IVYSPRING INTERNATIONAL PUBLISHER

Journal of Cancer

2017; 8(5): 786-792. doi: 10.7150/jca.17712

Research Paper

Hedgehog signaling pathway regulates ovarian cancer invasion and migration via adhesion molecule CD24

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Received: 2016.09.26; Accepted: 2016.11.27; Published: 2017.02.25

Abstract

Hedgehog (Hh) signalling plays an important role in cancer; however, its mechanism in ovarian cancer migration and invasion remains unclear. In the present study, we aimed to clarify the effect of the Hh signalling pathway on ovarian cancer migration and invasion through the regulation of CD24 expression, both *in vitro* and *in vivo*. Patients with ovarian cancer (n = 97) were recruited for this study. Evaluation of the explored the role parameters of patients indicated that CD24 expression was negatively associated with age, histological type and lymph node metastasis ($p>0.05$), but was positively associated with the clinical stage and pathological grading ($p<0.05$).The *in vitro* results indicated that the activator (sonic hedgehog, Shh) and inhibitor (GANT61) of Hh signalling significantly enhanced and reduced CD24 expression, respectively, at both the gene and protein levels ($p<0.05$).The addition of Shh significantly enhanced cellular migration and invasion of SKOV3 cells *in vitro* ($p<0.05$) Down regulation of CD24 using siRNA inhibited the tumour-promoting effects of Shh, and the *in vivo* results confirmed that GANT61 significantly inhibited CD24 expression and reduced tumour growth ($p<0.01$). In conclusion, the expression of CD24 can be regulated by Hh signalling, and downregulation of CD24 could play an important role in inhibiting ovarian cancer progression.

Key words: Hedgehog signalling; CD24; Ovarian cancer; Cellular migration; Cellular invasion.

Introduction

Ovarian cancer is a common cause of morbidity and mortality in women, estimated to affect 7.91 per 100,000 women, with an age-adjusted rate of 5.35 per 100,000 women in China between 1999 and 2010[1]. Several factors play an important role in the development of ovarian cancer, such as increasing age, nulliparity, early menarche or late menopause, and BRCA1 or BRCA2 mutations. Due to the location of tumours in the pelvis, the malignancies are often large and advanced at the time of diagnosis[2]. Almost75% of women with ovarian cancer are diagnosed at stage III or IV, which have 10-year survival rates of 21% and less than5%, respectively[3].

Despite the prevalence of ovarian cancer, its molecular etiology remains mostly unknown. Therefore, it is of great importance to clarify the association of key proteins with ovarian cancer migration and invasion[4]. In a previous study, we used a cDNA microarray to identify unique downstream targets of the glioma-associated oncogene (GLI) gene, including the adhesion molecule CD24, known to be associated with carcinoma progression [5]. Hedgehog (Hh) signalling has been reported to be reactivated in metastases of ovarian, prostatic, gastric, esophageal and pancreatic carcinomas in a ligand-dependent or -independent manner[6, 7]. Transcription factors of the Gli family (Gli1, Gli2 and Gli3) can regulate Hh target genes by

binding to specific elements in their promoter sequences, promoting cellular proliferation, cellular survival, stemness and cell fate determination in a variety of organs [8, 9].

In the present study, we firstly investigated the relationship of CD24 expression with clinic pathological features and prognosis in human ovarian cancer. We then explored the role of CD24 in ovarian cancer cell migration and invasion via upregulating or downregulating its expression, both *in vitro* and *in vivo*.

Methods

Patients and tissue samples

Patient samples were obtained after written informed consent had been obtained, in accordance with the ethics committee of the participating institutes and the Declaration of Helsinki. This study was approved by the Institutional Review Board of the Second Affiliated Hospital of Nanchang University.

Tissue samples were collected from 97 patients with ovarian epithelial cell carcinoma who underwent surgical resection at the Second Affiliated Hospital of Nanchang University between 2007 and 2010 (age range 18–55 years). All patients were histopathological diagnosed based on clinical protocols, and none had received pre-surgery chemotherapy or immunotherapy.

