Co-expression of Lewis y antigen and CD147 in epithelial ovarian cancer is correlated with malignant progression and poor prognosis

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Abstract. CD147 is a highly glycosylated transmembrane protein expressed on the surface of tumor cells. In the present study, the expression and clinical significance of the Lewis y antigen and CD147 in epithelial ovarian cancer (EOC) were analyzed, and the function and correlation in between the expression of Lewis y and CD147 were evaluated using immunohistochemical staining, reverse transcription-quantitative polymerase chain reaction analysis, immunocytochemical staining, immunoprecipitation and western blotting. The results showed that the expression of CD147 was higher in EOC tissues and correlated with a higher tumor burden. Lewis y and CD147 exhibited similar expression patterns and their expression was positively correlated. The results of the immunofluorescence and immunoprecipitation experiments showed that Lewis y and CD147 colocalized in the cell membrane and cytoplasm. Lewis y antigen, but not Lewis x or sialyl Lewis x, was predominantly expressed in the highly glycosylated form of CD147. These changes occurred at the post-transcriptional level. As an important component of CD147, Lewis y promoted CD147-mediated cell adhesion and the expression of matrix metalloproteinase 2. In conclusion, Lewis y antigen and CD147 were significantly upregulated in ovarian tumors, and the altered expression of Lewis y may cause changes in CD147. The two molecules are associated with carcinogenesis and the development of ovarian cancer, and Lewis y antigen is a component of the CD147 structure.

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Introduction

Epithelial ovarian cancer (EOC) has the highest mortality rate and the poorest prognosis among female malignant tumors (1); however, a clear and reliable biological marker for early treatment is lacking (2). Understanding the mechanism underlying tumor metastasis and invasion may provide novel strategies and therapeutic targets for the early testing and diagnosis of EOC. There is a close correlation between glycoconjugates of the cell membrane and biological characteristics, including cell canceration, invasion and metastasis (3). Glycoconjugates are involved in several important biological processes, including adhesion, recognition of cells and signal transduction (4). The Lewis y antigen is an oligosaccharide containing two fucoses that belongs to the blood group A, B and H antigen family. In ~75% of EOC cases, Lewis y antigen is overexpressed to varying degrees, and its expression is associated with prognosis (5). In our previous studies, the genetic transfection technique was used to transfect the α1,2-fucosyltransferase (α1,2-FT, FUT1) gene into the RMG-I ovarian cancer cell line to establish a cell line exhibiting high expression of Lewis y antigen (RMG-I-hFUT); this cell line was used to show that Lewis y can promote malignant cell behavior by increasing proliferation, adhesion, invasion, metastasis, drug resistance and *in vitro* tumor rate (6-8). In addition, it was shown that the Lewis y antigen serves an important role in the occurrence, development, invasion and metastasis of EOC.

The invasion and metastasis of tumor cells involves cell adhesion molecules and protease-mediated degradation of the extracellular matrix. The extracellular matrix metalloproteinase inducer EMMPRIN or CD147 can alter the microenvironment of carcinoma cells by inducing matrix metalloproteinases (MMPs), angiogenic factors of carcinoma and substratum cells. It can also modulate the anchor-independent growth of carcinoma cells. Previous studies have shown that CD147 is involved in several processes, including promoting the metastasis of carcinoma cells, drug resistance, invasion and other aspects of malignancy (9-11). CD147 has been identified as an important marker of an unfavorable prognosis in ovarian carcinoma. Its expression is significantly correlated with cell signaling molecules, including Akt and

extracellular signal-regulated kinase (ERK). CD147 promotes the development of ovarian carcinoma by inducing the production of MMPs and modulating tumor growth, angiogenesis, signal transduction and drug-resistance (12-14).

The molecular weight of CD147 varies between 31 and 65 kDa depending on the degree of glycosylation and the level of Lewis x antigen (15,16). CD147 glycosylation is required for inducing the expression of MMP (15,17,18). However, the mechanism underlying the effect of glycosylation on regulating CD147 function remains to be fully elucidated. The present study examined the expression and correlation between the Lewis y antigen and CD147 in EOC using immunohistochemical staining of tissue specimens, and examined the function and mechanism of Lewis y in CD147-mediated cell adhesion. The RMG-I-hFUT cell line stably overexpressing Lewis y was used to investigate the molecular basis of the pathogenesis, progression and biological treatment of ovarian cancer.

Materials and methods

Ethics statement. Samples were fully encoded to protect patient confidentially. The present study was approved by the Ethical Committee of Shengjing Hospital of China Medical University (Shenyang, China; approval no. 2013PS66K). The Ethics Committee waived the requirement for patient consent, as the patient information was withheld.

Patients and tissue samples. A total of 140 paraffin-embedded ovarian tissue samples were obtained from surgical procedures performed between 2000 and 2012 in the Department of Obstetrics and Gynecology of China Medical University Shengjing Hospital. All tissue sections were diagnosed by two specialists independently. There were 60 cases of primary EOC, including 30 serous, 22 mucinous, three endometrioid and five clear-cell carcinoma; in addition to 30 ovarian borderline tumors, 30 ovarian benign tumors and 20 normal ovarian tissues (from normal ovarian specimens resected following cervical carcinoma surgery). The average age of the patients was 46.97 (16-81) years. The average age of the malignant group was 50.62 (16-73) years with a median age of 53 years. The average age of the borderline group was 39.41 (22-77) years with a median age of 36 years. The average age of the benign group was 46.00 (22-81) years with a median age of 44 years. The average age of the normal group was 48.71 (37-59) years with a median age of 50 years. There were no statistically significant differences between the groups (Table I; $P>0.05$). According to the pathological grading, there were 21 well-differentiated, 21 moderately differentiated and 18 poorly differentiated cases. The group included 39 patients with stage I-II disease and 21 with stage III-IV disease, according to the International Federation of Gynecology and Obstetrics staging system for ovarian cancer (19); 12 patients had lymph node metastases. All cases were primary tumors with complete clinical pathological data and without chemotherapy prior to surgery.

Immunohistochemical staining and quantification. All ovarian tissue samples were obtained as successive 5-μm-thick sections. The expression of Lewis y and CD147 in ovarian carcinoma tissues was analyzed by immunohistochemical streptavidin-biotin-peroxidase (SP) staining. Positive and negative immunohistochemistry controls were routinely used. Primary antibodies against Lewis y and CD147 (both from Abcam, Cambridge, UK; cat. no. F3, ab3359; cat. no. ab666) were used at a dilution of 1:100. Staining was performed according to the instructions of the SP kit (Boshide Biotech Co., Ltd. Wuhan, China). The samples were considered positive if there were buffy granules in the cell membrane and cytoplasm. Immunohistochemical signals were calculated by quantifying positively stained cells under a light microscope (Olympus Corporation, Tokyo, Japan). According to the chromatosis intensity, no pigmentation, light yellow, buffy and brown were scored as 0, 1, 2 and 3, respectively. The number of cells with chromatosis was scored in five high-power fields from each section as follows: <5% = 0, 5-25% = 1, 26-50% = 2, 51-75% = 3, and >75% = 4. The number of cells with chromatosis was multiplied by the intensity to yield the following scores: 0-2 = (-), 3-4 = (+), 5-8 = (++) and 9-12 = (+++). Two observers examined the sections independently.

