ROR1 expression correlated with poor clinical outcome in human ovarian cancer

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The receptor-tyrosine-kinase-like orphan receptor 1 (ROR1) is a transmembrane protein belongs to receptor tyrosine kinase (RTK) family. This study aimed to examine the expression of ROR1 in human ovarian cancer and investigate the relationship between its expression and the prognosis of ovarian cancer patients. In this present study, one-step quantitative reverse transcription-polymerase chain reaction (15 ovarian cancer samples of high FIGO stage, 15 ovarian cancer samples of low FIGO stage and nine normal ovary tissue samples) and immunohistochemistry by tissue microarrays (100 ovarian cancer samples and 50 normal ovary samples) were performed to characterize expression of the ROR1 gene in ovarian cancer. Kaplan-Meier survival and Cox regression analyses were executed to evaluate the prognosis of ovarian cancer. The results of qPCR and IHC analysis showed that the expression of ROR1 in ovarian cancer was significantly higher than that in normal ovary tissues (all p < 0.05). Survival analysis showed that ROR1 protein expression was one of the independent prognostic factors for disease-free survival and overall survival (both p < 0.05). The data suggest that ROR1 expression is correlated with malignant attributes of ovarian cancer and it may serve as a novel prognostic marker in ovarian cancer.

Ovarian cancer is the most lethal gynecologic malignancy among women worldwide, and its incidence has been increasing persistently in Asian countries, including China. Approximately over 200,000 new cases of ovarian cancer occurred worldwide in 2011. Ovarian cancer generally originates from the malignant transformation of the ovarian surface epithelium, which is a single continuous layer of epithelial cells surrounding the ovary. The majority of ovarian cancer patients are diagnosed at advanced stages because of asymptomatic characteristics and lack of susceptible detection at early stage. Currently, surgery is still necessary for appropriate staging of ovarian cancer and for improving chemotherapy results and survival rate. Chemotherapy is an important strategy in the treatment of ovarian cancer. Platinum-taxane combination has been used as the reference standard for the first-line chemotherapy of postsurgical ovarian cancer. Although the standard platinum-taxane regimen shows effectiveness with a response rate of 80% in advanced ovarian cancer patients, most of these patients relapse because of drug resistance. Therefore, the identification of novel and specific biomarkers that have clinicopathologic and prognostic significance in ovarian cancer is remarkably important.

The receptor-tyrosine-kinase-like orphan receptor 1 (ROR1) is a transmembrane protein that belongs to the receptor tyrosine kinase (RTK) family. ROR1 consists of an extracellular frizzled-like, cysteine-rich domain, an extracellular, membrane proximal kringle domain, and an intracellular tyrosine-kinase-like domain. ROR1 protein is evolutionarily conserved among various species, and it is primarily expressed during embryogenesis. ROR1-deficient mice do not display any morphological abnormalities of the skeleton or heart or face, but they die within 24 h after birth probably because of respiratory failure. Although the exact biological function of ROR1 is not fully understood, an increasing number of studies indicated that ROR1 is highly associated with human...
cancers and ROR1 may serve as a potential target for cancer therapy. Moreover, there is emerging data suggesting that the Wnt/β-catenin pathway plays an important role in carcinogenesis of all ovarian cancer subtypes. Wnt5a, a substantial ligand of ROR1, also participates in the ROR1-dependent signaling pathway in enhancing cancer cell growth. Hence, we assume that there may be an intriguing relationship between ROR1 expression and certain clinicopathological significance of ovarian cancer. The potential of ROR1 as a candidate for molecular-targeted therapy of ovarian cancer requires further investigation.

In this present research, we detected the expression of ROR1 mRNA in fresh ovarian cancer tissue via one-step quantitative reverse transcription-polymerase chain reaction (qPCR). Subsequently, we examined the expression of ROR1 protein in ovarian cancer with tissue microarray (TMA) by immunohistochemistry (IHC) analysis. Finally, we evaluated the correlation of ROR1 expression with the clinicopathologic features and survival of ovarian cancer.

