

# Frequent overexpression of ROR1 in triple-negative breast cancer

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## Research Article

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# Abstract

## Purpose

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) belongs to a family of receptor tyrosine kinases with oncogenic properties. Therapeutic agents targeting ROR1 have yielded encouraging efficacy in several malignancies including triple-negative breast cancer (TNBC). Therefore, we aimed to investigate ROR1 expression in TNBC for future clinical trial design.

## Materials and Methods

148 metastatic and 49 matched primary samples were analyzed. ROR1 expression and immune cell densities were assessed via immunohistochemistry (IHC). Single-cell RNA-sequencing (scRNA-seq) data were additionally collected and analyzed to verify the cell types in which ROR1 is highly expressed.

## Results

66.9% of the metastatic lesions presented with ROR1 expression. ROR1 was more abundantly expressed in metastatic lesions than in matched primary samples (43/49 vs. 35/49,  $P = 0.045$ ). ROR1-negative in metastatic tissues were significantly correlated with better disease-free survival (DFS) [hazard ratio (HR) = 0.570, 95% CI = 0.385–0.843,  $P = 0.004$ ] and overall survival (OS) (HR = 0.308, 95% CI = 0.144–0.657,  $P = 0.001$ ), while the same trend was observed for the primary tissues ( $P < 0.001$  and  $P = 0.015$  respectively). Notably, high levels of ROR1 expression were also associated with more intratumoral CD3<sup>+</sup> and CD8<sup>+</sup> T-cells infiltration. CD3 and CD8 expression was more abundant in metastatic tissues than in primary tumors. scRNA-seq analysis demonstrated that ROR1 was markedly enriched in malignant cells.

## Conclusion

ROR1 is highly abundant in metastatic TNBC with poor prognosis and increased T-cells infiltration. Given the promising results of targeted ROR1 therapy in advanced disease, specific trials that target ROR1 are warranted.

## 1. Introduction

Receptor tyrosine kinase-like orphan receptor 1 (ROR1), a transmembrane receptor tyrosine kinase protein, belongs to the ROR family, which consists of ROR1 and ROR2(1). During embryogenesis, ROR1 plays important roles in multiple physiological processes by mediating signaling in the Wnt-related integration site (WNT) signaling pathway, including the regulation of cell division, proliferation, migration, and chemotaxis(2). Although ROR1 is abundantly expressed during embryonic and fetal development, its

expression level decreases during childhood and adult development. However, ROR1 expression is markedly upregulated in a variety of tumors, making this protein an ideal drug target for cancer therapy, including that of breast cancer(3).

ROR1 has been implicated as an oncogenic driver in many tumors and is broadly linked to cell proliferation, stemness(4), epithelial–mesenchymal transition (EMT)(5) and other metastatic abilities(6). WNT/ROR1 signaling involves the formation of complexes between ROR1 and ROR2 or between ROR1 and Frizzled (FZD) receptors after binding to noncanonical WNT ligands (e.g., WNT5A)(2). WNT5A drives the formation of ROR1/ROR2 heterooligomers to activate signaling, and neither ROR1 nor ROR2 alone efficiently triggered the expected downstream cascade(7).

A previous study revealed that the ROR1 expression level was notably elevated in poorly differentiated tumors, including triple-negative breast cancer (TNBC), and was associated with unfavorable OS(8). In 2017, Balakrishnan A et al. found that 57% of TNBCs exhibited strong ROR1 expression, whereas only 12% of estrogen receptor (ER)- and progesterone receptor (PR)-positive breast cancers showed ROR1 expression, and no ROR1 expression was detected in human epidermal growth factor receptor 2 (HER2)-positive breast cancers(9). Similarly, data from ISPY-2 trial indicated that ROR1-high expression was more prevalent among TNBC patients, while the expression levels of ROR2 were lowest in this subtype(10). Furthermore, resistance to paclitaxel in TNBC may be due to the abundance of cancer stem cells (CSCs) in the tumor microenvironment, which activates downstream signaling pathways through upregulating the expression of ROR1 to increase migration and resistance to paclitaxel(11). To overcome therapeutic failure, approaches to target ROR1, including small-molecule inhibitors, monoclonal antibodies or antibody–drug conjugates, are emerging. Combining paclitaxel chemotherapy with ROR1 blockade substantially suppressed tumor growth inhibition in a TNBC patient-derived xenograft (PDX) model(11).