Cell line and cell culture. The RMG-I-hFUT cell line, which is characterized by high expression of the FUT1 gene and Lewis y antigen, was established by transfecting the pcDNA3.1(-)-HFUT-H expression vector (containing the FUT1 gene) into RMG-I cells (a human ovarian clear cell carcinoma cell line, donated by Professor Iwamori Masao of Tokyo University (Tokyo, Japan) (7). The RMG-I and RMG-I-hFUT cells were cultured in DMEM (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% FBS (HyClone; GE Healthcare Life Sciences) at 37˚C in 5% CO_2 and saturated humidity.

Cells in the exponential growth phase were used in the subsequent experiments. A total of $1x10^5$ cells in 1 ml were inoculated into a 6-well plate in serum-free medium. For the inhibition assay, the final concentration of Lewis y antibody was 20 μg/ml, the duration of treatment was 1 h at 37˚C in 5% CO_2. In the case of CD147 antibody treatment, the CD147 antibody was added to the culture medium at 10 μg/ml and the cells were incubated for 1 h at 37˚C in 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RMG-I and RMG-I-hFUT cells at an exponential phase of growth were treated with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA; 1 ml per $1x10^7$ cells) to extract total RNA. Complementary DNA (cDNA) was synthesized according to the manufacturer's protocol of the RNA reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: 37˚C for 15 min, 85˚C for 5 sec, 4˚C for 5 min. The primers used were as follows: CD147, forward 5'-GACTGGGTACAAGATCAC-3' and reverse 5'-GCCTCCATGTTCAGGTTCTCAA-3'; FUT1, forward 5'-AGGTCATCCCTGAGCTGAAACGG-3' and reverse 5'-CGCCTGCTTCACCACCTTCTTG-3'. The real-time PCR reaction conditions were as follows: Denature at 95˚C for 30 sec, 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec in a 20-μl reaction mixture containing 10 μl SYBR® Premix Ex Taq™ (2X), 0.4 μl PCR forward primer (10 μmol/l), 0.4 μl PCR reverse primer (10 μmol/l), 2 μl cDNA and 7.2 μl dH_2O. The Light Cycler PCR system (Roche Diagnostics, Mannheim, Germany) was used for real-time PCR amplification and Cq value detection. The melting curves were analyzed

Table I. Ovarian tissue patient features.

Feature	Overall	Malignant	Borderline	Benign	Normal
Cases (n)	140	60	30	30	20
Age, years (mean ± SD)[a]	46.97±10.2	50.62±13.7	39.41±8.6	46.00±11.3	48.71±12.2
Age, years [median (range)]	51 (16-81)	53 (16-73)	36 (22-77)	44 (22-81)	50 (37-59)

[a]P>0.05 among groups, determined using one-way analysis of variance. SD, standard deviation.

following amplification. All PCR was performed in triplicate. The data were analyzed using the Cq method (20). The results were considered significant when at least a 2-fold difference in expression levels was detected.

Western blot analysis. The RMG-I-hFUT and RMG-I cells were washed twice with cold PBS, treated with cell lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 100 mM NaF, 200 μM Na_3VO_4 and 10 μg/ml each aprotinin, leupeptin, phenylmethanesulfonyl fluoride and pepstatin] and centrifuged at 14,000 x g for 15 min at 4˚C. The protein concentration in the supernatant was detected using the Coomassie brilliant blue method. The supernatant was treated with 1X SDS-PAGE loading buffer at 100˚C for 5 min for protein denaturation. Subsequently, 50 μg of each sample was separated by 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, blocked with 5% fat-free milk powder at room temperature for 2 h, and incubated with primary antibody in TBST/1% non-fat milk at 4˚C overnight, followed by incubation with the appropriate secondary HRP-labeled IgG at room temperature for 2 h and visualization using an ECL reagent. The experiment was repeated three times. The protein bands were visualized using the Molecular Imager system GDS8000b (UVP, Inc., Upland, CA, USA). Total protein levels were normalized to the expression of GAPDH on the same membrane, and the bands were quantified using ImageJ software v1.8.0 (National Institutes of Health, Bethesda, MD, USA).

The primary antibodies were as follows: Mouse anti-human CD147 monoclonal antibody (cat. no. ab666, 1:1,000) and rabbit anti-human MMP-2 monoclonal antibody (cat. no. ab92536, 1:1,000) from Abcam. Mouse anti-human GAPDH monoclonal antibody (cat. no. sc-47724, 1:2,000) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The secondary HRP-labeled antibodies (goat anti-mouse IgG-HRP, cat. no. sc-2005, 1:2,000; goat anti-rabbit IgG-HRP, cat. no. sc-2004, 1:2,000) were from Santa Cruz Biotechnology, Inc.

Co-immunoprecipitation assay. The protein was extracted from the cells prior to and following transfection. Following protein quantification, 6,000 μg of each lysate was added to 1 μg of CD147 monoclonal antibody and agitated at 4˚C overnight, followed by the addition of 40 μl Protein A+G-agarose and agitation at 4˚C for 2 h. The samples were then centrifuged at 2,500 g for 5 min at 4˚C and washed three times with lysis buffer as described above to collect the precipitate. The precipitated protein was mixed with 60 μl of 2X SDS-PAGE loading buffer at 100˚C for 5 min for denaturation. The supernatant (20 μl) was then subjected to SDS-PAGE. The Lewis y (cat. no. F3, ab3359, 1:500; Abcam)/Lewis x (cat. no. ab20137, 1:500; Abcam)/sLewis x (cat. no. sc-32243, 1:500; Santa Cruz Biotechnology, Inc.) antibodies were used to detect the antigens. The remaining steps were the same as described for the western blot analysis above. The protein for cellular location was extracted from the cells prior to and following transfection according to the Membrane Protein Extraction kit's instructions. The other steps were the same as described above. Mouse anti-human CD147 monoclonal antibody (cat. no. ab666, 1:200; Abcam) was used to detect the antigen. The densitometry of the protein bands was performed using ImageJ software v1.8.0 (National Institutes of Health).

Confocal laser scanning microscopy. In brief, mouse anti-human Lewis y antibody (cat. no. F3, ab3359; Abcam) and rabbit anti-human CD147 antibody (cat. no. ab188190; Abcam) were diluted to 1:100 as primary antibody solutions; goat anti-rabbit tetramethylrhodamine red fluorescence-labeled secondary antibody (cat. no. sc-2492; Santa Cruz Biotechnology, Inc.) and goat anti-mouse fluorescein isothiocyanate green fluorescence-labeled secondary antibody (cat. no. sc-2859; Santa Cruz Biotechnology, Inc.) were diluted to 1:200. The cells were blocked using normal goat serum for 30 min, treated with primary antibody solutions at 37˚C for 1 h, and cultured at room temperature overnight. Following washing with PBS, the cells were incubated with secondary antibody solution at 37˚C for 1 h, stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 min, and then observed under a confocal laser scanning microscope (C1-SI; Nikon Corporation, Tokyo, Japan). The data were collected by a computer for digital imaging. For the negative controls, PBS replaced the primary antibodies.