Results

Clinical features of 100 ovarian cancer patients. The main clinicopathologic characteristics of ovarian cancer patients are shown in Table 1. The age of the 100 patients with ovarian carcinoma ranged from 21 years to 82 years (mean age, 50.8 years). The tumor diameter of 64 patients was \( \geq 5 \) cm, whereas that of the remaining 36 patients was \(<5 \) cm. In terms of the distribution of FIGO stage, 56 patients were at stages I and II, while 44 patients were at stages III and IV. Regarding the histologic tumor grade, 25 patients were at grade 1, 50 were at grade 2, and 25 were at grade 3. The distribution of histological type was as follows: 76 patients had serous-papillary type, 6 had clear cell type, 10 had mucinous type, and 8 had endometrioid type. The serum CA-125 level of 52 patients was \( \geq 35 \) U/ml, whereas that of the other 48 patients was \(<35 \) U/ml. A total of 63 patients presented positive ascites syndrome while the remaining 37 showed negative ascites. There were 35 patients encountered positive lymph node metastasis and the other 65 showed negative lymph node metastasis. 43 patients experienced cancer relapse while the other 57 did not. All 100 patients with ovarian cancer shared an overall survival rate of 39% and a disease-free survival rate of 28%.

Analysis of ROR1 mRNA expression in ovarian cancer by qPCR. Three reference genes, peptidylprolyl isomerase A (PPIA), β-actin (ACTB), and TATA-Box binding protein (TBP), were used as internal control in qPCR test. Total RNA was extracted from the 30 ovarian cancer tissues (15 high FIGO stage and 15 low FIGO stage) and subjected to one-step qPCR to evaluate the expression of ROR1 mRNA. We also detected samples from nine normal ovary tissues for comparing the expression of the mRNA with that of non-cancerous tissue. When normalized to PPIA, the expression of ROR1 mRNA (means ± SEM) in ovarian cancer of high stage, low stage and corresponding normal ovary were 4.02 ± 0.785, 3.20 ± 0.515 and 1.14 ± 0.292 respectively. The expression of ROR1 mRNA in high (t = 2.745, p = 0.002) and low (t = 2.910, p = 0.022) stage ovarian cancer samples was significantly higher than in non-cancerous tissues. Similarly, when normalized to ACTB and TBP, the ROR1 expression in ovarian cancer samples (both high and low stage) was statistically higher than that of in normal ovary samples (Figure 1).

Detection of ROR1 protein expression in ovarian cancer by IHC. IHC was employed to investigate the expression of ROR1 in ovarian cancer.

| Groups                  | No. | + | %  | χ²      | p value |
|-------------------------|-----|---|----|---------|---------|
| Age (years)             |     |   |    |         |         |
| ≥60                     | 54  | 31| 57.4| 0.2749  | 0.600   |
| <60                     | 46  | 24| 52.2|         |         |
| Tumor diameter (cm)     |     |   |    |         |         |
| ≥5                      | 64  | 36| 56.3| 0.1122  | 0.738   |
| <5                      | 36  | 19| 52.8|         |         |
| FIGO stage              |     |   |    |         |         |
| I + II                  | 56  | 25| 44.6| 5.5162  | 0.019*  |
| III + IV                | 44  | 30| 68.2|         |         |
| Tumor grade             |     |   |    |         |         |
| Grade 1                 | 25  | 8 | 32.0| 7.2323  | 0.027*  |
| Grade 2                 | 50  | 32| 64.0|         |         |
| Grade 3                 | 25  | 15| 68.0|         |         |
| Histological type       |     |   |    |         |         |
| Serous-papillary        | 76  | 40| 53.1| 4.0174  | 0.260   |
| Clear cell              | 6   | 4 | 66.7|         |         |
| Mucinous                | 10  | 8 | 80.0|         |         |
| Endometrioid            | 8   | 3 | 37.5|         |         |
| Serum CA-125 level (U/ml)|     |   |    |         |         |
| ≥35                     | 52  | 29| 55.8| 0.0259  | 0.872   |
| <35                     | 48  | 26| 54.2|         |         |
| Ascites                 |     |   |    |         |         |
| Positive                | 63  | 30| 47.6| 3.7479  | 0.053   |
| Negative                | 37  | 25| 67.6|         |         |
| Lymph node metastasis   |     |   |    |         |         |
| Positive                | 35  | 25| 71.4| 5.8719  | 0.015*  |
| Negative                | 65  | 30| 46.2|         |         |
| Relapse status          |     |   |    |         |         |
| Positive                | 43  | 21| 48.8| 1.1576  | 0.282   |
| Negative                | 57  | 34| 59.6|         |         |