All stained sections were independently evaluated and scored by two pathologists with no prior knowledge of the clinic pathological outcome of the patients. The mean percentage of positive cells were scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%). The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). The final histological (h) scores were obtained for each patient by multiplying the score for the percentage of positive cells and the intensity score. Protein expression levels were further analysed by classifying the h values as negative (-) for scores of 0–3, low positive (+) for scores 4–6, medium positive(++)for scores 7–9, or strongly positive (+++) for scores >10 [10].

Histopathological analysis

The excised samples were opened along the lesser curvature, and the longitudinal section was fixed in a 10%neutral buffered formalin solution, then embedded in paraffin and processed for histopathological analysis [11]. All samples were stained with haematoxylin and eosin (H&E). Histopathological evaluation was performed by a histopathologist with no prior knowledge of the identity of the samples.

Immunofluorescence and immunohistochemistry

The resected tumours were fixed in 10% buffered formalin, embedded in paraffin and mounted on slides. After deparaffinization and rehydration, the tumour sections were incubated in 3% hydrogen peroxide to suppress endogenous peroxidase activity. Antigen retrieval was achieved by microwaving the sections in 10 mM sodium citrate solution (pH 6.0). Sections were then blocked by incubating with 2.5% horse serum, and then antibodies were applied overnight at 4^{o}C. Antibody detection was achieved using an avidin-biotin complex system (Vector Laboratories, Burlingame, CA). Slides were stained with 3,3′-diaminobenzidine, then washed and counterstained with hematoxylin. Slides were subsequently dehydrated, treated with xylene and mounted.

Cell culture

The human ovarian carcinoma-derived SKOV3 cell line was cultured in RPMI-1640 medium, supplemented with foetal bovine serum (FBS; 10%) and penicillin/streptomycin (100 U/ml of each), and cultured in a 5% humidified CO_2atmosphere at 37^{o}C.

Cell migration and invasion assays

Cell migration was measured using a scratch assay. The SKOV3 cells were plated onto 6-well plates to create a confluent monolayer. The cell monolayer was scraped in a straight line using a pipette tipto create a “scratch”. Cells were washed once with the growth medium, then RPMI1640 medium containing 2% FBS was added. The cells were treated with N-Shh conditional medium plus either GANT61 inhibitor or DMSO for 36 hours. Cells were photographed usinga phase-contrast microscope at 0, 12, 24, 36 and 48 hours. The wound area was quantified using Institutes of Health (NIH) Image-Pro Plus software.

The cell invasion assay was performed in Transwell® plates (8-mm pore size, 6.5-mm diameter; Corning Life Sciences, Lowell, MA) which had been pre-coated with Matrigel®basement membrane matrix (1 mg/ml; BD Biosciences, Franklin Lakes, NJ) according to the manufacturer’s instructions. Briefly, highly invasive ovarian cancer SKOV3 cells (5 × 10^4 cells/well) were plated into the upper chamber of the system, and cultured with RPMI1640 supplemented with 2% FBS. The bottom wells in the system were filled with 500 ml N-Shh conditional medium supplemented with 2% FBS. Where applicable, the GLI2 antibody or control IgG (25 mg/ml) was added to the top chamber. After24 hours of incubation, any uninvaded cells in the upper chamber were removed, and cells that had invaded through the Matrigel®

matrix membrane were stained with crystal violet for 30 minutes. The number of invading cells was counted under an inverted microscope and photographed.

