-actin complex. Proteomics. **8**(16), 3221–3228 (2008)
18. Dong, L.Q., et al.: Proteogenomic characterization identifies clinically relevant subgroups of intrahepatic cholangiocarcinoma. Cancer Cell. **40**(1), 70– (2022)
19. Li, J., et al.: Precision N-glycoproteomics reveals elevated LacdiNAc as a novel signature of intrahepatic cholangiocarcinoma. Mol Oncol (2021)
20. Weinstein, J.N., et al.: The Cancer Genome Atlas Pan-Cancer analysis project. Nat. Genet. **45**(10), 1113–1120 (2013)
21. Li, P., et al.: Comparative Proteomic Analysis of Polarized Human THP-1 and Mouse RAW264.7 Macrophages. Front. Immunol. **12**, 700009 (2021)
22. Shen, J., et al.: StrucGP: de novo structural sequencing of site-specific N-glycan on glycoproteins using a modularization strategy. Nat. Methods. **18**(8), 921–929 (2021)
23. Szklarczyk, D., et al.: STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. **47**(D1), D607–D613 (2019)
24. Dennis, G., et al.: *DAVID: Database for annotation, visualization, and integrated discovery*.Genome Biology, **4**(9). (2003)
25. Khan, S.A., et al.: Global trends in mortality from intrahepatic and extrahepatic cholangiocarcinoma. J. Hepatol. **71**(6), 1261–1262 (2019)
26. Li, J., et al.: *Precision N-glycoproteomics reveals elevated LacdiNAc as a novel signature of intrahepatic cholangiocarcinoma*. Molecular Oncology
27. Dang, L.Y., et al.: Recognition of Bisecting N-Glycans on Intact Glycopeptides by Two Characteristic Ions in Tandem Mass Spectra. Anal. Chem. **91**(9), 5478–5482 (2019)
28. Yuan, W., et al.: Quantitative analysis of immunoglobulin subclasses and subclass specific glycosylation by LC-MS-MRM in liver disease. J. Proteom. **116**, 24–33 (2015)
29. Chen, Z.S., Gu, J.: Immunoglobulin G expression in carcinomas and cancer cell lines. Faseb J. **21**(11), 2931–2938 (2007)
30. Zauner, G., et al.: Glycoproteomic Analysis of Antibodies. Mol. Cell. Proteom. **12**(4), 856–865 (2013)
31. Pietraszek, K., et al.: Lumican: A new inhibitor of matrix metalloproteinase-14 activity. FEBS Lett. **588**(23), 4319–4324 (2014)
32. Bartsch, F., et al.: *Influence of Lymphangio (L), Vascular (V), and Perineural (Pn) Invasion on Recurrence and Survival of Resected Intrahepatic Cholangiocarcinoma*.Journal of Clinical Medicine, **10**(11). (2021)
33. Gu, J., et al.: A Mutual Regulation between Cell-Cell Adhesion and N-Glycosylation: Implication of the Bisecting GlcNAc for Biological Functions. J. Proteome Res. **8**(2), 431–435 (2009)
34. Strumane, K., Berx, G., Van Roy, F.: *Cadherins in Cancer*, in *Cell Adhesion*, J. Behrens and W.J. Nelson, Editors. Springer Berlin Heidelberg: Berlin, Heidelberg. p. 69–103. (2004)
35. Niu, H.Y., Qu, A.N., Guan, C.Y.: Suppression of MGAT3 expression and the epithelial-mesenchymal transition of lung cancer cells by miR-188-5p. Biomedical J. **44**(6), 678–685 (2021)
36. Yoshimura, M., et al.: Aberrant glycosylation of E-cadherin enhances cell-cell binding to suppress metastasis. J. Biol. Chem. **271**(23), 13811–13815 (1996)
37. Deng, Q.Y., et al.: Expression of N-Acetylglucosaminyltransferase III Promotes Trophoblast Invasion and Migration in Early Human Placenta. Reproductive Sci. **26**(10), 1373–1381 (2019)
38. Yoshimura, M., Taniguchi, N.: *Suppression of lung metastasis of B16 mouse melanoma cells by introduction of N-acetylglucosaminyltransferase III gene.* Nihon rinsho. Japanese J. Clin. Med. **53**(7), 1786–1790 (1995)
39. Takahashi, M., et al.: Core fucose and bisecting GlcNAc, the direct modifiers of the N-glycan core: their functions and target proteins. Carbohydr. Res. **344**(12), 1387–1390 (2009)
40. Czubryt, M.P.: *Inhibition of collagen synthesis*. (2016)