*p < 0.05.

Table 1 | Relationship of high ROR1 expression with clinicopathological characteristics in ovarian cancer
cancer. High ROR1 expression was detected in 55 of 100 (55%) ovarian cancer tissues, whereas only 6 cases of 50 normal ovary tissues (12%) exhibited ROR1 expression. A significant difference in ROR1 expression was found between ovarian cancer tissues and normal ovary tissues (p < 0.001). Positive staining was mainly localized in the cytoplasm and partly localized in the nucleus of cancer cells. The observed typical IHC staining shapes for ROR1 expression in ovarian cancer are shown in Figure 2.

Relationship between ROR1 expression and clinicopathological parameters. The relationship between ROR1 protein expression and the clinicopathological parameters of 100 ovarian cancer patients was demonstrated in Table 1. High ROR1 expression was associated with FIGO stage (p = 0.019), tumor grade (p = 0.027) and positive lymph node metastasis (p = 0.015). In contrast, no significant correlation was discovered between ROR1 expression and other clinical parameters, such as age, tumor diameter, histological type, serum CA-125 level, ascites condition and relapse status.

Survival analysis. According to univariate analysis, the disease-free survival of the ovarian cancer patients was correlated with ROR1 expression (p = 0.005), FIGO stage (p = 0.001), and lymph node metastasis (p = 0.001), whereas the overall survival was correlated with ROR1 expression (p = 0.001), FIGO stage (p = 0.001), tumor grade (p = 0.009), and lymph node metastasis (p = 0.001) in overall survival (Tables 2 and 3). Multivariate analysis using Cox regression model indicated that ROR1 protein expression and FIGO stage may serve as independent prognostic factors for disease-free survival (both p < 0.05) and overall survival (both p < 0.05) (Tables 2 and 3). Kaplan-Meier survival curves also demonstrated that ovarian cancer patients with high ROR1 expression and advanced FIGO stage presented a significantly unfavorable disease-free survival time and overall survival time (Figure 3).

Discussion

Despite the substantial advances in surgery and chemotherapy for ovarian cancer over the last few decades, therapeutic failure and disease progression are still quite frequent. Searching for novel and validated biomarkers correlated with the clinical and prognostic characteristics of ovarian cancer is important for diagnosis and treatment. A few studies have suggested several molecular biomarkers of ovarian cancer. In our previous research, we have identified several oncogenic biomarkers, constructed related human monoclonal antibody-based drugs and tested their anti-tumor effectiveness. Similarly, we have been searching a novel and valuable marker which associates with ovarian cancer and could be used as a potential candidate for targeted therapy with monoclonal antibodies.