Reverse-transcription polymerase chain reaction

SKOV3 cells were seeded into 24-well plates (5 × 10^4 cells per well) for 24 hours, after which N-Shh or GANT61 were added to downregulate or upregulate the Hh signalling pathway, respectively. RNA was isolated using Trizol solution (Life Technologies, Grand Island, NY). After removal of genomic DNA using DNAse I (Ambion, Austin, TX), 2.4 μg of total RNA from SKOV3 cells were reverse-transcribed to cDNA using a commercially available kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed with a 7900HT fast real-time PCR system (ABI, Foster City, CA) using 2×SYBR Green master mix (Bio-Rad, Richmond, Calif). Forty cycles were performed as follows: 95°C for 30 seconds, 60°C for 30 seconds, preceded by 1 minute at 95°C for polymerase activation with the following primers (q-PCR **GAPDH** sense primer 5′- CAGGGCTGCTT TTAACTCTGGT -3′, antisense primer 5′- GATTTTG GAGGGAATCTCGCT -3′; q-PCR **CD24** sense primer 5′- GCTAAACGGATTCCAAAGAG-3′, antisense primer 5′- CTGGGCGACAAAGTGAGA -3′).

Western blotting

Whole-cell lysates were prepared using cell lysis buffer supplemented with protease inhibitor cocktail and 1 mM PMSF[12]. Protein concentrations were measured and then separated by polyacrylamide gel electrophoresis. After electro transferring to polyvinylidene difluoride membranes, non-specific binding sites were blocked with 5% non-fat milk in TBST for 1 hour at room temperature. Membranes were incubated with primary antibodies overnight at 4°C, washed with TBST, and then incubated with the appropriate HRP-conjugated secondary antibody for 1 hour at room temperature. Immune complexes were visualised using enhanced chemiluminescence. Consistent loading and transfer were confirmed by probing the same membrane with an anti-GAPDH antibody.

siRNA transfection

CD24-miRNAi expression vectors were generated using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (K4936-00; Invitrogen, Carlsbad, CA). The oligonucleotide sequences for the miRNAi constructs are presented in Table S1. SKOV3/DDP cells were plated at a density of 18,000 cells/cm^2. Transfection was conducted 24 hours after plating, using the RNAi-Max transfection reagent and different concentrations and scrambled siRNA sequences or negative control.

Xenograft tumour model and antitumor effect of CD24 *in vivo*

SKOV3 cells (5 × 10^7) were subcutaneously implanted into the flank of BALB/c nu/nu mice. Tumours were allowed to grow until they reached a median size of 100 mm^3, then the mice were randomly assigned to receive a subcutaneous injection of either GANT61 (25 mg/kg) in solvent (corn oil:ethanol, 4:1) or an equal volume of solvent only for 15 days. The subcutaneous injections were initiated 4 days after tumour inoculation, and repeated every other day. At the end of the experiment, tumours were excised, weighed, and then prepared for H&E staining and immunohistochemically (IHC) analyses.

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Second Affiliated Hospital of Nanchang University.

Statistical analysis

The statistical significance of the relationships between CD24 expression and the clinic pathological parameters was evaluated using Wilcoxon and Kruskal-Wallis tests and Spearman's rank correlation. The data are presented as mean ± SD, and $p<0.05$ was considered statistically significant [10, 13, 14].

Results

Correlation between the clinicopathological features and CD24expression

To explore the clinical significance of CD24, 97 patients with ovarian epithelial cell carcinoma were recruited. The histopathological evaluation revealed a significant increase in inflammation in ovarian serous carcinomas compared to normal ovaries and benign ovarian tumours (Fig.1).The proportion of cells that positively expressed CD24 was 55%in borderline tumours and80.95% in malignant tumours, which was significant higher than the21.43% positive cells in benign tumours ($p<0.01$). There was no significant difference observed between serous cystadeno-carcinoma and mucinous cystadenocarcinoma (Table 1 and Fig. 1).

