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# Prognostic role of Wnt7a expression in ovarian carcinoma patients

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Wnt7a is a secreted glycoprotein that regulates normal cellular proliferation and differentiation as well as tumorigenesis and progression. The aim of this study was to investigate the expression and prognostic significance of Wnt7a in ovarian carcinoma. Wnt7a expression was immunohistochemically examined in normal ovaries (n=15), benign tumors (n=50) and ovarian carcinomas (n=78). The correlation of Wnt7a expression with clinicopathological parameters and survival was evaluated. Wnt7a expression was higher in ovarian carcinomas compared to normal ovaries and benign tumors ($p<0.001$ and $p=0.001$, respectively). Wnt7a positive expression was significantly correlated with serous subtype ($p<0.001$), elder age ($p=0.017$), advanced stage ($p<0.001$), high grade ($p=0.001$), a high degree of ascitic fluid volume ($p=0.015$) and high CA125 expression ($p=0.025$). Wnt7a was found to be a significant prognostic factor in univariate and multivariate analysis. High Wnt7a expression in ovarian cancer may be associated with poor prognosis.

*Key words: Wnt7a, expression, ovarian cancer, prognosis.*

Ovarian carcinoma is the most leading cause of death among gynecological malignancies worldwide, with a 5-year survival rate of 25%-30% in advanced stage disease [1]. Epithelial ovarian carcinomas (EOCs) originating from the ovarian surface epithelium account for over 90% of all ovarian carcinomas [2]. At present, patients have advanced stage when diagnosed because of the poor early symptoms. Over the past 40 years, despite advances in chemotherapeutic and surgical treatment, there has been little improvement in the overall 5-year survival rate for women with EOCs [3]. The early stage of diagnosis and therapy of ovarian carcinoma ultimately give patients the chance for long-term survival. Therefore, it is important to identify the prognostic factors of ovarian carcinoma in order to develop novel diagnostic and therapeutic strategies for improving the prognosis.

In recent years, several studies have shown that Wnt signal pathway plays an important role in the regulation of embryonic development and carcinogenesis [4-8]. At the surface of target cells, secreted Wnt proteins bind to specific receptor complex consisting of Frizzled (Fzd) and low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) to activate canonical pathway, resulting in the accumulation and translocation of β-catenin into the nucleus. Nuclear β-catenin commonly associates with the T cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors to modulate the target gene activity [9]. Non-canonical pathways induced by Wnt are β-catenin-independent, and act through multiple mechanisms [10]. Wnt signal pathway is complex and can display several distinct characteristics, depending on the Wnt proteins and the cell types [11, 12]. Abnormal activation of Wnt signal pathway associates with ovarian carcinogenesis and progression [13].

Wnt7a is a member of the Wnt protein family. Wnt7a was overexpressed in the cell lines of colorectal carcinoma, pancreatic carcinoma, gastric carcinoma and breast carcinoma [14]. However, Wnt7a has been reported to function as a tumor suppressor gene in non-small cell lung carcinoma [15]. In addition, the Wnt7a gene was also found to be down-regulated because of hypermethylation at high frequency in pancreatic carcinoma [16]. These results indicate that there may be a contrary effect of Wnt7a in different carcinomas.

Wnt7a regulates many kinds of cellular and developmental pathways that direct the prenatal growth of the female reproductive tract and maintain proper uterine function in the adult [17]. In the endometrium, Wnt7a functioned in cell-cell communication and was responsive to changes in levels of sex steroid hormones [18]. In endometrial carcinoma cell line (Ishikawa cells), ER antagonist reversed the reduction of Wnt7a, indicating that the down-regulation of Wnt7a in Ishikawa cells was partly mediated by the ER [19]. Overexpression of Wnt7a has been observed in invasive and low malignant potential (LMP) ovarian tumors compared with benign and normal ovarian tissues. In vitro, stable expression of Wnt7a in an ovarian carcinoma cell line OVCAR3 resulted in increased migration and invasive capacity [20]. It was recently reported that Wnt7a was also exclusively up-regulated in malignant ovarian carcinomas and widely expressed in highly metastatic EOC cell lines [21]. These results indicate that Wnt7a plays an

important role in ovarian tumor development and progression. However, Wnt7a expression status and its association with prognosis in gynecological carcinomas has not been evaluated. The present study aimed to investigate Wnt7a expression in ovarian carcinoma and to evaluate its relationship to the clinicopathological features and patient survival.