RTKs have significant functions in cell differentiation, proliferation, angiogenesis, and migration. In this regard, RTK-like ROR1 is an evolutionarily conserved, type-I membrane protein that is primarily expressed during embryogenesis, and it is significant for the morphogenesis of several organs. Although the exact function of ROR1 in malignancies is not well understood, an increasing number of studies have reported that ROR1 expression is highly correlated with the development, progression, and metastasis of various types of human cancers. At the same time, the possible mechanism of ROR1 characteristics is being explored continuously. ROR1 reportedly interacts with casein kinase 1 epsilon to activate phosphoinositide 3-kinase-mediated AKT phosphorylation and cAMP-response-element-binding protein, which are associated with enhanced tumor-cell growth. Also, Wnt5a, a ligand of ROR1, also
participates in the ROR1-dependent signaling pathway in enhancing cancer cell growth\textsuperscript{[12,13]}. Constitutive silencing of ROR1 tumor cells impairs significant proliferation \textit{in vitro} and induces remarkable inhibition of tumorigenesis \textit{in vivo}; and the critical function for ROR1 in malignant phenotypes is sustained by the Met oncogene\textsuperscript{[15,35]}. Treatment with specific antibody for ROR1 in tumor cells induces the downmodulation of vimentin and inhibits cancer migration and invasion in cells as well as tumor metastasis in mice. ROR1 may regulate epithelial-mesenchymal transition and metastasis; antibodies that target ROR1 can inhibit cancer progression and metastasis\textsuperscript{[14]}. Nevertheless, the potential relationship between ROR1 expression and ovarian cancer remains obscure. The present study is the first to investigate the association between ROR1 and ovarian cancer.

| Table 2 | Univariate and multivariate analysis of prognostic factors in ovarian cancer for disease-free survival |
|---|---|---|---|---|---|
| **ROR1 expression** | **Univariate analysis** | **Multivariate analysis** |
| High versus Low | 2.15 | 0.005* | 1.265–3.656 | 2.90 | 0.001* | 1.639–5.128 |
| Age | 0.80 | 0.397 | 0.483–1.335 | 0.29 | 0.001* | 0.168–0.485 |
| ≥60 years versus <60 years | 1.01 | 0.968 | 0.588–1.738 | 0.65 | 0.164 | 0.358–1.190 |
| Tumour diameter | 0.93 | 0.778 | 0.545–1.576 | 0.65 | 0.110 | 0.379–1.103 |
| ≥5 cm versus <5 cm | 1.16 | 0.581 | 0.688–1.948 | 1.16 | 0.581 | 0.688–1.948 |
| FIGO stage | 0.32 | 0.001* | 1.910–5.533 | 1.32 | 0.001* | 1.910–5.533 |
| Stage I and II versus Stage III and IV | 3.25 | 0.001* | 1.910–5.533 | 1.32 | 0.001* | 1.910–5.533 |
| Tumor grade | 1.73 | 0.559 | 0.664–1.587 | 1.73 | 0.559 | 0.664–1.587 |
| Grade 1 versus Grade 2 and 3 | 0.93 | 0.778 | 0.545–1.576 | 0.93 | 0.778 | 0.545–1.576 |
| Histological type | 0.65 | 0.110 | 0.379–1.103 | 0.65 | 0.110 | 0.379–1.103 |
| Serous versus Nonserous | 1.16 | 0.581 | 0.688–1.948 | 1.16 | 0.581 | 0.688–1.948 |
| Serum CA-125 level | 0.93 | 0.778 | 0.545–1.576 | 0.93 | 0.778 | 0.545–1.576 |
| ≥35 U/ml versus <35 U/ml | 0.32 | 0.001* | 1.910–5.533 | 0.32 | 0.001* | 1.910–5.533 |
| Positive versus Negative | 3.25 | 0.001* | 1.910–5.533 | 3.25 | 0.001* | 1.910–5.533 |
| Ascites | 1.73 | 0.559 | 0.664–1.587 | 1.73 | 0.559 | 0.664–1.587 |
| Positive versus Negative | 0.93 | 0.778 | 0.545–1.576 | 0.93 | 0.778 | 0.545–1.576 |
| Lymph node metastasis | 0.65 | 0.110 | 0.379–1.103 | 0.65 | 0.110 | 0.379–1.103 |
| Positive versus Negative | 4.47 | 0.001* | 2.552–7.821 | 4.47 | 0.001* | 2.552–7.821 |
| Relapse status | 2.21 | 0.364 | 0.209–1.306 | 2.21 | 0.364 | 0.209–1.306 |
| Positive versus Negative | 0.72 | 0.235 | 0.423–1.235 | 0.72 | 0.235 | 0.423–1.235 |