Cell adhesion assay. The 96-well plates were coated with 60 μg/ml collagen IV or 12 μg/ml laminin (50 μl/well). The plates coated with 3 mg/ml polylysine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1% BSA (Sigma-Aldrich; Merck KGaA) were used as maximal and minimal adhesion controls, respectively. Following incubation for 2 h at 37˚C, the plates were washed twice with PBS, and blocked again with 1% BSA for 2 h. The cells were digested with 0.25% trypsin, centrifuged at 1,000 x g for 5 min at room temperature, and mixed with serum-free DMEM culture medium to prepare single-cell suspensions. The cells were diluted to $5x10^4$/ml, added to coated plates (100 μl/well) and cultured at 37˚C in 5% CO_2 for 2 h. Following washing to remove non-adherent cells,

the plates were fixed with 4% paraformaldehyde for 30 min, stained with 0.5% crystal violet (100 μl/well) for 2 h, and then washed twice with cold PBS. The absorbance at 597 nm (A_{597} absorbance represents the adhesive cells) was detected using a microplate reader. The experiment was repeated three times.

Statistical analysis. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Quantitative data are presented as the mean ± standard deviation, and qPCR data are expressed as the mean ± standard error of the mean. Positive ratios were evaluated using the χ^2 test. Student's t-test was used for comparisons between two groups and one-way analysis of variance with the LSD or Bonferroni post hoc test was used for comparisons between more than two groups. The correlation between Lewis y antigen and CD147 in ovarian cancer was examined using a χ^2 test. Survival was analyzed using Kaplan-Meier curves, and significant differences among clinicopathological variants and immunomarkers were tested using the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Lewis y antigen and CD147 in the groups of ovarian tissues. Lewis y antigen was upregulated in the 60 EOC samples analyzed, the expression of which was high in the membrane and occasional in the cytoplasm. The positive expression rate was 88.33%, which was higher than that of the borderline group (60.00%; P<0.05) and the benign group (33.33%; P<0.01); the expression rate of Lewis y in the borderline group was higher than that of the benign group, however, the difference did not reach statistical significance (P>0.05). The expression of Lewis y antigen was negative in the 20 normal ovarian tissues (Fig. 1A-D; Table II).

The expression pattern of CD147 was similar to that of Lewis y antigen, with high expression in the cell membrane and occasional expression in the cytoplasm. The positive expression rates were 80.00, 50.00, 23.30 and 5.00% in the malignant, borderline, benign and normal groups, respectively. The highest positive rate was that of the malignant group, which was higher than the rates of the borderline, benign and normal ovarian groups (P<0.05); the positive expression rate of the borderline group was higher than that of the benign and normal groups (P<0.05); there was no significant difference in the positive expression rate between the benign and normal groups (P>0.05) (Fig. 1E-H; Table II).

Correlation between the expression of Lewis y antigen and CD147 and the clinicopathological parameters of ovarian cancer. The positive expression rate of the Lewis y antigen was 90.00% in ovarian serous cystadenocarcinoma and 81.82% in ovarian mucinous cystadenocarcinoma, with no significant difference between the two (P>0.05). In endometrioid and clear cell carcinomas, high expression rates of Lewis y were observed. Positive expression of Lewis y was present in 95.24% of patients with stages III-IV ovarian cancer, and was higher than that of patients with stage I-II ovarian cancer (84.62%), however, this difference was statistically significant (P>0.05). The positive expression rates of

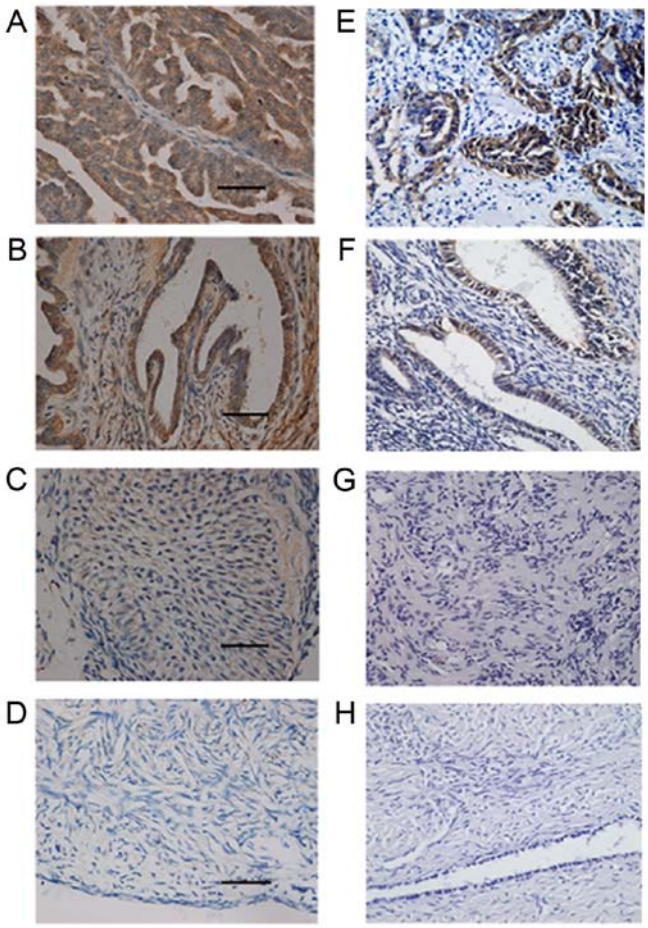

Figure 1. Immunohistochemical staining. Immunohistochemical staining of Lewis y in (A) ovarian malignant tumor, (B) borderline tumor, (C) benign tumor and (D) normal ovarian tissue. CD147 in (E) ovarian malignant tumor, (F) borderline tumor, (G) benign tumor and (H) normal ovarian tissue. Original magnification, x400.

Lewis y antigen in the well-, moderate, and poorly differentiated groups were 80.95, 85.71 and 100%, respectively. The degree of differentiation was inversely correlated with the positive expression rate, although the differences between the groups were not statistically significant (P>0.05). The positive rate of Lewis y in the lymphatic node metastasis group (100.0%) was higher than that in the non-metastasis group (85.43%), although the difference was not significant (P>0.05) (Table III).

The positive expression rates of CD147 in ovarian serous cystadenocarcinoma and ovarian mucinous cystadenocarcinoma were 86.67 and 73.33%, respectively, which were not significantly different (P>0.05). CD147 was detected in 20 cases of stage III-IV EOC (95.24%), and its expression was significantly higher than that of stage I-II EOC (71.79%) (P<0.05). The positive expression rates of CD147 in the well-, moderate, and poorly differentiated groups were 71.43, 76.19 and 94.24, respectively. The degree of differentiation was inversely correlated with the positive expression rate of CD147, although the difference was not statistically significant (P>0.05). The positive rate of CD147 in the lymphatic node metastasis group (100.0%) was higher than that in the non-metastasis group (75.00%) and this difference was statistically significant (P<0.05) (Table III).

Table II. Expression of Lewis y and CD147 in different ovarian tissues.

		Lewis y antigen					CD147				
Group	Cases (n)	-	+	++	+++	Positive (%)	-	+	++	+++	Positive (%)
Malignant	60	7	15	20	18	53 (88.33)[a]	12	15	17	16	48 (80.00)[a]
Borderline	30	12	6	11	1	18 (60.00)[b]	15	6	7	2	15 (50.00)[b]
Benign	30	20	6	4	0	10 (33.33)	23	4	3	0	7 (23.33)
Normal	20	20	0	0	0	0 (0)	19	1	0	0	1 (5.00)

[a]P<0.05 and P<0.01 compared with the borderline and benign group, respectively; [b]P<0.05 compared with the benign group. P-values were determined using the χ^2 test.