## Patients and methods

*Patients and tissue specimens.* 78 ovarian carcinomas, 15 normal ovaries and 50 benign ovarian tumors (25 serous cystadenomas, 25 mucous cystadenomas) were selected from The Tumor Hospital of Harbin Medical University between 2000 and 2004 after informed consent. All patients underwent surgical treatment, and before surgery without any chemotherapeutic or radiotherapeutic treatment. Eighty-two percent received postoperative chemotherapy. The median age at diagnosis was 52.5 years (range, 23 to 80 years). Histological subtype and differentiation grade were assigned according to World Health Organization (WHO) criteria and were further reviewed by two experienced pathologists. The surgical staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO) system. Progression-free survival (PFS) was defined as the time interval between the date of surgery and the date of identification of progressive disease. And overall survival (OS) was defined as the time interval between the date of surgery and the date of death.

*Immunohistochemistry.* Serial sections of 4 μm were deparaffinized with xylene and rehydrated in serial dilutions of ethanol. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide at room temperature for 10 min. After that, the sections were microwaved for 20min in 10 mM trisodium citrate buffer (PH 7.0) to retrieve antigens, then blocked with normal goat serum for 30 min. The sections were incubated overnight at 4˚C with the primary antibodies for Wnt7a (goat anti-Wnt7a, diluted 1:80, AF3008; R&D, Minneapolis, USA). After washing, the signal was amplified by incubating the sections with secondary antibodies for 45 minutes at 37˚C; anti-goat IgG-polymer horseradish peroxidase (HRP; Polink-2 Plus HRP Goat DAB kit, D43-6; GBI, Mukilteo, WA). The slides were counterstained with hematoxylin. Endometrial carcinomas were used as a positive control for Wnt7a. For negative controls the primary antibody was replaced with PBS.

*Staining evaluation.* All slides were examined by two experienced pathologists, who were blinded to patient outcome. The Wnt7a expression level was classified based on the total combined scores of staining extent (the percent of positive staining tumor cells) together with the staining intensity. The percentage of positive tumor cell was graded and scored as follows: 0 (none), 1 (≤25%), 2 (26-50%), 3 (51-75%), 4 (≥76%). The staining intensity was graded and scored as follows: 0 (no staining), 1 (very weak), 2 (weak), 3 (moderate), 4 (strong). The final score (0-16) was calculated multiplying the positive proportion score by staining intensity score. Scores≥4 were considered positive.

*Western blot analysis.* Four tissues of ovarian carcinoma and two tissues of normal ovary were homogenized in modified RIPA buffer and 1 mM PMSF mixture. After centrifugation at 12,000 g for 15 min, the supernatant was obtained. Then, the proteins were subjected to 10% SDS-PAGE separation, and transferred to nitrocellulose membrane by semidry electrophoresis. The blots were blocked in PBST (1×PBS, 0.1% Tween 20, 5% nonfat dried milk) overnight at 4˚C, then incubated with primary antibodies for Wnt7a (goat anti-Wnt7a, diluted 1:100, AF3008; R&D, Minneapolis, USA) for 1 h at room temperature. Secondary IgG antibodies (diluted 1:5000; GBI, Mukilteo, WA) were incubated for 1 h at room temperature as well. Finally, immunoreactive proteins were stained using 3, 3'-diaminobenzidine tetrachloride. Membranes were then washed with PBST for 5 h at room temperature and followed with a mouse anti-β-actin antibody (diluted 1:500, sc-69879; Santa, California, USA) as an internal control.