Currently, TNBC poses significant treatment challenges because of the lack of identifiable targets. Therefore, in the present study, we aimed to investigate the frequency of ROR1 overexpression in primary and metastatic lesions from TNBC patients. Given the pivotal role of immune cells in shaping responses to monoclonal antibody/chimeric antigen receptor modified T (CAR-T) cell therapy, we also comprehensively profiled the tumor immune microenvironment to determine its immune cell composition. We further explored the expression profiles of ROR1 in single-cell RNA sequencing (scRNA-seq) datasets to lend further support to our conclusions, identify relevant cell clusters and gain further biological insight to guide future trial designs.

## **2. Materials and Methods**

### **2.1 Patient cohort**

Patients who were diagnosed with TNBC between March 2005 and July 2022 at the Sun Yat-sen University Cancer Center (SYSUCC) were identified. Eligible patients were aged  $\geq 18$  years with

histologically confirmed TNBC, with available response assessments, and had adequate cardiac, bone marrow, and hepatic function apart from organ function affected by the disease. Formalin-fixed and paraffin-embedded (FFPE) material available for scientific purposes was obtained from the pathology department. Clinical data were extracted retrospectively using medical records. Telephone follow-up was conducted every 6 to 12 months until death or loss to follow-up. Assessment of tumor response according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 was performed via computed tomography (CT) every 2 cycles of systemic treatment or every 3 months following radical surgery(12). This study was approved by the Ethics Committees of SYSUCC (approval number: B2023-368-01) in accordance with the Declaration of Helsinki. Written informed consent was waived because of the retrospective nature of the study.

## 2.2 Immunohistochemistry (IHC)

Sequential histological tumor sections 4  $\mu\text{m}$  thick were cut from a representative FFPE tumor block and used for IHC analysis. The tissue sections were deparaffinized in xylene, hydrated in ethyl alcohol, washed in phosphate-buffered saline (PBS), and subjected to antigen retrieval. Endogenous peroxidase activity was blocked with 3%  $\text{H}_2\text{O}_2$  for 10 minutes. Immunohistochemical staining was performed by using the following antibodies overnight at 4°C: anti-ROR1 (Origene, #TA806841, 1:2000), anti-CD3 (ZS, #ZA-0503, 1:200), anti-CD8 (ZS, #ZA-0508, 1:250), anti-CD68 (ZS, #ZM-0060, 1:100) and anti-CD163 (ZS, #ZM-0428, 1:100). A protocol including specific conditions for each antibody is provided in **Table S1**. The sections were then washed twice in PBS for 3 minutes each, then incubated with secondary antibodies for 1 hour at room temperature. After washing in PBS, staining was developed using 3,3'-diaminobenzidine (DAB).

All stained images were scanned by a digital pathological section scanner (KFBIO, Ningbo Jiangfeng BioInformation Technology Co., Ltd., China) at a magnification of 40 $\times$  and analyzed via K-viewer software (version 1.5.3.1; KFBIO). Two independent pathologists examined and evaluated the IHC-stained slides. The histological score (H-score) was calculated to evaluate the expression of ROR1. The H-score was determined by multiplying the membrane staining intensity (0, negative; 1, weak; 2, moderate; 3, strong) by the percentage of positively stained cells (1–100%). The immune cell CD3, CD8, CD68 and CD163 subsets were evaluated by randomly selecting three areas (1  $\text{mm}^2$ ), counting the number of positive cells at 200 $\times$  magnification and taking the average of the three fields as the final positive cell density score. Representative IHC images for these markers are presented in Fig. 1A and Fig. 3A.

## 2.3 Bioinformatic Analysis

The scRNA-seq data of breast cancer tissues were obtained from the GEO dataset (GSE150660 and GSE176078) via the Tumor Immune Single-cell Hub (TISCH) database (<http://tisch.comp-genomics.org/home/>)(13). Raw UMI counts were normalized with Seurat and scaled to 10,000 per cell.. A uniform analysis algorithm (MAESTRO) was adopted for each dataset to perform quality control, clustering, and cell-type annotation. The GSE150660 dataset contains 10,605 cells from cerebrospinal

fluid of 3 breast cancer patients with leptomeningeal metastasis, whereas the GSE176078 dataset consists of 89,471 cells from 26 primary breast cancer tissues (10 TNBCs). The scRNA-seq was conducted by the 10× Genomics platform. The infiltration abundances of CD8<sup>+</sup> T cells were estimated with TIMER2 (<http://timer.cistrome.org>) via multiple immune deconvolution methods.