Moreover, the relationship between CD24 expression and ovarian epithelial cancer indicated that CD24 expression was negatively associated with patient age, histological type and lymph node metastasis ($p>0.05$), but was positively associated with the clinical stage and pathological grading

(p<0.05; Table 2). The proportion of cells positive for CD24 expression in stages III and IV was 94.59%, whereas this was only 61.54% in stages I and II (p <0.005). A high pathological grading (G1) indicated a lower positive rate of CD24 expression (68.97%) than lower pathological grading (G2/G3, 91.18%; 0.025<p<0.05).

Table 1. Expression of CD24 in different ovarian tissues using immunohistochemistry.

	n	CD24 positive		X^2	P
		n	%		
Serous cystadenocarcinoma	50				
Benign	8	2	25.00	10.45	0.005 < p < 0.01
Borderline	10	5	50.00		
Malignant	32	26	81.25		
Mucinous cystadenocarcinoma	47				
Benign	6	1	16.67	9.85	0.005 < p < 0.01
Borderline	10	6	60.00		
Malignant	31	25	80.65		
Total benign	14	3	21.43		
Total borderline	20	11	55.00	20.00	< 0.005
Total malignant	63	51	80.95		

Table 2. Correlation between the protein expression of CD24 and clinicopathological parameters in patients with epithelial ovarian cancer (n=63).

Characteristics	n	CD24 positive		X^2	p
		n	%		
Age					
≤50	17	13	76.47	0.0358	0.75 < p < 0.90
> 50	46	38	82.61		
Histologic type					
Serous	32	26	81.25	0.00374	p > 0.90
Mucinous	31	25	80.65		
Pathological grading					
G1	29	20	68.97	5.01	0.025 < p < 0.05
G2/G3	34	31	91.18		
Clinical stages					
I/II	26	16	61.54	8.78	p < 0.005
III/IV	37	35	94.59		
Lymph node metastasis					
Yes	37	31	83.78	0.127	0.5 < p < 0.75
No	26	20	76.92		

Effect of CD24 on ovarian cancer cell migration and invasion

To confirm the cDNA microarray results obtained in our previous study, we examined the effect of Shh (activator of Hh signalling) and GANT61 (inhibitor of Hh signalling) on CD24 expression at both gene and protein levels. As shown in Fig. S1, addition of Shh significantly enhanced the CD24 expression both at the gene and protein levels (p<0.05), and GANT61 significantly inhibited CD24 expression (p<0.05).

When Shh was added, the activator of Hh signalling significantly enhanced the cellular migration of SKOV3 compared with the control group at both 24 and 48 hours (p<0.01). Furthermore, Shh at 25 and 50% concentrations significantly enhanced the invasion of SKOV3 cells (p<0.05; Fig. 2).

BlockingCD24 inhibits ovarian cancer cell migration and invasion *in vitro*

To further determine the effect of CD24 on ovarian cancer cells, a plasmid containing three different fragments that interfere with CD24 was constructed and tested in the SKOV3 cell line. Fragment3 was chosen asit demonstrated the best silencing effect, as determined by Western blot analysis (Table S1 and Fig. S2). We then evaluated the effect of direct silencing of CD24 expression on cell migration and invasion (Fig. 3). Results from the scratch test at 12 hours indicated no obvious changes in cellular migration. However, addition of Shh in the miR-control-N-Shh group resulted in significantly enhanced migration of SKOV3 cells at 24 and 36hours (p<0.05), and silencing CD24 in the miR-CD24-N-Shh group counteracted the effect of Shh (Fig.3a). Similarly, the Transwell results demonstrated that Shh significantly enhanced the cellular migration and invasion of SKOV3 in the control group. The downregulation of CD24 by fragment 3 eliminated its promoting effects, suggesting an important role of CD24as a downstream target of Hh signalling.

BlockingCD24 inhibits ovarian cancer growth *in vivo*

As GANT61 was found to significantly reduce the expression of CD24 (Fig.S1), this inhibitor was used to block the expression of CD24 *in vivo*. As shown in Fig.4, the IHC results indicated that blocking CD24 by GANT61reduced CD24 expression in the ovarian cancer tumours, and GANT61 successfully reduced the growth of tumours compared with the control on day 8 (p<0.01).