Table III. Association between Lewis y and CD147 expression and pathological features.

		Lewis y antigen			CD147		
Feature	Cases (n)	Positive cases (n)	Rate (%)	P-value	Positive cases (n)	Rate (%)	P-value
Pathological type							
Serous	30	27	90.00	>0.05	26	86.67	>0.05
Mucous	22	18	81.82		14	63.64	
Endometrioid	3	3	100.00		3	100.00	
Clear cell	5	5	100.00		5	100.00	
FIGO stage							
I-II	39	33	84.62	>0.05	28	71.79	<0.05[a]
III-IV	21	20	95.24		20	95.24	
Differentiation level							
Well	21	17	80.95	>0.05	15	71.43	>0.05
Moderate	21	18	85.71		16	76.19	
Poor	18	18	100.00		17	94.44	
Lymphatic metastasis							
No	48	41	85.42	>0.05	36	75.00	<0.05[a]
Yes	12	12	100.00		12	100.00	

[a]Statistically significant differences. P-values were determined using the χ^2 test. FIGO, International Federation of Gynecology and Obstetrics.

Relevance of the expression of Lewis y and CD147 in ovarian cancer. Of the 60 ovarian cancer tissues samples, 46 were positive for the expression of both Lewis y and CD147 and five were negative for both. A positive, significant correlation between Lewis y and CD147 was observed in ovarian cancer (χ^2=9.71, P<0.01; Table IV).

Table IV. Relevance of the expression of Lewis y and CD147 in ovarian cancer.

	CD147		
Lewis y	Positive (n)	Negative (n)	Total (n)
Positive	46	7	53
Negative	2	5	7
Total	48	12	60

χ^2 test, χ^2=9.71, P<0.01.

Survival analysis. In the 60 patients with EOC, four were lost to follow-up, and the remaining 56 patients were regularly followed up to April 2017, with a follow-up time of 12-86 months, and 27 cases of mortality. Kaplan-Meier analysis of the patient survival rates showed that the survival rate of patients with a high expression of CD147 was lower than that of patients with a low expression of CD147 (log-rank P=0.005, Fig. 2A). Similarly, the survival rate of patients with a higher expression of Lewis y antigen was lower than of patients with lower expression (log-rank P=0.005, Fig. 2B). The mortality rate of patients with pathological stages III-IV (Fig. 2C) and lymph node metastasis (Fig. 2D) was significantly higher than that of patients with pathological stages I-II and without lymph node metastasis (P=0.00023 and 0.00036, respectively).

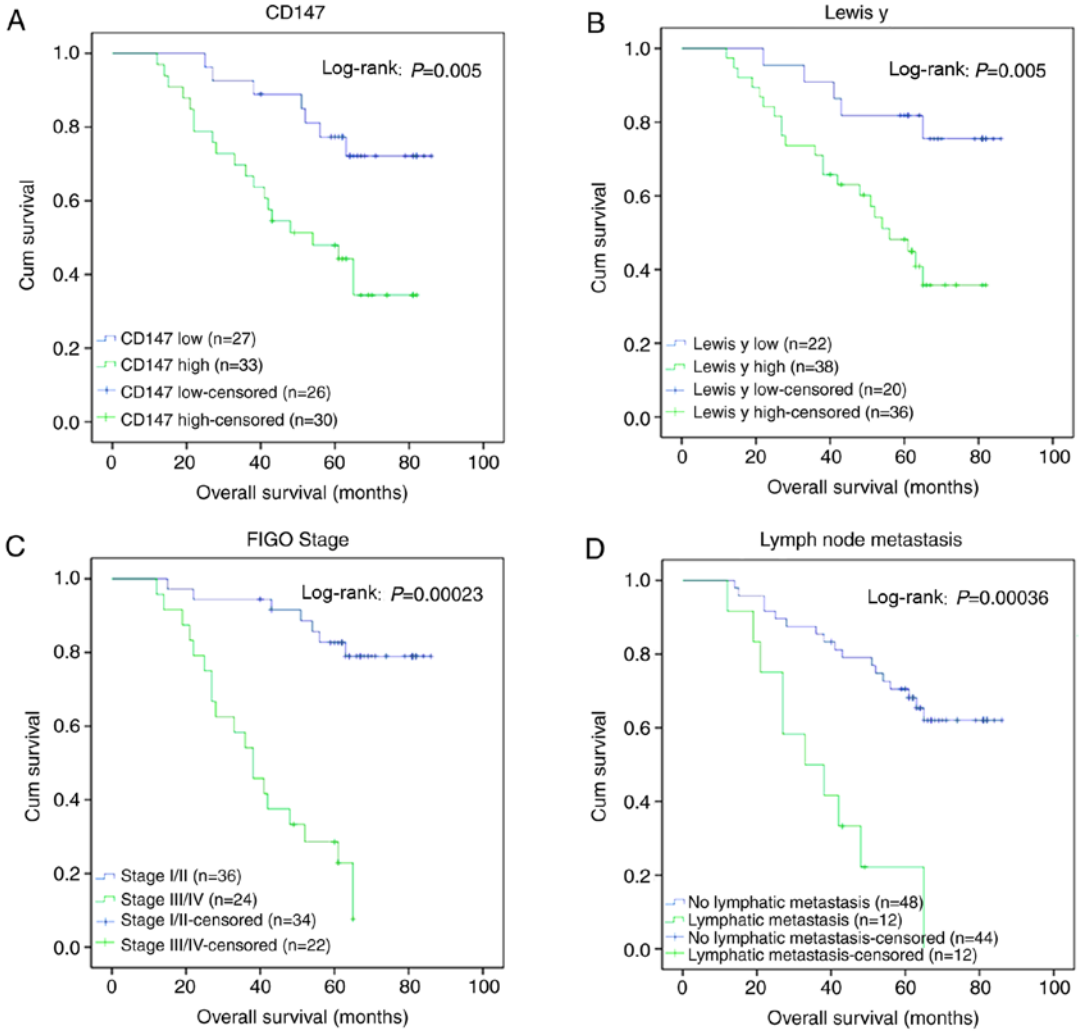

Figure 2. Association between CD147 and Lewis y antigen expression and survival rates of patients with ovarian cancer. Kaplan-Meier survival analysis revealed that a (A) high expression of CD147 and (B) Lewis y, (C) late surgical stage and (D) lymph node metastasis were independent risk factors for overall survival. log-rank P=0.005, 0.005, 0.00023 and 0.00036, respectively. FIGO, International Federation of Gynecology and Obstetrics.

Expression of Lewis y antigen and CD147 in ovarian cancer cells. The RT-qPCR results are shown in Fig. 3A. The mRNA expression of CD147 was lower in the RMG-I-hFUT cells than in the RMG-I cells, although the difference was not significant (P>0.05). The mRNA expression of FUT1 was 3.07-fold higher in the RMG-I-hFUT cells than in the RMG-I cells (P<0.05).

The immunocytochemical staining revealed that positive CD147 staining was predominantly located in the cell membrane of the RMG-I cells, where it was detected as light-yellow granules. The average optical density was 0.107±0.001. Positive CD147 staining in the RMG-I-hFUT cells was widely located in the membrane and cytoplasm, and was detected as brown granules. The average optical density was 0.287±0.002, which was significantly higher than that of the RMG-I cells (P<0.05; Fig. 3B). The expression pattern was comparable between Lewis y and CD147, with expression mainly in the cell membrane and occasionally in the cytoplasm. The expression of Lewis y antigen was significantly higher in the RMG-I-hFUT cells than in the RMG-I cells (P<0.05; Fig. 3B).