## 2.4 Statistical analysis

Continuous variables are presented as medians with ranges, whereas categorical variables are expressed as percentages. The cutoff for biomarkers were determined using the median value. Clinicopathological variables associated with ROR1 expression were analyzed using the chi-square test or Fisher's exact test. Differences between groups were tested using a two-tailed Mann–Whitney test or Kruskal–Wallis test. McNemar test or paired t-test were applied for the paired categorical or quantitative comparisons respectively. Disease-free survival (DFS) was defined as the interval from surgery to disease relapse (local or distant relapse or death from any cause). Overall survival (OS) was calculated from the date of recurrence/metastasis to death from any cause or the last follow-up. The Kaplan–Meier method was used to construct DFS and OS survival curves with the log-rank test. A two-sided *P* value < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism, Version 9.4.0, IBM SPSS Statistics, Version 26.0, and the R statistical environment v.4.1.1.

## 3. Results

### 3.1 Patient characteristics

A total of 181 patients with TNBC were identified, of whom 21 had incomplete clinical information and 12 did not have available pathological specimens. Finally, 148 patients were included in this study (**Figure S1**). Of these, matched primary and metastatic samples were available from 49 patients. The clinical-pathological characteristics of the patients are summarized in **Table 1**. The median age of the patients was 51 years (range from 29 to 75). 27 (18.2%) patients were at initially De novo stage IV and the common metastatic sites included the lymph nodes (65.5%), bone (32.4%), lung (38.5%), chest wall (28.4%), liver (17.6%), and brain (6.8%). A total of 66.2% of the patients received (neo)adjuvant systemic treatment, and 35.8% received adjuvant radiotherapy. At the last follow-up, 33 (22.3%) patients had died of TNBC. Patient characteristics according to the ROR1 expression level are summarized separately in Table 1.

### 3.2 ROR1 is frequently expressed in metastatic lesions

ROR1 immunostaining was heterogeneously distributed across tumor tissues, with expression intensities ranging from negative to low or high (**Figure 1A**). The median H score was 135 among all samples, and cases were classified as negative (total score = 0), low (total score 1–135) or high (total score 136–300). In the absence of an established clinical threshold, this exploratory categorization was adopted to better delineate the biological relevance of ROR1 expression levels. Overall, 99/148 (66.9%) metastatic tissues were ROR1-positive (**Figure 1B; Table 1**), with high expression detected in 50

metastatic samples (33.8%). Notably, 58/96 (60.4%) HER2-negative metastatic samples exhibited ROR1 expression, and 41/52 (78.8%) HER2-low metastatic samples were positive for ROR1 ( $P = 0.023$ ; **Figure 1C**).

### 3.3 ROR1 expression is more frequent in metastatic lesions than in matched primary tumors

Matched tumor tissues for the analysis of ROR1 were available from 49/148 (33.1%) patients. The expression level of ROR1 was significantly different between primary and metastatic tumor tissues. Any level of ROR1 expression was more commonly observed in metastatic lesions than in primary breast tumors (43/49 vs. 35/49;  $P = 0.045$ ; **Figure 1D**), and ROR1-high expression was more frequently observed in metastatic lesions than in primary breast tumors (49.0% vs. 26.5%;  $P = 0.018$ ). Among the HER2-low patients, 22/35 (62.9%) were also positive for ROR1 in metastatic tumors, whereas 11/42 (26.2%) were positive in primary tumors (**Figure 1E**).

### 3.4 Correlation of ROR1 with survival time

At the last follow-up date of May 21, 2023, the median follow-up time of the whole cohort was 46.4 months [95% confidence interval (CI), 36.2–56.5]. Kaplan–Meier survival curves revealed that the median DFS [hazard ratio (HR) = 0.570, 95% CI = 0.385–0.843,  $P = 0.004$ , **Figure 2A**] and OS (HR = 0.308, 95% CI = 0.144–0.657,  $P = 0.001$ , **Figure 2B**) were longer in patients with ROR1-negative. When categorized by expression level, patients with ROR1-high levels had worse DFS and OS ( $P$  all < 0.001, **Figure 2C–D**). In the primary tissues of 49 patients, the same trend was also observed ( $P < 0.001$  and  $P = 0.015$  respectively, **Figure S2A–B**). Univariate and multivariate analyses revealed that ROR1-negative independently predicted favorable OS (Table S2).