Discussion

Ovarian cancer is a common gynaecological cancer. Many studies have confirmed that approximately 25% of all cancer deaths are related to aberrant activation of the Hh pathway [15]. The Hh signalling pathway is a cascade involving Hh, Ptch, Smo and Gli. This signalling pathway can effectively regulate developmental processes in mammals, and plays an important role in regulating the development of embryos, cell differentiation, proliferation, tissue polarity, formation of tumour stem cells and so on[16, 17]. However, the molecular mechanism of this pathway in ovarian cancer is not well understood.

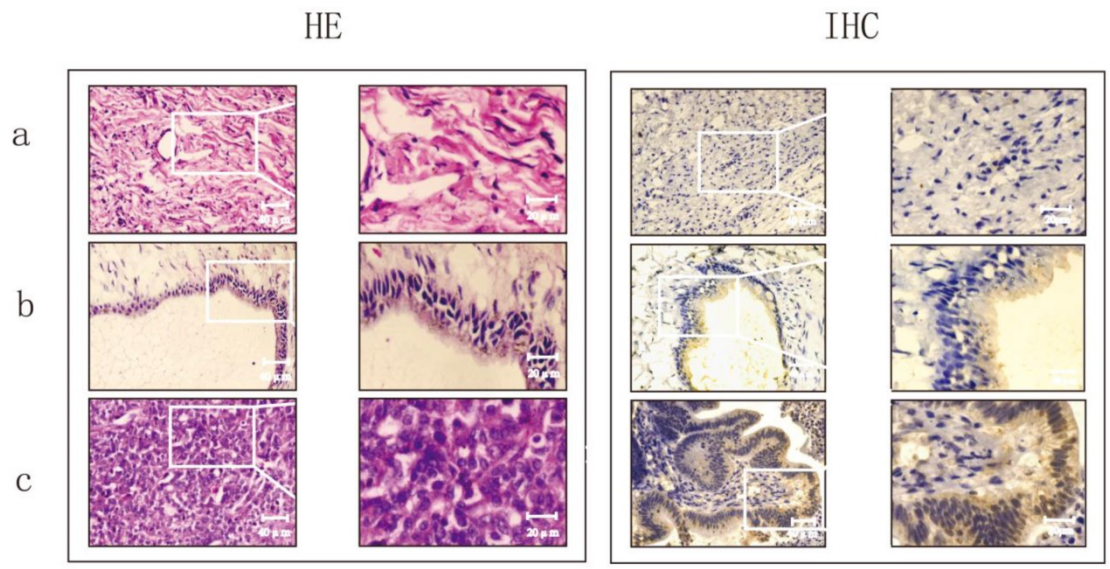

Figure 1. Evaluation of the histomorphology and protein expression of CD24 in normal ovaries, ovarian benign tumor and ovarian serious carcinoma using HE staining and immunohistochemistry (IHC).