The expression of CD147 determined by western blotting was similar to that detected by immunocytochemical staining. The expression of CD147 was 2.43-fold higher in the RMG-I-hFUT cell line than in the RMG-I cells (P<0.05; Fig. 3C).

Co-expression of Lewis y antigen and CD147 in ovarian cancer cells. Co-expression of Lewis y antigen and CD147 in the RMG-I cell line was detected using immunoprecipitation. The Lewis y antigen was predominantly expressed in the highly glycosylated form of CD147 (40-60 kDa, Fig. 4A). An unidentified 26-kDa form of CD147 was also found containing the Lewis y antigen structure (Fig. 4A). No expression of CD147 or Lewis y antigen was present in the negative control (Fig. 4A).

The expression level of CD147 was higher following transfection than prior to transfection. The unidentified 26-kDa band detected in all samples showed changes in expression in accordance with the highly glycosylated form of CD147 (40-60 kDa; P<0.05, Fig. 4B). The lower glycosylated form of CD147 (36 kDa) was weakly expressed in the total protein lysates and in the CD147 immunoprecipitation samples (Fig. 4B, lanes 5-8), whereas it was undetected in the protein membrane and cytoplasmic immunoprecipitation samples and

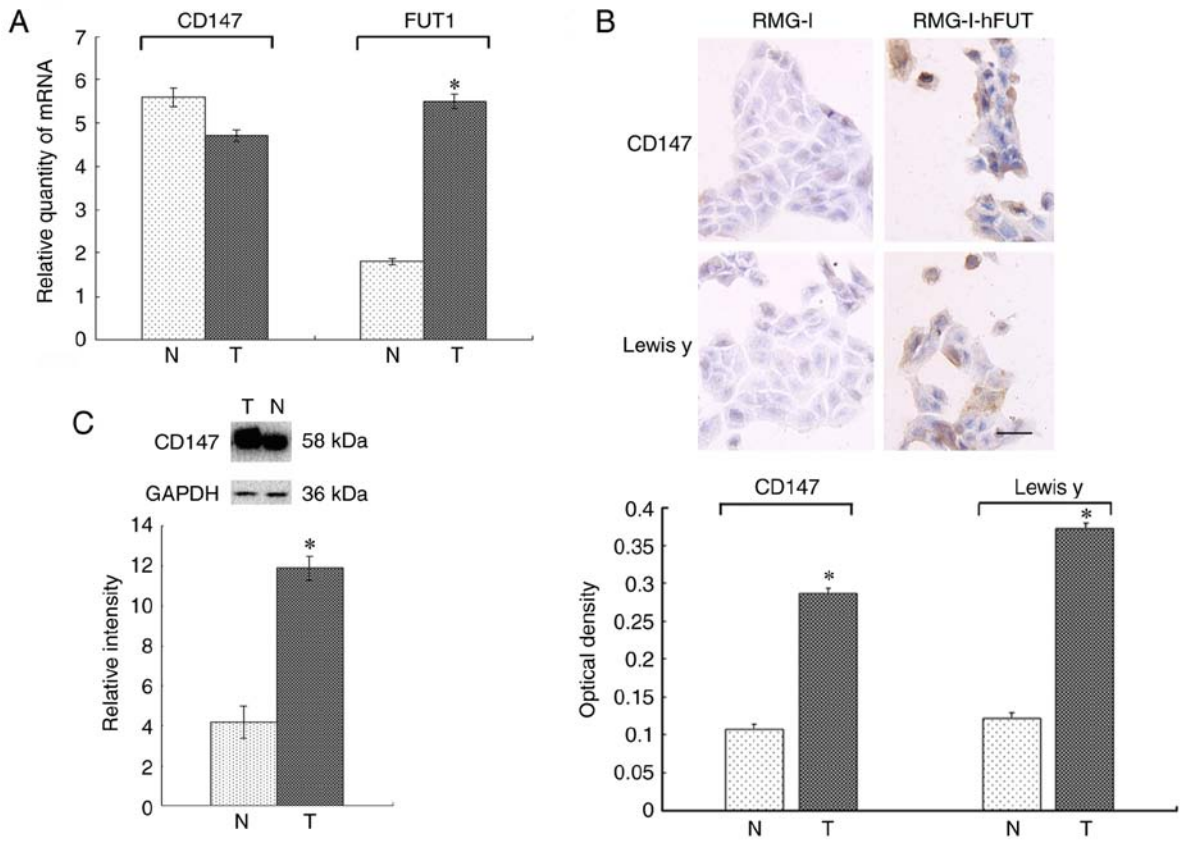

Figure 3. Expression of Lewis y antigen and CD147 in RMG-I and RMG-I-hFUT ovarian cancer cells. (A) Reverse transcription-quantitative polymerase chain reaction detection of the mRNA expression of CD147 and FUT1 in the two cell lines. (B) Immunocytochemical staining detection of the expression of Lewis y antigen and CD147 in the two cell lines. Original magnification, x400. (C) Western blot detection of the expression of CD147 in the two cell lines. *P<0.05 compared with RMG-I. FUT1, α1,2-fucosyltransferase; N, RMG-I cells; T, RMG-I-hFUT cells.