The qPCR result with three reference genes all confirmed that the ROR1 mRNA expressions in ovarian cancer tissues were higher than those in normal ovary tissues. The data is consistent with the previous reports, which showed that the mRNA of ROR1 was highly upregulated in cancer cell lines\textsuperscript{[11,13,15]}. For further analysis, we prepared TMA with ovarian cancer specimens. IHC analysis revealed higher ROR1 protein expression in ovarian cancer tissues than that in normal ovary tissues. This result is similar to the studies of various malignancies that indicated high expression of ROR1 in cancer tissues\textsuperscript{[11–13]}. Besides, high ROR1 expression in ovarian cancer was correlated with certain clinical pathologic parameters, including FIGO stage, tumor grade and positive lymph node metastasis. Kaplan-Meier analysis demonstrated that the life span of patients with pos-

| Table 3 | Univariate and multivariate analysis of prognostic factors in ovarian cancer for overall survival |
|---|---|---|---|---|---|
| **ROR1 expression** | **Univariate analysis** | **Multivariate analysis** |
| High versus Low | 3.04 | 0.001* | 1.757–5.246 | 3.54 | 0.001* | 1.990–6.289 |
| Age | 0.70 | 0.163 | 0.421–1.157 | 0.30 | 0.015* | 0.113–0.790 |
| ≥60 years versus <60 years | 1.33 | 0.311 | 0.766–2.313 | 0.30 | 0.015* | 0.113–0.790 |
| Tumour diameter | 0.72 | 0.235 | 0.423–1.235 | 0.72 | 0.235 | 0.423–1.235 |
| ≥5 cm versus <5 cm | 0.21 | 0.001* | 0.122–0.378 | 0.30 | 0.015* | 0.113–0.790 |
| FIGO stage | 0.44 | 0.009* | 0.241–0.820 | 1.15 | 0.715 | 0.544–2.433 |
| Stage I and II versus Stage III and IV | 0.21 | 0.001* | 0.122–0.378 | 0.30 | 0.015* | 0.113–0.790 |
| Tumor grade | 0.44 | 0.009* | 0.241–0.820 | 1.15 | 0.715 | 0.544–2.433 |
| Grade 1 versus Grade 2 and 3 | 1.36 | 0.250 | 0.806–2.292 | 2.01 | 0.141 | 0.795–5.060 |
| Histological type | 0.44 | 0.009* | 0.241–0.820 | 2.01 | 0.141 | 0.795–5.060 |
| Serous versus Nonserous | 0.30 | 0.015* | 0.113–0.790 | 0.30 | 0.015* | 0.113–0.790 |
| Serum CA-125 level | 0.72 | 0.235 | 0.423–1.235 | 0.72 | 0.235 | 0.423–1.235 |
| ≥35 U/ml versus <35 U/ml | 0.44 | 0.009* | 0.241–0.820 | 4.47 | 0.001* | 2.552–7.821 |
| Ascites | 1.36 | 0.250 | 0.806–2.292 | 1.36 | 0.250 | 0.806–2.292 |
| Positive versus Negative | 0.21 | 0.364 | 0.209–1.306 | 0.21 | 0.364 | 0.209–1.306 |
| Lymph node metastasis | 0.72 | 0.221 | 0.421–1.221 | 0.72 | 0.221 | 0.421–1.221 |
| Positive versus Negative | 4.47 | 0.001* | 2.552–7.821 | 4.47 | 0.001* | 2.552–7.821 |
| Relapse status | 2.01 | 0.141 | 0.795–5.060 | 2.01 | 0.141 | 0.795–5.060 |
| Positive versus Negative | 0.72 | 0.221 | 0.421–1.221 | 0.72 | 0.221 | 0.421–1.221 |