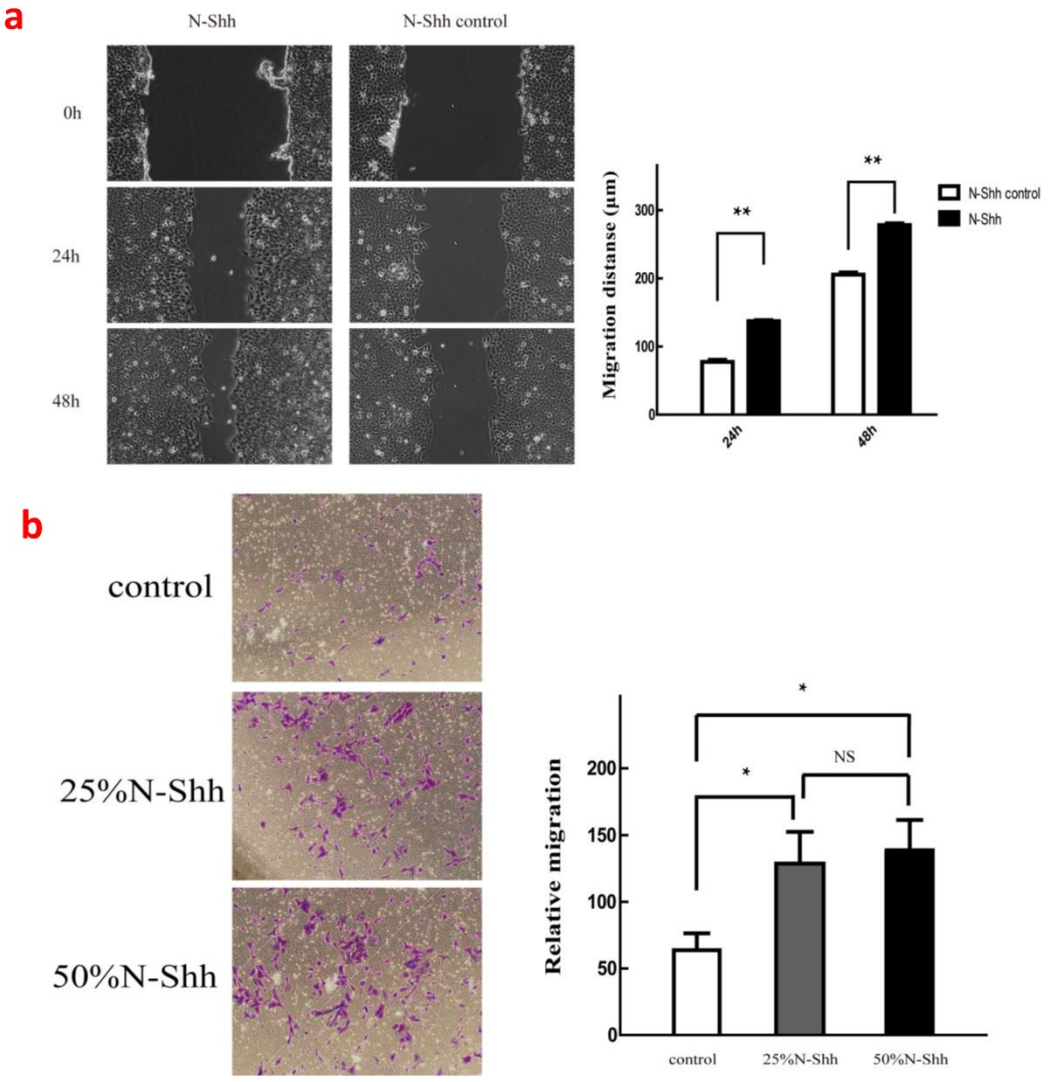

Figure 2. (a), Effects of 50% N-Shh on cancer cell migration; (b), Effects of 25% and 50% N-Shh on cancer cell invasion.