### 3.5 ROR1 expression is correlated with the inflammatory tumor microenvironment composition

We investigated the density of 4 immune cell markers per  $\text{mm}^2$ . Representative IHC images for low- or high-density intratumoral tissue-infiltrating lymphocytes (marked by CD3 or CD8) and tissue-infiltrating macrophages (marked by CD68 or CD163) are shown in **Figure 3A**. Next, we analyzed the distribution of immune markers and their correlations. Compared with patients with negative or low ROR1 expression, those with high ROR1 expression exhibited significantly more intratumoral CD3<sup>+</sup> ( $263 \pm 159$  vs.  $512 \pm 238$  vs.  $662 \pm 292$  cells/ $\text{mm}^2$ ,  $P < 0.001$ , **Figure 3B**) and CD8<sup>+</sup> ( $89 \pm 66$  vs.  $177 \pm 99$  vs.  $259 \pm 109$  cells/ $\text{mm}^2$ ;  $P < 0.001$ , **Figure 3C**) T cells. However, no significant difference was observed between the CD68<sup>+</sup> ( $96 \pm 95$  vs.  $109 \pm 83$  vs.  $105 \pm 75$  cells/ $\text{mm}^2$ ,  $P = 0.695$ , **Figure 3D**) and CD163<sup>+</sup> ( $45 \pm 48$  vs.  $60 \pm 60$  vs.  $62 \pm 50$  cells/ $\text{mm}^2$ ,  $P = 0.195$ , **Figure 3E**) macrophage densities in patients with different levels of ROR1 expression. Notably, CD3 and CD8 expression in the intratumoral areas of the metastatic tissues was also higher than that in the primary tumors, regardless of the level of ROR1 expression ( $P$  all < 0.05, **Figure 4A–F**).

### 3.6 Identification of ROR1<sup>+</sup> cell clusters in metastatic and primary tissues

Given the prognostic value and unusual distribution of ROR1 in TNBC tissues, we explored the cell types in which they are enriched through scRNA-seq. The scRNA-seq data in the GSE150660 dataset were obtained from cerebrospinal fluid of 3 breast cancer patients with leptomeningeal metastasis. A total of 16 cell clusters and 7 cell types were identified (**Figure 5A–B**). ROR1 was markedly enriched in malignant cells, especially in the C3 cluster (**Figure 5C**). Cell–cell interaction (CCI) analysis revealed that ROR1<sup>+</sup> malignant cells interact mainly with other malignant cell clusters and CD8<sup>+</sup> T cells (**Figure 5D–E**), corroborating our IHC findings. We also analyzed another scRNA-seq dataset, GSE176078, in which primary specimens from 26 breast cancer patients were obtained. The results showed that 39 cell clusters and 11 cell types are identified in the breast cancer tissue samples (**Figure S3A–B**). However, ROR1 was predominantly enriched in CAFs and endothelial cells, especially in the C7 cluster (**Figure S3C**), rather than in malignant cells. This may explain why compared with matched primary tumors, metastatic lesions exhibit more frequent ROR1 expression.

To support the above results, we analyzed the correlation between ROR1 expression and CD8<sup>+</sup> T-cell infiltration levels in breast cancer tissues on the basis of three bulk RNA-seq datasets. As shown in **Figure S3D**, analysis of the TCGA BRCA cohort by TIMER algorithms revealed that ROR1 expression is negatively correlated with the purity but positively correlated with the infiltration level of CD8<sup>+</sup> T cells. These exploratory analyses require further confirmation.

## 4. Discussion

ROR1 is currently of particular interest in the context of many types of tumors, including breast cancer, and represents a promising therapeutic target for personalized therapeutic approaches. In the current study, we explored the expression of ROR1 and the immune microenvironment of primary tumors and metastatic lesions in TNBC patients and then evaluated their prognostic value. To our knowledge, this study provides preliminary evidence that ROR1 is frequently expressed in metastatic TNBC patients, with a significantly higher rate and intensity in metastatic lesions than in matched primary tumors. Moreover, high ROR1 expression is correlated with worse DFS and OS and a more robust inflammatory tumor microenvironment (TME).