*Statistical analysis.* The Chi-square test or Fisher exact test was used to analyze the distribution of Wnt7a positive expression according to several clinicopathological features from cases and to examine the correlation between the expression of Wnt7a and these clinicopathological features (age, FIGO stage, histology, grade, residual tumor, ascitic fluid volume, CA125). For univariate analysis, survival curves were plotted using the Kaplan-Meier method and differences between survival curves were tested using the log-rank test. For multivariate analysis, Cox proportional-hazard model was performed to evaluate the independence of Wnt7a expression as prognostic factor against other variables. Differences at $p<0.05$ were considered statistically significant. All statistical analyses were performed using SPSS software Version 13.0.

## Results

*Clinical profiles of the patients.* Of 78 patients, 35 (44.9%) were FIGO stage I+II, and 43 (55.1%) were stage III+IV. 22 (28.2%) were G1, and 56 (71.8%) were G2+G3. Histological subtypes of the tumor included 41 (52.6%) cases of serous ovarian carcinoma, 37 (47.4%) cases of non-serous ovarian carcinoma (17 were mucinous carcinoma, 14 were endometrioid carcinoma, and 6 were clear cell carcinoma) (Table 1). At the end of the follow-up period, 32 (41.0%) patients were alive, 46 (59.0%) patients had died of disease, and none of the patients were lost to follow-up. The median follow-up time was 48.5 months (range 2-101).

*Expression of Wnt7a in normal ovaries, benign tumors and ovarian carcinomas.* Wnt7a immunostaining was observed mainly in the cytoplasm (Fig. 1). Wnt7a expression in ovarian carcinomas was positive in 51 of 78 patients (65.4%) and negative in 27 of 78 patients (34.6%) as defined by the staining evaluation above. In Table 2, a significant association was observed among the groups (normal ovaries, benign tumors and ovarian carcinomas) for Wnt7a expression. The proportion of Wnt7a positive expression was significantly higher for ovar-

**Table 1. Expression of Wnt7a and its relationship with clinicopathological parameters of ovarian carcinoma**

| Variables | No. of cases | Wnt7a (%) | | p-value* |
|---|---|---|---|---|
| | | Negative | Positive | |
| All cases | 78 | 27(34.6) | 51(65.4) | |
| Age | | | | 0.017 |
| ≤50 years | 32 | 16(50.0) | 16(50.0) | |
| >50 years | 46 | 11(23.9) | 35(76.1) | |
| FIGO stage | | | | <0.001 |
| I+II | 35 | 20(57.1) | 15(42.9) | |
| III+IV | 43 | 7(16.3) | 36(83.7) | |
| Grade | | | | 0.001 |
| G1 | 22 | 14(63.6) | 8(36.4) | |
| G2+G3 | 56 | 13(23.2) | 43(76.8) | |
| Histological subtype | | | | <0.001 |
| Serous | 41 | 6(14.6) | 35(85.4) | |
| Non-serous | 37 | 21(56.8) | 16(43.2) | |
| Residual tumor | | | | 0.748 |
| ≤2 cm | 65 | 23(35.4) | 42(64.6) | |
| >2 cm | 13 | 4(30.8) | 9(69.2) | |
| Ascitic fluid volume | | | | 0.015 |
| ≤100 ml | 29 | 15(51.7) | 14(48.3) | |
| >100 ml | 49 | 12(24.5) | 37(75.5) | |
| CA125 | | | | 0.025 |
| ≤50 U/ml | 13 | 8(61.5) | 5(38.5) | |
| >50 U/ml | 65 | 19(29.2) | 46(70.8) | |

FIGO, International Federation of Gynecology and Obstetrics; *Chi-square test.