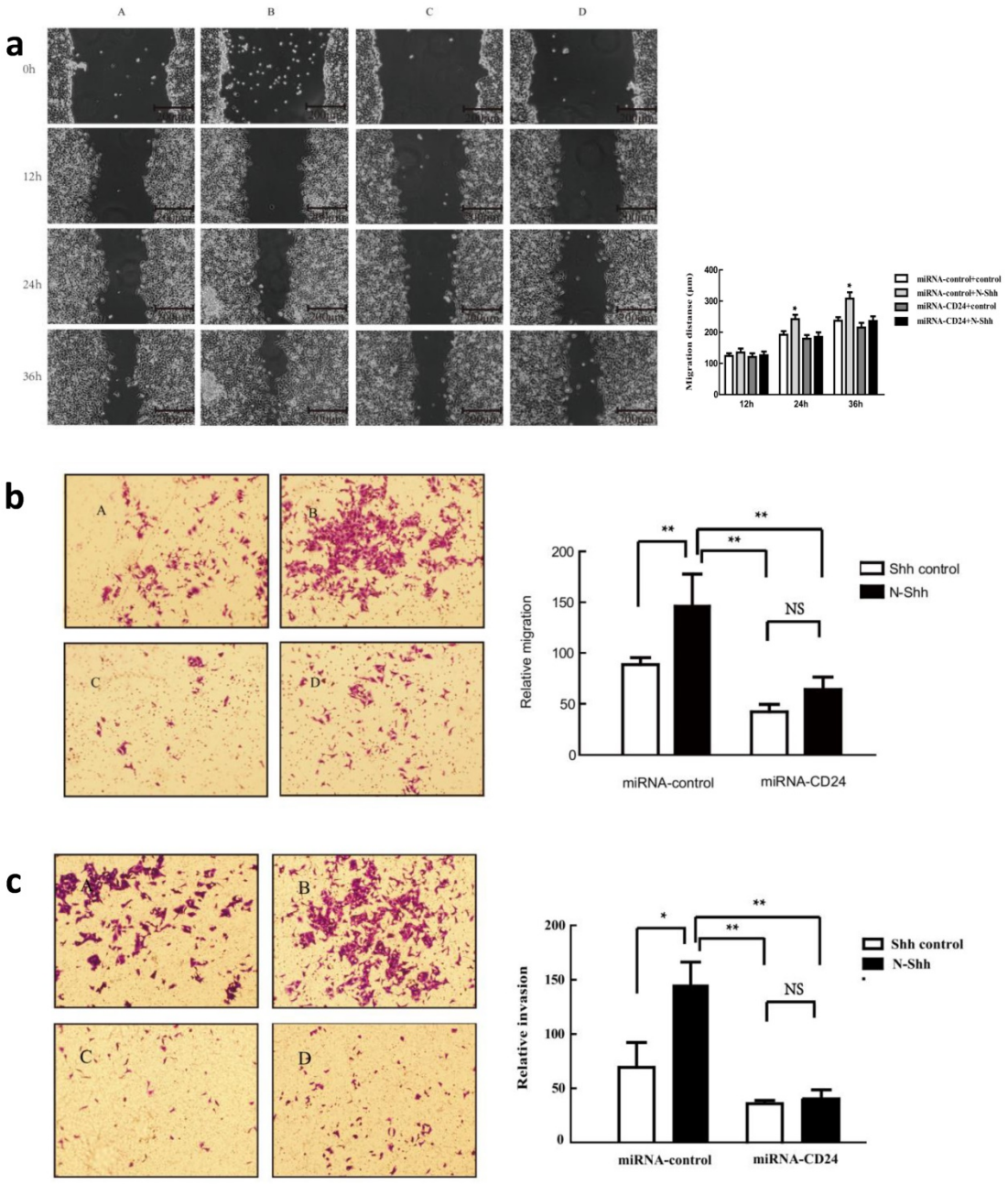

Figure 3. Effects of down-regulation of CD24 on ovarian cancer cell migration (a, b) and invasion (c). (A-D), transfeced with miR-control-control, miR-control-N-Shh, miR-CD24-control and miR-CD24-N-Shh.

In our previous studies, we identified that blocking the Hh signalling pathway was associated with significantly downregulated CD24 expression *in vitro*. CD24 is glycoprotein expressed on the surface of most B lymphocytes and differentiating neuroblasts. In neoplasia, the expression of CD24 has not only been describedin haematological malignancies, but also in a large variety of solid tumours, such as renal cell carcinoma, small cell lung cancer, nasopharyngeal carcinoma, hepatocellular carcinoma, bladder carcinoma, glioma and breast cancer[17–19]. To date, little is known about the role of CD24 in ovarian cancer via Hh signalling.