A previous study showed that strong ROR1 staining could be detected more frequently in grade III breast invasive ductal carcinomas than in grade I and II invasive ductal carcinomas(8). Cui et al. examined ROR1 expression in 14 breast cancer cell lines and reported that all basal-type cell lines expressed ROR1, whereas luminal-type cell lines generally did not(5). Parker et al. used the annotated I-SPY2 transcriptome data from a cohort of nearly 1000 patients with newly diagnosed high-risk early breast cancer and found that ROR1 expression levels was prevalent in TNBC subtype. Although not correlated with pathological complete response (pCR), high-level expression of ROR1 distinctly identified breast cancer patients with different tumor subtypes with adverse outcomes(10). In our study, approximately two-thirds of metastatic samples presented with detectable ROR1 expression of any intensity, whereas almost half of them had high expression, as defined by H-scores > 135. Our findings were also consistent with those of previous studies in that strong ROR1 expression correlated with tumor recurrence and was

associated with shorter DFS and OS(5, 8, 14). Given the lack of systemic treatment options in advanced TNBC thus far, our data may support the further development of ROR1-directed therapies.

Moreover, compared with HER2-negative subtypes, the HER2-low subtype presented more frequent ROR1 expression. A recent study reported the mechanistic regulation of the ROR1/HER3/lncRNA signaling axis in modulating the Hippo–YAP pathway(15). YAP1-mediated EGFR overexpression and chemoresistance have recently been described in esophageal cancer(16). T-DM1-mediated HER2 inhibition increases the expression of ROR1 through YAP1 activation, thus conferring therapeutic resistance in a critical subset of cells(17). The connection between ROR1 and HER2, along with the specific underlying mechanism, warrants further investigation in the future. A synergistic therapeutic strategy may exhibit significant efficacy, thereby providing a novel therapeutic approach to benefit breast cancer patients.

ROR1 was expressed more frequently in metastatic tissues than in matched primary tumors from the same patients, — a pattern that contrasts with the behavior of other immune-related biomarkers in metastatic TNBC, such as PD-L1, which has been reported to decrease in expression at recurrent and metastatic sites relative to the corresponding primary tumors(18). This divergent trajectory of ROR1 upregulation during metastatic progression underscores its distinct biological role and supports the importance of ROR1 signaling in tumor occurrence, development and metastasis. ROR1 expression is highly restricted to undifferentiated tumors, and it promotes tumor resistance and metastasis with the promotion of epithelial-to-mesenchymal transition (EMT)(19). A previous study also reported high-level ROR1 or ROR2 was associated with high-level expression of genes involved in EMT(10). The induction of ROR1 expression results in a proportional increase in CSC enrichment, thereby leading to a further increase in the risk of tumor recurrence(20).

To gain more insight into the link between ROR1 expression and the inflammatory tumor microenvironment composition in TNBC, we determined the density of four immune cell types. Our results demonstrated that patients with ROR1-high expression had significantly more intratumoral CD3<sup>+</sup> and CD8<sup>+</sup> T cells, but no difference was observed between the CD68<sup>+</sup> and CD163<sup>+</sup> macrophage densities in patients with different levels of ROR1 expression. Compared with that in the primary tumors, the expression of CD3 and CD8 in the intratumoral areas of the metastatic tissues was also greater regardless of the level of ROR1 expression. Recent evidence has suggested that TILs are associated with a favorable prognosis of TNBC patients(21). High numbers of tumor-associated macrophages are significantly associated with increased angiogenesis, worse breast cancer-specific survival and shorter disease-free intervals(22).

The CD3<sup>+</sup> T-cell population, which encompasses all T-cell subsets, including cytotoxic T lymphocytes (CTLs), T helper (Th) cells, and regulatory T cells (Tregs), can orchestrate antitumor immunity both directly via CTL-mediated killing and indirectly through the secretion of cytokines(23). CD8<sup>+</sup> cytotoxic T cells infiltrate the tumor core or invading site and play essential roles in killing cancer cells upon activation(24). The significantly higher expression of CD3 and CD8 in metastatic lesions than in primary

tumors in our study suggested a complex tumor microenvironment in metastatic tissues which may exhibit enhanced anti-tumor effect. However, we did not evaluate the functional characterization of the T cells, further research is needed to investigate the expression level of TNF- $\alpha$ , IFN- $\gamma$  or other functional markers.