**Table 2. Expression of Wnt7a in normal ovaries, benign tumors and ovarian cancers**

| | No. of cases | Wnt7a (%) | | p-value* |
|---|---|---|---|---|
| | | Negative | Positive | |
| Normal ovaries | 15 | 13(86.7) | 2(13.3) | <0.001 |
| Benign tumors | 50 | 32(64.0) | 18(36.0) | 0.001 |
| Ovarian cancers | 78 | 27(34.6) | 51(65.4) | |

Normal ovaries vs ovarian cancer: p<0.001; Benign tumors vs ovarian cancer: p=0.001; *Chi-square test.

ian carcinomas (65.4%) compared to normal ovaries (13.3%) (p<0.001) and benign tumors (36.0%) (p=0.001).

To validate the immunohistochemical results above, 6 cases of ovarian tissues, including 4 ovarian carcinomas and 2 normal ovaries, were subjected to detect the Wnt7a expression levels by western blot analysis. Wnt7a protein was detected as ~48 kDa bands. As shown in Fig. 2, expression of Wnt7a increased in ovarian carcinomas, which paralleled the immunohistochemical results, although Wnt7a was expressed constitutively in normal ovary.

*Expression of Wnt7a and its relationship with clinicopathological features of ovarian carcinoma.* Table 1 showed the