In the present study, 97 patients with ovarian epithelial cell carcinoma were recruited. Histopathological evaluation and IHC images revealed a significant increase in inflammation and the number of CD24-positivecellsin ovarian serous carcinoma than in normal ovaries or benign ovarian tumours (Fig.1). CD24 expression was negatively associated with patient age, histological type and lymph node metastasis ($p>0.05$), but was positively associated with the clinical stage and pathological grading ($p<0.05$; Table 2), indicating that CD24possesses a strong association with ovarian cancer. Moreover, the *in vitro* and *in vivo* results

confirmed that the upregulation of CD24 expression significantly enhanced cellular migration and invasion of SKOV3 cells ($p<0.05$; Fig. 2), and downregulation of CD24 expression reduced the growth of tumours compared with the control group ($p<0.01$; Fig.4).

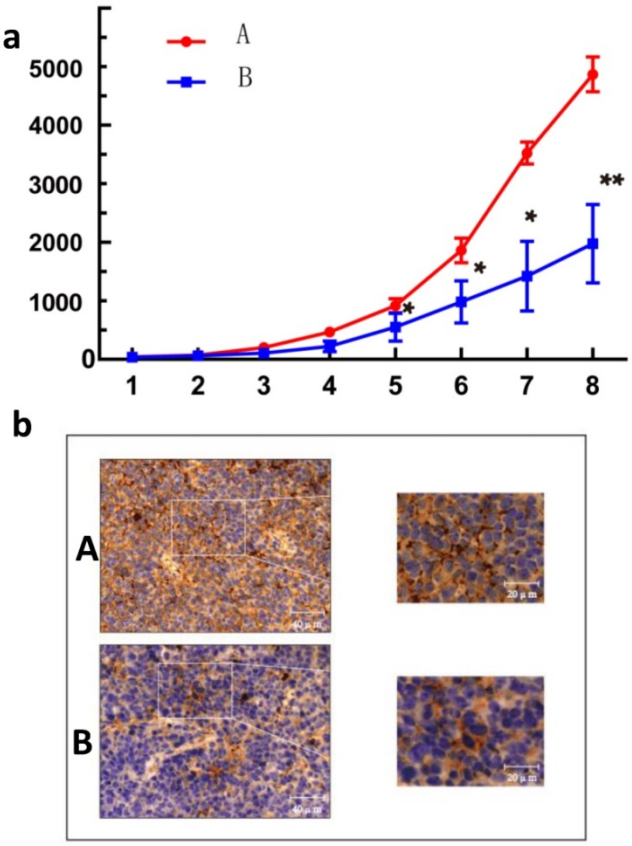

Figure 4. Effect of CD24 blockade on growth of human ovarian cancer cells in vivo. (a) Treatment with GANT61 led to a significant growth inhibition of xenografted human ovarian cancer tumors. n = 8; *, P<0.05; **, P<0.01. (b) Immunohistochemical image of tumor tissue section. A, control group; B, treatment with GANT61 diminished expression of CD24.

Many target genes have been found to be regulated by the Hh pathway, however, only few studies in the literatures have aimed to clarify the connection between CD24 and the Hh signalling pathway. In a previous study, we discovered that the adhesion molecule CD24 may be the downstream target gene of Hh signalling using a bioinformatics approach. In the current study, we have confirmed the key role of the Hh signalling pathway on the expression of CD24. In summary, our findings highlight the potential role of CD24 expression in Shh-stimulated cellular migration and invasion, both *in vitro* and *in vivo*. We also present a novel molecular mechanism responsible for Hh signalling-mediated ovarian cancer cell migration and invasion via CD24 expression, which may be useful for the treatment of ovarian cancer.

Supplementary Material

Supplementary figures and tables.
http://www.jcancer.org/v08p0786s1.pdf

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 81460392, 81503364, 31560264) and the Jiangxi Government (20161ACB20014, 20151BAB205045, 20153BCB23041, 20151BAB205001).

Conflicts of interest

All authors declare that they have no conflicts of interest.

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