Specifically, patients with ROR1-high expression had higher densities of CD3<sup>+</sup> and CD8<sup>+</sup> T cells than those with negative or low ROR1 expression did. However, the link between ROR1 expression and T cells infiltration has not been thoroughly investigated. Prior research has demonstrated that WNT5A drives inflammation and chemotaxis via ROR1–AKT–p65 axis, creating a pro-inflammatory milieu. Subsequent TLR–MyD88–p50 signaling in myelomonocytic cells then reprograms mononuclear phagocytes toward a tolerogenic state(25). Furthermore, WNT5A– $\beta$ -catenin signaling drives an immunosuppressive program characterized by elevated PD-L1, and blocking this pathway enhances anti-PD-L1 efficacy(26). This might explain why the expression of ROR1 can lead to an increase in the level of T cells infiltration, and future studies are warranted to elucidate the precise molecular mechanisms involved.

To date, several therapeutic strategies targeting ROR1, including monoclonal antibodies (mAbs), antibody-drug conjugate (ADC), CAR-T cell therapy and small-molecule inhibitors have been developed and evaluated in clinical trials and preclinical studies(27–30). Despite promising results in some solid tumors, the mechanisms by which these compounds and immune cells exert their effects must be delineated. Furthermore, the correlation between ROR1 expression and the response to ROR1-targeted therapy is currently under investigation.

The current study has several limitations. First, this was a single-center retrospective study with a small sample size and should be further verified in a larger multicenter cohort. Second, the density of immune cells was detected by IHC, while the relative proportions of immune cells measured by transcriptome data may be more precise. Third, the conventional scoring method in our research does not explicitly distinguish between malignant and stromal compartments. Finally, due to the small sample size and multiple metastatic sites, whether ROR1 expression differed by metastatic site could not be demonstrated. Nevertheless, this study represents a comprehensive work on ROR1 expression and the microenvironment composition in TNBC thus far which including matched primary and metastatic samples.

In conclusion, our data highlight the role of ROR1 expression in TNBC especially in metastatic lesions, may warrant further clinical development of ROR1-targeting therapeutics in patients.

## **Declarations**

## **Ethics Approval and Consent to Participate**

The study protocol was approved by the Institutional Review Board of the Sun Yat-Sen University Cancer Center (approval number: B2023-368-01), and the study was performed in accordance with the principles

of the Declaration of Helsinki. Informed consent was waived by the ethics committee because this was a retrospective, non-interventional study collecting secondary data. All methods were performed in accordance with the relevant guidelines and regulations.

## Consent for publication

Not applicable.

## Conflicts of Interest

The authors declare no competing interests.

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## Author Contribution

Limin Chen and Na Wang: Writing—original draft, data curation, formal analysis, visualization. Mei Li: Methodology, data curation. Huailiang Wu, Mengqian Ni, Qianyi Lu : Data curation, validation. Fei Xu: Conceptualization, resources. Ruoxi Hong, Shusen Wang: Conceptualization, investigation, supervision, writing—reviewing and editing. All authors read and approved this manuscript.

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## Data Availability

The sc-RNA data analyzed in this study are available in GEO database under the accession number GSE150660 and GSE176078. The immune infiltration estimation was performed in the TIMER2

(<http://timer.cistrome.org>). All the other data generated during the current study are available from the corresponding author upon reasonable request.

## Table 1

Table 1 is available in the Supplementary Files section.

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## Figures

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### Figure 1

Frequent overexpression of ROR1. **(A)**. Representative IHC images of ROR1 grouped by staining intensity into negative, weak, moderate and strong groups. Scale bar, 100  $\mu$ m. **(B)**. The proportion of ROR1 expression in metastatic tissues. **(C)**. Correlation between HER2 IHC score and ROR1 expression in metastatic tissues. **(D)**. Sankey diagram demonstrating the transformation of ROR1 expression between matched primary tumors and corresponding metastatic lesions. **(E)**. The proportion of ROR1 expression in matched primary tumors and corresponding metastatic lesions.