*p < 0.05.
ROR1 expression was shorter than that of patients with negative expression. Univariate analysis showed that ROR1 expression, FIGO stage, and lymph node metastasis were correlated with life span (both in disease-free survival and overall survival) of ovarian cancer patients. Multivariate analysis further confirmed that high ROR1 expression and advanced FIGO stage independently predicted unfavorable disease-free survival and overall survival of ovarian cancer patients. Further studies that enroll a larger number of clinical samples of ovarian cancer tissues are necessary to confirm our findings concerning the effectiveness of ROR1 expression on the prognosis of human cancers. In addition, a fully human anti-ROR1 antibody is undergoing construction and evaluation by our group.

In conclusion, to the best of our knowledge, this is the first study to report that high ROR1 expression is correlated with aggressive malignant phenotype of ovarian cancer. Our data indicate that ROR1 may serve as a novel prognostic marker for ovarian cancer and targeting ROR1 may provide a promising strategy for targeted therapy in ovarian cancer treatment.

**Methods**

**Patient specimens.** A total of 100 paraffin-embedded tissue samples of ovarian cancer were collected from the archives of the Department of Pathology, Nanjing Maternal and Children Care Hospital Affiliated to Nanjing Medical University, between July 2003 and July 2010. 50 normal ovary samples from hysterectomy specimens resected for non-ovarian disease in our hospital were collected for control in IHC analysis. The stage of tumors was evaluated according to the International Federation of Gynecology and Obstetrics (FIGO) system. Tumors were graded according to the Silverberg grading system. All the cases were reevaluated for grade and histological type by two independent pathologists (LX and FSL). When a conclusion differed, the final decision was made by consensus. The original clinical data were obtained from hospital medical records, including patient age, tumor size, FIGO stage, tumor grade, histological type, serum CA-125 level, ascites, lymph node metastasis, relapse status, disease-free survival and overall survival. Besides, another 15 ovarian cancer tissue samples of high FIGO stage (FIGO III–IV), 15 ovarian cancer tissue samples of low FIGO stage (FIGO I–II) and nine normal ovary tissue samples were collected for qPCR analysis. None of the patients had received radiotherapy, chemotherapy or immunotherapy before surgery. Written informed consent was obtained from the patients for publication of this study and any accompanying images. Study protocol was approved by the Ethics Committee of Nanjing Maternal and Children Care Hospital Affiliated to Nanjing Medical University and all experiments were performed in accordance with approved guidelines of Nanjing Medical University.

**One-step qPCR test.** Total RNA from ovarian cancer tissues and normal ovary tissues were extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s guidelines. One-step qPCR analysis was performed with a SensiMixTM One-Step Kit (Quantace, London, UK) using a Real Time PCR system (Bio-Rad IQ5, Hercules, CA, USA) according to the standard protocol. Total RNA extraction, quality control, and one-step qPCR were performed as previously described36. The primers for ROR1 which designed by Primer Express Software were as follows: forward primer 5'- TAA TCG GAG AGC AAC TTCA -3' and reverse primer 5' - TGT AGT AAT CAG CGG AGT AA -3'. Three reference genes, peptidylprolyl isomerase A (PPIA), β-actin (ACTB), and TATA-Box binding protein (TBP), were employed to standardize the analysis of the target gene in qPCR test. The primers of three reference genes were as follows: PPIA forward primer GTG GTG TTT GGC AAA GTG AA, reverse primer TCG AGT TGT CCA CAG TCA GC;
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Author contributions
Y.M. and J.Z. designed the study; H.L.Z. and J.R.Q. collected the tissue samples and carried out the initial analysis; F.S.I., X.N., J.T.I. and L.X. performed the iHc analysis; H.L.Z. J.R.Q., C.P.Y., D.Z.Y., J.L.G. and D.W.Z. collected clinical data and participated in the evaluation of the iHc; Y.M. drafted the manuscript; Y.M., F.S.L. and J.Z. supervised the study. All authors read and approved the final manuscript.

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