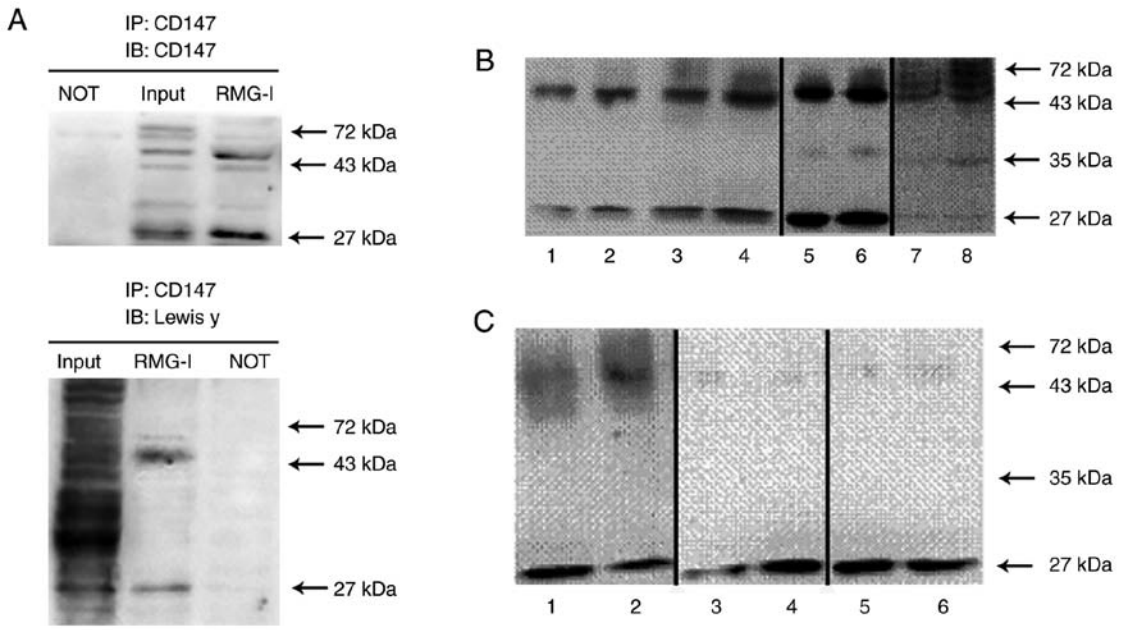

Figure 4. Co-expression of Lewis y antigen and CD147 in ovarian cancer cells. Bands 1, 3, 5 and 7, RMG-I cells; 2, 4, 6, and 8, RMG-I-hFUT cells. (A) Cell lysates from RMG-I cells were immunoprecipitated with anti-CD147 antibody, then immunoblotted with anti-CD147 and anti-Lewis y antibodies. (B) Bands 1-6, CD147 levels in precipitation samples following the addition of 1 μg anti-CD147 antibody to 1,000 μg of protein. Bands 1 and 2, CD147 levels in equal cytoplasmic precipitation samples; bands 3 and 4, CD147 levels in equal membrane precipitation samples; bands 5 and 6, CD147 levels in total protein precipitation samples; bands 7 and 8, CD147 levels in 150 μg total protein precipitation samples. The samples of bands 1-4, 5/6, and 7/8 were fresh samples collected at different times, and the experiment was conducted immediately following sample collection. (C) Bands 1-6, levels of glycosylated CD147 following the addition of 1 μg CD147 antibody to 6,000 μg of protein. The samples of bands 1/2, 3/4, and 5/6 were transferred onto polyvinylidene difluoride membranes and the membranes were incubated with different primary antibodies. Bands 1 and 2, Lewis y antigen level; bands 3 and 4, Lewis x antigen level; bands 5 and 6, sLewis x antigen level. NOT, anti-IgG antibody (negative control).

Table V. Determination of the adhesive abilities, represented by the absorbance at 597 nm, of the RMG-I and RMG-I-hFUT cell lines on collagen IV and laminin.

	Adhesive ability (collagen IV)		Adhesive ability (laminin)	
Group	RMG-I	RMG-I-hFUT	RMG-I	RMG-I-hFUT
Negative control	1.198±0.090	2.191±0.042[a]	1.582±0.142	2.403±0.047[a]
Lewis y antibody	0.550±0.011[b]	0.595±0.023[b]	0.573±0.009[b]	0.594±0.036[b]
CD147 antibody	0.667±0.050[b]	0.689±0.040[b]	0.877±0.026[b]	0.926±0.034[b]
IgM control	1.549±0.113	2.068±0.076[a]	1.416±0.082	2.259±0.151[a]

[a]P<0.05 compared with RMG-I; [b]P<0.05 compared with untreated cells. P-values were determined using the independent t-test.

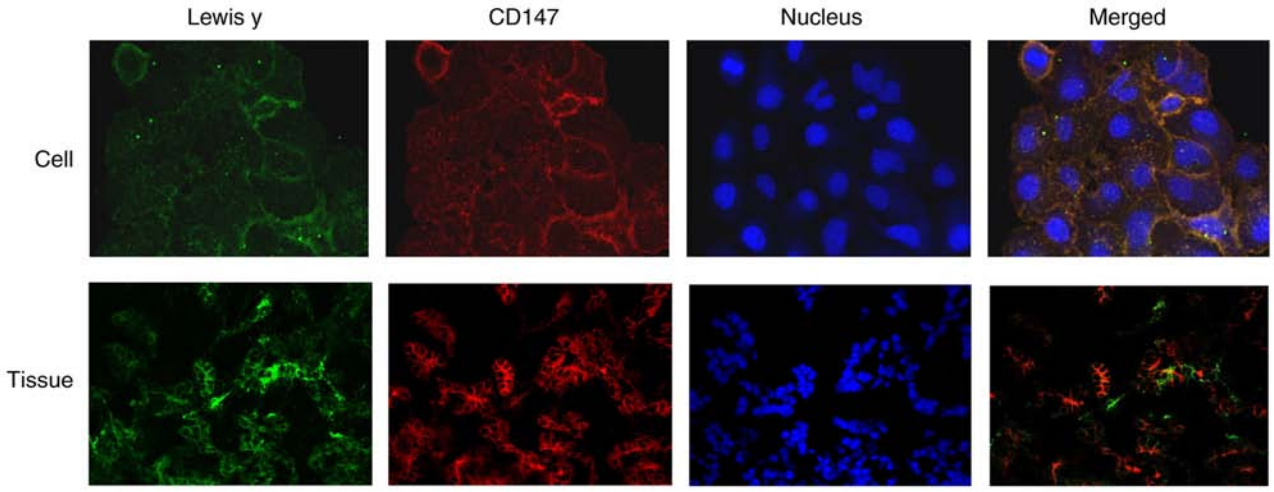

Figure 5. Immunofluorescence imaging. Double-labeling immunofluorescence showed the colocalization of CD147 and Lewis y antigen in the RMG-I-hFUT cell line and ovarian malignant tumor (original magnification, x400).

in the Lewis y antigen immunoprecipitation samples (Fig. 4B, lanes 1-4). Cellular colocalization experiments showed that the highly glycosylated form and the 26-kDa form of CD147 were expressed in the cell membrane and cytoplasm in the RMG-I and RMG-I-hFUT cell lines. In addition, the expression of CD147 was higher in the membrane and cytoplasm of the RMG-I-hFUT cells than in the RMG-I cells (P<0.05; Fig. 4B, lanes 1-4).

The expression levels of the glycosylated antigen CD147 in cells prior to and following transfection were examined by immunoprecipitation. The results showed that Lewis y antigen was predominantly expressed in the highly glycosylated form of CD147 and its expression was higher following cell transfection than prior to transfection (P<0.05; Fig. 4C, lanes 1 and 2). Under the same conditions, Lewis x and sialyl Lewis x showed weak expression in the highly glycosylated form of CD147 (Fig. 4C, lanes 3-6). The Lewis y, Lewis x, and sialyl Lewis x antigens were expressed at high levels in the 26 kDa form of CD147. The expression of Lewis y antigen in the 26 kDa CD147 was lower in the RMG-I-hFUT cells than in the RMG-I cells (P<0.05, Fig. 4C, lanes 1 and 2). Both sialyl Lewis x and Lewis y showed higher expression levels in the 26 kDa form of CD147 in cells prior to transfection, whereas Lewis x showed higher expression levels in cells following transfection (Fig. 4C, lanes 3-6).

In the double fluorescence confocal experiment, Lewis y (green) and CD147 (red) were predominantly located in the cell membrane and partly in the cytoplasm; the green and red fluorescent signals were higher at the edge of the cells. As shown in Fig. 5, most of the green and red fluorescence overlapped, as shown by the yellow fluorescence, indicating the colocalization of CD147 and Lewis y.

Immunofluorescence double labeling showed the CD147 antigen as red fluorescence in EOC tissues, and the fluorescence was primarily detected in the cell membrane. The green fluorescence corresponded to Lewis y, which was also primarily detected in the cell membrane, with occasional signal in the cytoplasm. The blue fluorescence indicated the nuclei stained by DAPI. Image analysis software was used to analyze the three fluorescence signals, and yellow fluorescence appeared in the position of the red and green signals, indicating the colocalization of Lewis y antigen and CD147 (Fig. 5).