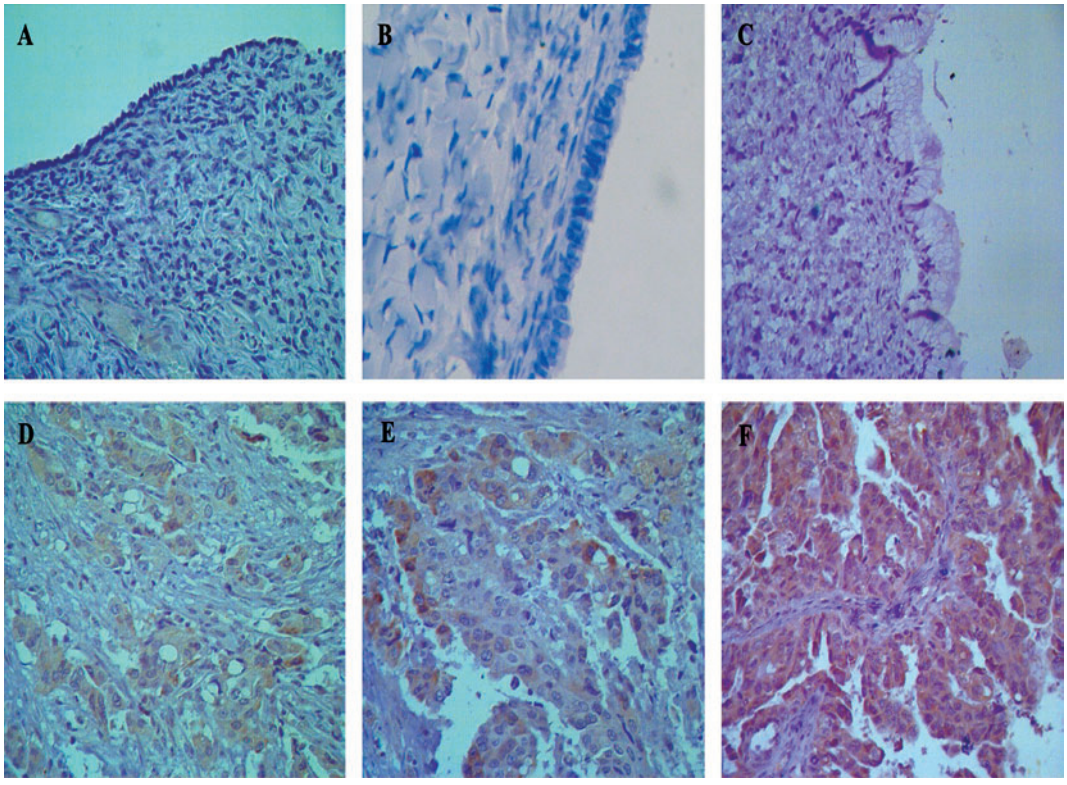


**Figure 1. Immunohistochemical staining of Wnt7a in normal ovary, benign ovarian tumor and ovarian carcinoma. A, Negative staining of normal ovarian epithelium (×400); B, Negative staining of serous cystadenoma (×400); C, Negative staining of mucinous cystadenoma (×400); D-F, Ovarian carcinoma with increasing immunostaining intensity (D, Weak; E, Moderate; F, Strong) (×400).**

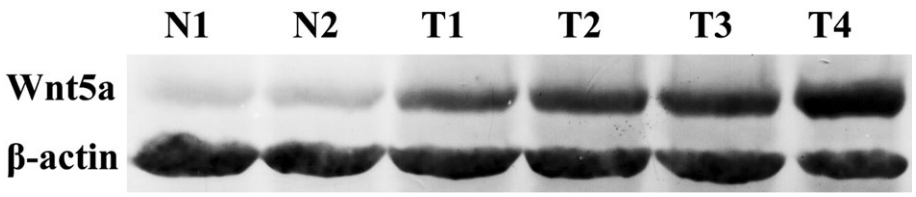


**Figure 2. Wnt7a expression in normal ovaries and ovarian carcinomas as detected by western blot analysis. Lane N1, N2: normal ovaries. Lane T1-T4: ovarian carcinomas. β-actin served as a loading control.**

relationship between Wnt7a expression and clinicopathological features in ovarian carcinomas. Wnt7a expression showed correlation with histological subtype (p<0.001), age (p=0.017), FIGO stage (p<0.001), grade (p=0.001), ascitic fluid volume (p=0.015) and CA125 (p=0.025). However, Wnt7a positivity was not associated with residual tumor (p=0.749).

*Correlation of Wnt7a expression with OS and PFS.* In univariate analysis, histological subtype, stage, grade, ascitic fluid volume, CA125 and Wnt7a expression were found to be associated with poor OS and PFS. The mean survival time for patients with positive Wnt7a expression was lower than that for those with negative Wnt7a expression (38±4 months vs. 86±6 months, p<0.001). The median survival time of Wnt7a positive patients was 30 months, while it was not defined for Wnt7a negative patients. The 5-year OS rates of patients with positive and negative Wnt7a expression were 23.5% and 79.5%, respectively. And the 5-year PFS rates of patients with positive and negative Wnt7a expression were 8.3% and 63.1%, respectively. Both OS and PFS in patients positive for Wnt7a expression were significantly lower than those in patients who were negative for Wnt7a expression (OS, PFS; p<0.001) (Table 3). The OS and PFS curves according to Wnt7a expression was shown in Fig. 3 by Kaplan-Meier method. Finally, a multivariate analysis with the Cox proportional-hazard model was conducted incorporating the variables in histological subtype, stage, grade, ascitic fluid volume, CA125 and Wnt7a expression (Table 4). Only Wnt7a expression retained an independent prognostic value for poor OS and PFS (HR, 3.717; 95%CI, 1.265-10.921; p=0.017 and HR, 3.572; 95%CI, 1.488-8.575; p=0.004).