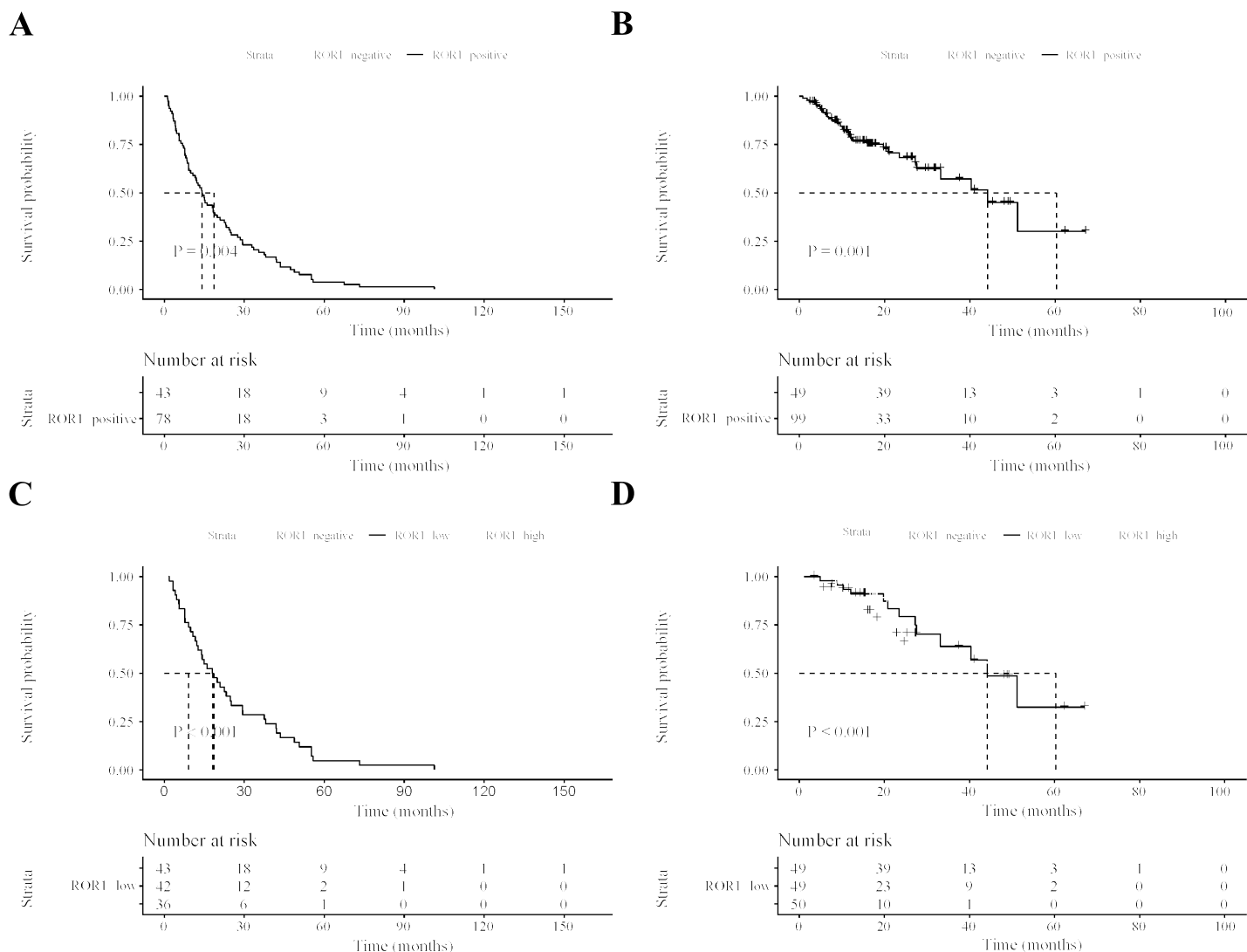


**Figure 2**

Survival analysis according to ROR1 expression in metastatic lesions of patients. DFS **(A)** and OS **(B)** in ROR1-negative patients versus ROR1-positive patients. DFS **(C)** and OS **(D)** among the ROR1-negative, ROR1-low and ROR1-high groups.