Determination of the adhesive ability of the RMG-I and RMG-I-hFUT cell lines on collagen IV and laminin. To examine the adhesive ability of the RMG-I and RMG-I-hFUT cells, 96-well plates were coated with 60 μg/ml of collagen IV or 12 μg/ml of laminin. The adhesive values of the RMG-I-hFUT cell line on collagen IV and on laminin were

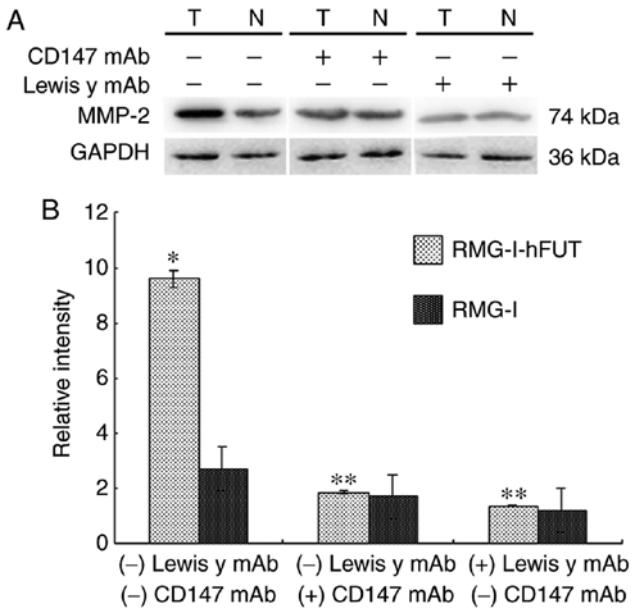

Figure 6. Effect of FUT1 transfection on the expression of MMP-2, and the effect of anti-Lewis y antibody and anti-CD147 antibody on the expression of MMP-2. Western blot detection of the expression of MMP-2 in RMG-I and RMG-I-hFUT cells, and in the absence and presence of anti-Lewis y antibody and anti-CD147 antibody, respectively. (A) Representative western blots of MMP-2 in the cell lines. (B) Densitometric quantification of the protein expression (n=3). For the inhibition assay, the final concentration of anti-Lewis y antibody was 20 μg/ml and the final concentration of anti-CD147 antibody was 10 μg/ml. The duration of treatment was 1 h. *P<0.05, vs. RMG-I; **P<0.05, vs. RMG-I or RMG-I-hFUT cells without anti-Lewis y antibody or anti-CD147 antibody treatment. FUT1, α1,2-fucosyltransferase; MMP-2, matrix metalloproteinase; mAb, monoclonal antibody; N, RMG-I cells; T, RMG-I-hFUT cells.

2.191±0.042 and 2.403±0.047, respectively. These values were significantly higher than those of the RMG-I cell line, which were 1.198±0.090 and 1.582±0.142, respectively (P<0.05; Table V). However, treatment with the anti-Lewis y monoclonal antibody significantly decreased the adhesive abilities of the RMG-I and RMG-I-hFUT cells to 46.0 and 27.2%, respectively, on collagen IV (P<0.05; Table V), and to 36.2 and 24.7%, respectively, on laminin (P<0.05; Table V). Treatment with anti-CD147 monoclonal antibody yielded similar results. The adhesive abilities of the RMG-I and RMG-I-hFUT cells on collagen IV decreased to 55.7 and 31.4%, respectively (P<0.05, Table V), and on laminin to 55.4 and 38.5%, respectively (P<0.05, Table V). Compared with the corresponding controls, there was no significant difference in the cell adhesive abilities prior to or following treatment (P>0.05, Table V).

Expression of MMP-2 in ovarian cancer cells. The extracellular matrix (ECM) is a major barrier to tumor metastasis. MMPs are important enzymes that degrade the ECM. MMP-2, which can hydrolyze the main component type IV collagen, serves an important role in the invasion and metastasis of malignant tumors. As shown in Fig. 6A and B, the expression of MMP-2 was upregulated by 3.64-fold over the untransfected value in the RMG-I-hFUT cells (P<0.01). To determine whether the upregulation of MMP-2 was associated with increased expression of the Lewis y antigen on the cell surfaces of CD147 and CD147, the cells were treated with anti-Lewis y antibody and anti-CD147 antibody, respectively. As shown in Fig. 6A and B, in the presence of the anti-Lewis y antibody and CD147 antibody, the expression of MMP-2 and the differences in expression intensities between the two cell lines were significantly decreased.

Discussion

The main metastatic pathway of ovarian cancer is intraperitoneal dissemination and adhesion. Invasion and metastasis of malignant carcinoma are complex processes with multiple elements and steps, including infiltration of the primary neoplasm, degradation of the basement membrane, invasion into the blood vessels of tumor cells and the invasion of tumor cells into target tissues (21). CD147 is not only a cell surface adhesion molecule that mediates cell adhesion, it is also an inducer or extracellular MMPs, which are important in tumor invasion and metastasis. Zhang *et al* (22) showed that CD147 can stimulate the expression of MMP in hepatocellular carcinoma cells, modulate the secretion of MMPs from surrounding fibroblasts, and promote the infiltration and metastasis of tumor cells. CD147 is upregulated in several types of tumor, including endometrial carcinoma, bone giant cell tumor and urinary tumors. The expression of CD147 in certain tumors increases in correlation with the malignancy of tumors, and CD147 is correlated with the infiltration and metastasis of tumors (23). Jin *et al* (24) reported that CD147 is upregulated in malignant ovarian carcinoma and is closely associated with stage and differentiation of serous cystadenocarcinoma. Sillanpää *et al* (25) showed that, in contrast to other types of ovarian carcinoma, serous cystadenocarcinoma exhibits a low expression of CD147 that is associated with tumor stage. In the present study, the expression of CD147 was positively correlated with the malignancy of tumors, and the positive expression rate of CD147 increased with clinical stage, although it was not associated with the histological type or degree of differentiation.

Carbohydrate chains in the cell membrane are an important medium of communication among cells and between cells and the external environment, and they are involved in cell signal transduction pathways. Fucose is the final form in the synthesis of carbohydrates, and following the glycosylation of fucose, carbohydrate chains usually stop synthesis. The Lewis y antigen is a difucosylated oligosaccharide and an important marker for in determining the diagnosis and prognosis of several types of cancers (26). Our previous and present studies have been committed to examination of the role of Lewis y antigen in the development of ovarian cancer and its mechanism of action. Our previous studies showed that Lewis y antigen, a tumor-associated antigen, promotes the proliferation, adhesion, invasion, metastasis and drug resistance in ovarian cancer cell lines (6-8). In the present study, the analysis of tissue specimens showed that Lewis y antigen was overexpressed in ovarian carcinoma, with a positive expression rate of 88.33%, which was higher than that in the borderline and benign groups (60.00 and 33.33%; P<0.05 and P<0.01, respectively). In addition, the expression of Lewis y was positively correlated with the grade of malignancy (P<0.05) and disease stage. The results were not only consistent with our previous results (27), but also further verified the original results on the

original basis. In addition to the recollection of samples, the follow-up time of the patients was extended. The present study focused on the association between the levels and structures of CD147 and Lewis y in ovarian tissues, determining whether CD147 has Lewis y glycosylation modification, and examining the role of co-expressed CD147 and Lewis y in the development of ovarian cancer.