**Table 3. Univariate survival analysis of PFS and OS in 78 patients with ovarian carcinoma**

| | No. of cases | Estimated 5-year PFS (%) | p-value* | Estimated 5-year OS (%) | p-value* |
|---|---|---|---|---|---|
| Age | | | 0.078 | | 0.062 |
| ≤50 years | 32 | 40.3 | | 54.8 | |
| >50 years | 46 | 18.6 | | 34.8 | |
| FIGO stage | | | 0.016 | | 0.002 |
| I+II | 35 | 41.3 | | 62.9 | |
| III+IV | 43 | 20.3 | | 27.2 | |
| Grade | | | 0.003 | | 0.002 |
| G1 | 22 | 54.5 | | 70.8 | |
| G2+G3 | 56 | 19.3 | | 32.1 | |
| Histological subtype | | | 0.001 | | <0.001 |
| Serous | 41 | 10.9 | | 24.4 | |
| Non-serous | 37 | 43.8 | | 63.6 | |
| Residual tumor | | | 0.386 | | 0.989 |
| ≤2 cm | 65 | 32.9 | | 46.2 | |
| >2 cm | 13 | 15.4 | | 38.5 | |
| Ascitic fluid volume | | | 0.013 | | 0.005 |
| ≤100 ml | 29 | 48.3 | | 69.0 | |
| >100 ml | 49 | 17.9 | | 27.6 | |
| CA125 | | | 0.008 | | 0.012 |
| ≤50 U/ml | 13 | 55.4 | | 72.5 | |
| >50 U/ml | 65 | 21.4 | | 36.9 | |
| Wnt7a | | | <0.001 | | <0.001 |
| Negative | 27 | 63.1 | | 79.5 | |
| Positive | 51 | 8.3 | | 23.5 | |

FIGO, International Federation of Gynecology and Obstetrics; PFS, progression-free survival; OS, overall survival; *log-rank test.

## Discussion

Previous studies have evaluated the expression of Wnt protein in various human carcinomas through immunohistochemistry. In ovarian cancer, positive Wnt5a expression correlated to worse prognosis, compared to negative Wnt5a expression [22]. However, in colorectal cancer, there was no significant association between the Wnt2 overexpression and the survival of patients [23]. In the present study, we examined Wnt7a expression immunohistochemically in 78 ovarian carcinoma tissues, investigated the relationship between Wnt7a expression and the clinicopathological parameters of ovarian carcinomas, and found that Wnt7a was an independent prognostic factor.

First of all, we analyzed the state of Wnt7a expression by immunohistochemistry. The present data showed that Wnt7a was overexpressed in ovarian carcinomas, compared with little or no expression in normal ovaries and benign tumors. Then we analyzed the expression of Wnt7a in 4 cases of ovarian carcinoma and 2 cases of normal ovarian tissue by western blot analysis and found that the Wnt7a protein was dominantly expressed in ovarian carcinomas, which was consistent with immunohistochemical analysis. In agreement with our data, it was also reported that exclusively increased expression in

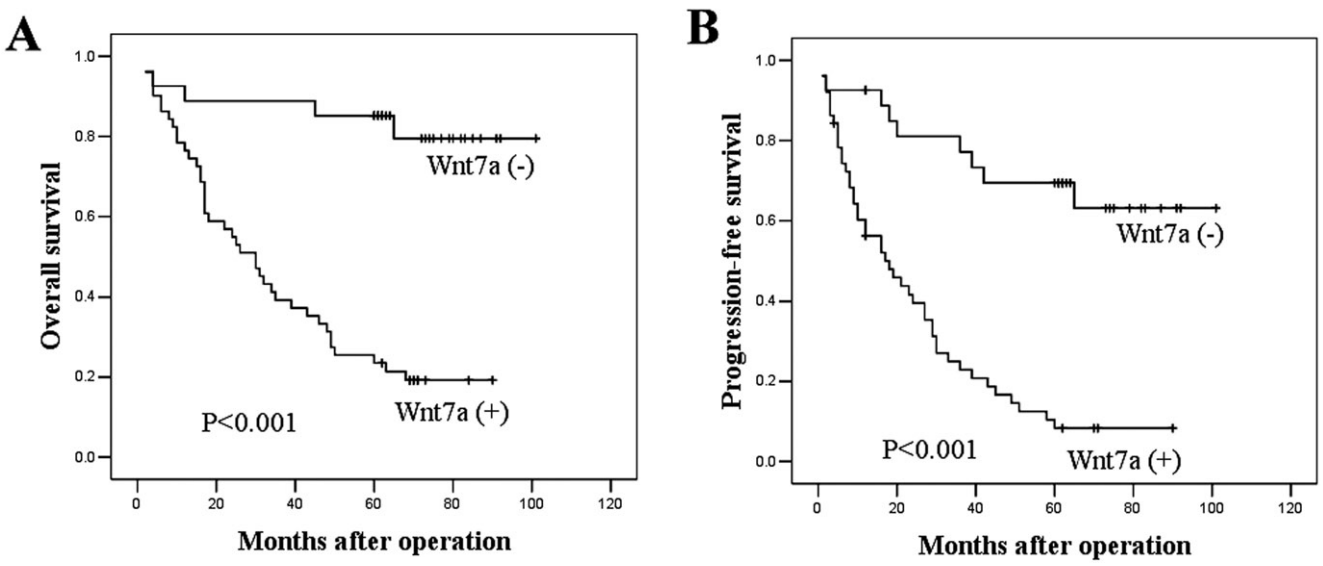


**Figure 3. (A) Kaplan-Meier curves for OS of ovarian carcinoma patients according to negative and positive expression of Wnt7a (log-rank analysis). (B) Kaplan-Meier curves for PFS of ovarian carcinoma patients according to negative and positive expression of Wnt7a (log-rank analysis).**

malignant ovarian tumors, very low or almost undetectable in the normal tissues, and moderately in the benign, suggesting that the role of the Wnt7a protein might be associated with the tumorigenesis in ovarian carcinoma [21]. Statistically, overexpression of Wnt7a correlated with a serous histological subtype, elder age, advanced clinical stage, a high degree of histological grade, a high production of ascitic fluid volume and high expression of CA125, but not with residual tumor of ovarian carcinomas. These results suggest that the high Wnt7a expression is associated with the disease progression of ovarian carcinoma, and raise the possibility that its expression reflects tumor aggressiveness.

Additionally, our multivariate analysis showed Wnt7a expression to be an independent predictor of OS and PFS. Consequently, overexpression of Wnt7a may be a prognostic factor for ovarian carcinomas. The mechanism of Wnt7a expression affecting the prognosis of patients is unclear at present. It has been reported that overexpression of Wnt7a in the ovarian carcinoma cell line OVCAR3 could result in increased cell migration and invasive capacity [20]. Similarly, Wnt7a might be involved in enhancing the ability of metastasis by regulating MMP7, MMP9 and CDH1 in ovarian carcinoma cells [21]. Another report has also shown that Wnt7a promoted the proliferation and migration of normal cornea cells by up-regulating MMP12 during wound healing [24]. Therefore, we speculate that Wnt7a overexpression in ovarian carcinoma may contribute to unfavorable biological behavior through increasing the MMPs expression. Owing to the complex of the Wnt signal transduction, Wnt7a signal pathway may be able to regulate many other downstream target proteins besides MMPs involved in the cell proliferation and migration of the ovarian carcinoma. Thus, the mechanism by which Wnt7a expression contributes to tumor progression of ovarian carcinoma remains to be worked out. Analysis of Wnt7a expression may be useful for estimating the prognosis of the patients with ovarian carcinoma.

**Table 4. Prognostic factors for progression-free and overall survival selected by Cox's multivariate proportional model analysis**

| | Progression-free survival | | | Overall survival | | |
|---|---|---|---|---|---|---|
| Variables | HR | 95%CI | p-value* | HR | 95%CI | p-value* |
| FIGO stage | 1.084 | 0.575-2.045 | 0.802 | 1.343 | 0.661-2.731 | 0.415 |
| Grade | 1.359 | 0.639-2.892 | 0.426 | 1.503 | 0.603-3.745 | 0.382 |
| Histological subtype | 0.801 | 0.418-1.534 | 0.504 | 0.594 | 0.286-1.234 | 0.163 |
| Ascitic fluid volume | 1.326 | 0.688-2.556 | 0.399 | 1.513 | 0.720-3.179 | 0.274 |
| CA125 | 1.777 | 0.652-4.839 | 0.261 | 1.683 | 0.464-6.102 | 0.428 |
| Wnt7a expression | 3.572 | 1.488-8.575 | 0.004 | 3.717 | 1.265-10.921 | 0.017 |