CD147, which was originally cloned as a carrier of the Lewis x antigen (28), is involved in a series of biological processes as a main substrate of N-acetylglucosamine glycosyltransferase V (9). The present results showed that the expression of CD147 increased significantly in correlation with the upregulation of Lewis y antigen following transfection with FUT1 ($P<0.05$). The results of the immunofluorescence and immunoprecipitation assays demonstrated that Lewis y was one of the components of CD147. In ovarian cancer cell lines, Lewis y was predominantly detected in the highly glycosylated 26 kDa form of CD147. In addition, compared with the parental cell lines, Lewis y was significantly upregulated in the highly glycosylated form of CD147 in the RMG-I-hFUT transfected cell line, whereas it was significantly downregulated in the 26 kDa form ($P<0.05$). Under the same conditions, Lewis x and sLewis x antigens were expressed at low levels in the highly glycosylated form of CD147. This indicates that, in the highly glycosylated form of CD147, most of the Lewis x antigen was changed into Lewis y antigen under the catalytic action of FUT1. Compared with sialytransferase, FUT1 exhibited a higher catalytic ability toward the substrate Lewis x, resulting in the formation of the main product Lewis y and low levels of sLewis x. This suggested that FUT1 had modification priority towards the highly glycosylated form of CD147 but not the 26 kDa form, due to the enrichment of the lactosaminoglycan constituent of the highly glycosylated form (29).

An unidentified 26 kDa protein band was detected in the total protein lysate of the RMG-I and RMG-I-hFUT cell lines. This band was observed in the cell membrane and the cytoplasm. As the molecular weight of the CD147 core protein is 27 kDa, this unknown 26 kDa protein band may be a form of membrane shedding (30) or the subtype basigin-3 (31). Loss of solubility is an important process in the functional regulation of several membrane proteins. A 22 kDa form of CD147 detected in the HT1080 and A431 human cancer cell lines was suggested to be a form of membrane shedding. Highly glycosylated CD147, which has a molecular weight of 22 kDa (the molecular weight is ~10 kDa post-deglycosylation), can still induce the production of MMPs (30). Belton *et al* (31) suggested that the 25 kDa form of basigin-3 is a critical subtype of basigin. In human tumor cells, the expression level of basigin-3 is <3% of that of basigin-2, a main subtype of basigin. In immunoprecipitation samples, basigin-3 is mainly expressed in the cytoplasm. Following treatment with recombinant human basigin, basigin-3 is expressed in the cell membrane and cytoplasm (31). The results of the present study showed that, although the 26 kDa form of CD147 was expressed in the cell membrane and cytoplasm, it was weakly expressed in the total protein samples. The immunoprecipitation assays showed that this protein was highly glycosylated and expressed the Lewis y, Lewis x and sLewis x antigens. As previously reported, CD147 is differentially expressed in different tissues and cell types (15). The membrane shedding form may be a soluble form of basigin-3, which requires further investigation.

The present cellular colocalization assay showed that both the highly glycosylated form and the 26 kDa form of CD147 were expressed in the cell membrane and in the cytoplasm. In addition, the two forms of CD147 were expressed at higher levels in the RMG-I-hFUT cells than in the RMG-I cells ($P<0.05$). Taylor *et al* (32) demonstrated that the release of a small proportion of activated CD147 from the cell surface of breast cancer into the culture medium was not associated with proteinase shearing action. As with cellular CD147, soluble CD147 maintained the original C- and N- termini.

A previous study suggested that soluble CD147 is released as a mechanism of cystic shedding (33). The tumor promoter phorbol 12-myristate 13-acetate, which activates the protein kinase C/Ca^{2+} and ERK1/2 signaling pathways, can significantly induce the expression of soluble CD147, suggesting that the cystic release of CD147 is controlled by cellular signal transduction (33). Our previous study also demonstrated that the Lewis y antigen can induce the phosphorylation of ERK, resulting in the malignant progression of ovarian cancer (34). In tumor cells, the production of CD147 is a positive feedback cascade response. Soluble CD147 may have other biological functions, as it is important in the production of distant fibroblasts and endothelial cells (35).

The results of the present study showed that the mRNA expression of CD147 was marginally decreased ($P>0.05$) in the transfected cells, whereas the protein expression of CD147 was significantly upregulated ($P<0.05$). This suggests that the changes in the expression levels of CD147 may be due to protein N-glycosylation rather than regulation at the transcriptional level. This may be associated with glycosylation-mediated changes in the function of relevant transport proteins (36) and the ubiquitin proteasome-induced inhibition of protein degradation (37).

The role of FUT1 glycosylation in the sugar chain of CD147 remains to be elucidated. Lewis y antigen was expressed at high levels in the highly glycosylated form of CD147, which can induce the expression of MMP (15). This suggests that Lewis y regulates the expression of CD147. The present study demonstrated that the expression of MMP-2 was significantly higher in the RMG-I-hFUT cell line than in the RMG-I cells, whereas its expression decreased significantly following treatment with Lewis y antibodies and CD147 antibodies.

Previous studies have shown that CD147 promotes tumor invasion by inducing tumor cell adhesion and spreading in integrin-dependent or anchorage-independent growth (38,39). In our previous study, it was demonstrated that Lewis y, as an important component of integrin $\alpha5\beta1$ and CD44, is involved in the process of cell spreading and promotes the adhesion and spread of transfected cells on fibronectin and hyaluronic acid (8,40). It has been suggested that the CD147 molecular chaperone MCT4 and integrin $\beta1$ can interact with each other and contribute to tumor metastasis. In addition, CD147, MCT4 and integrin $\beta1$ can regulate cell adhesion and migration by forming a supramolecular complex (41). The basement membrane serves important roles in tumor progression. Its main components, collagen IV and laminin, have been used as

substrates in cell adhesion experiments. The adhesive ability of FUT1-transfected cells on collagen IV or laminin improved significantly ($P<0.05$) and was markedly inhibited by anti-Lewis y and CD147 antibodies ($P<0.05$). In addition, the suppressive effect of anti-Lewis y was more marked than that of anti-CD147. This suggests that the effect of Lewis y antigen on upregulating the expression of CD147 is accompanied by the upregulation of relevant adhesive molecules, including integrins, which are involved in the regulation of cell adhesion.

In conclusion, the Lewis y antigen and CD147 were significantly upregulated in ovarian tumors, suggesting that they promote the development of each other. Lewis y antigen is an important component of the highly glycosylated CD147 molecule and can therefore induce the expression of CD147 and CD147-mediated MMP-2 in the RMG-I ovarian cancer cell line, resulting in increased tumor adhesion and metastasis. The overexpression of Lewis y antigen on the surface of ovarian cancer cells is a potential therapeutic target for the treatment of ovarian tumors.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JL and BL contributed to conception and design of the study. JL, QL and YW contributed to the acquisition, analysis and interpretation of the data, and were major contributors in writing the manuscript. QL, ML and YQ contributed to the acquisition of the data. QL and JG collected the clinical specimens. JL, YW and BL contributed to the revision of the manuscript. QL, ML, YQ and JG contributed to analysis and interpretation of the data and to revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Samples were fully encoded to protect patient confidentially. The study and its protocols were approved by the Research Ethics Committees of Shengjing Hospital Affiliated with China Medical University (no. 2013PS66K). The Ethics Committee waived the requirement for patient consent, as the patient information was withheld.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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