CI, confidence interval; HR, hazard ratio; *Cox regression test

Although Wnt7a contributed to invasion and metastasis in ovarian carcinoma cells, acting as a tumorigenic inducer [20], it had the opposite effect in some other carcinomas. In non-small cell lung carcinoma, Wnt7a was reported to function as a tumor suppressor gene. The anti-tumorgenic effects of Wnt7a interacting with the specific receptor Fzd9 was mainly mediated through ERK-dependent activation of the nuclear receptor gene PPARγ [15]. On the other hand, Wnt7a positively regulated E-cadherin in lung carcinoma cell. Loss of E-cadherin was thought to be involved in an epithelial-mesenchymal transition, resulting in increased tumorigenesis [25]. In addition, loss of E-cadherin was one of the most important events during carcinomas progression [26]. These results indicated that the effects of Wnt7a as a tumor suppressor could also be mediated via E-cadherin in lung carcinoma. These studies above suggest that the functions of Wnt7a on tumorigenesis and progression may be dependent on the different tumor types examined. We speculate that the mechanism of Wnt7a is possibly based on the Fzd receptor availability, because at least 10 members of the Fzd family have been identified. Therefore, investigation of the major Fzd receptor expression on the ovarian carcinoma cell surface, especially its interaction with Wnt7a, may help to reveal possible therapeutic approaches for tumorigenesis and invasion of ovarian carcinoma.

It has been reported that Wnt7a expression was sex hormone dependent. Estrogens decreased Wnt7a expression through its receptor ER in endometrial carcinoma Ishikawa cell line [19]. In accordance with endometrial carcinoma, an inverse correlation was apparent between Wnt7a and ERα expression in human uterine leiomyoma [27]. Another study detected that norethisterone up-regulated Wnt7a gene in endometrial epithelial cells, suggesting the up-regulation of Wnt7a may be a mechanism by which progestogens reduced endometrial carcinoma risk [28]. These previous results indicate that decreased Wnt7a associates with the development of sex hormone dependent endometrial carcinoma and leiomyoma. This raises the speculation that the expression of Wnt7a may be also correlated with the level of sex hormones and their receptors in ovarian carcinoma. To our knowledge, studies on the association of ER/PR expression with ovarian carcinoma are few and contradictory, although steroid hormones and receptors may have important influences on the tumorigenesis and progression of ovarian carcinomas [29-33]. Therefore, further investigation including evaluation of hormone receptor status in these patients is needed to specifically identify the pathological roles of Wnt7a in ovarian carcinoma cells.

Currently used molecular markers such as survivin, COX-2 and Snail have been identified, which show association with prognosis in ovarian cancer [34-36]. However, it is not yet clear whether such indicators may be most effective for clinical treatment and prognosis. Wnt7a as an upstream molecule of Wnt canonical pathway activates multiple downstream molecules, thus, Wnt7a may be more reliable and effective than clinicopathological factors in use for predicting prognosis of patients with ovarian cancer. Additionally, combined detection of Wnt7a together with currently used indicators would be worth investigating to enhance prognostic effectiveness.

In conclusion, we demonstrated that high Wnt7a expression correlated with disease progression and impaired clinical outcome in ovarian carcinoma patients. Furthermore, Wnt7a was an independent prognostic factor for OS and PFS. Clearly, additional studies are needed, however, these results indicate that Wnt7a is a promising prognosis indicator and may become a novel molecular target in the strategies for clinical evaluation and treatment of ovarian carcinoma.

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