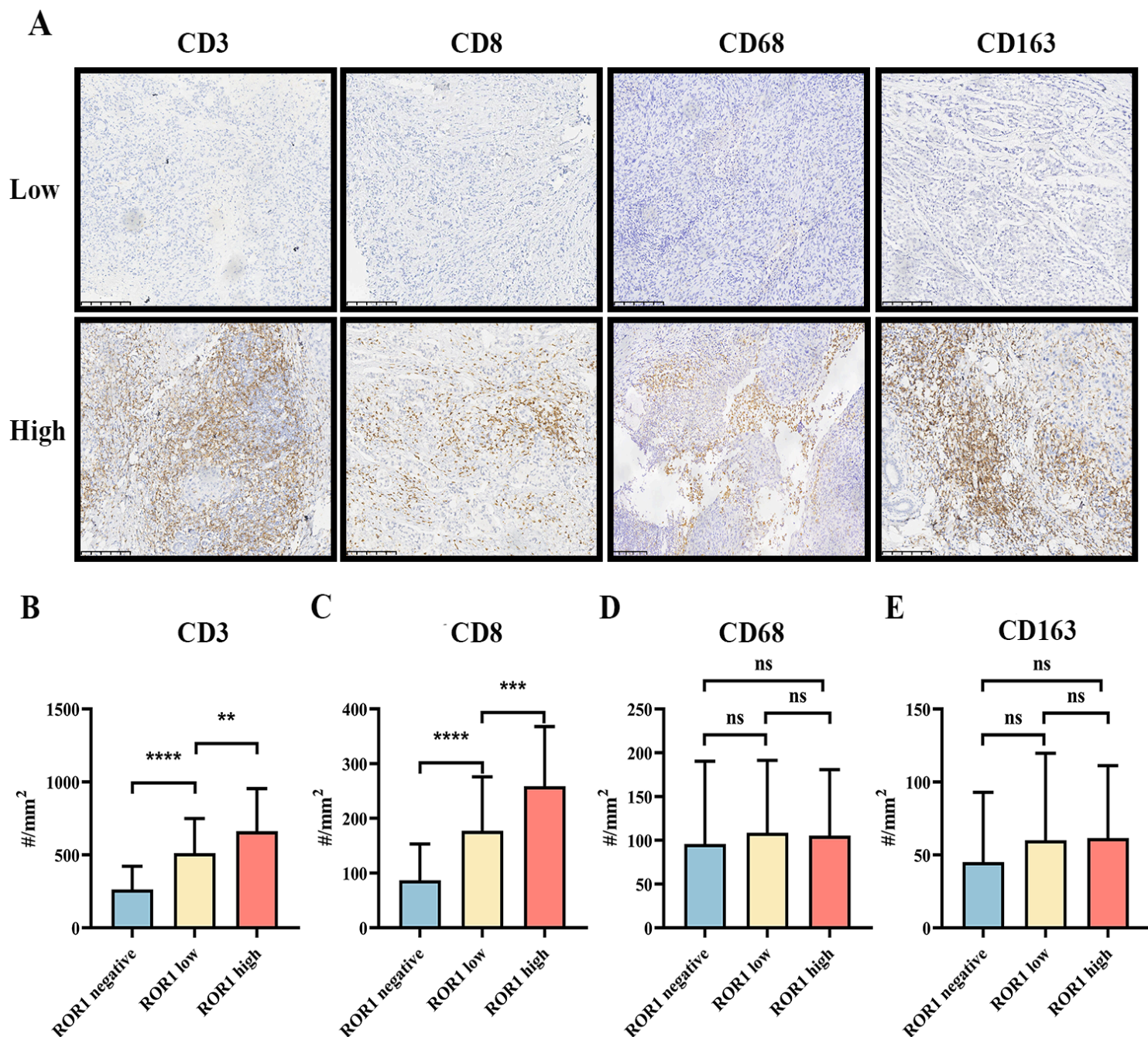


**Figure 3**

Association between immune cell infiltration level and ROR1 expression. **(A)**. Representative IHC images for low- and high-density immune cells marked by CD3, CD8, CD68 and CD163 expression in tumor tissues. Comparisons of intratumoral CD3<sup>+</sup> **(B)**, CD8<sup>+</sup> **(C)**, CD68<sup>+</sup> **(D)** and CD163<sup>+</sup> **(E)** immune cells among the ROR1-negative, ROR1-low and ROR1-high groups.

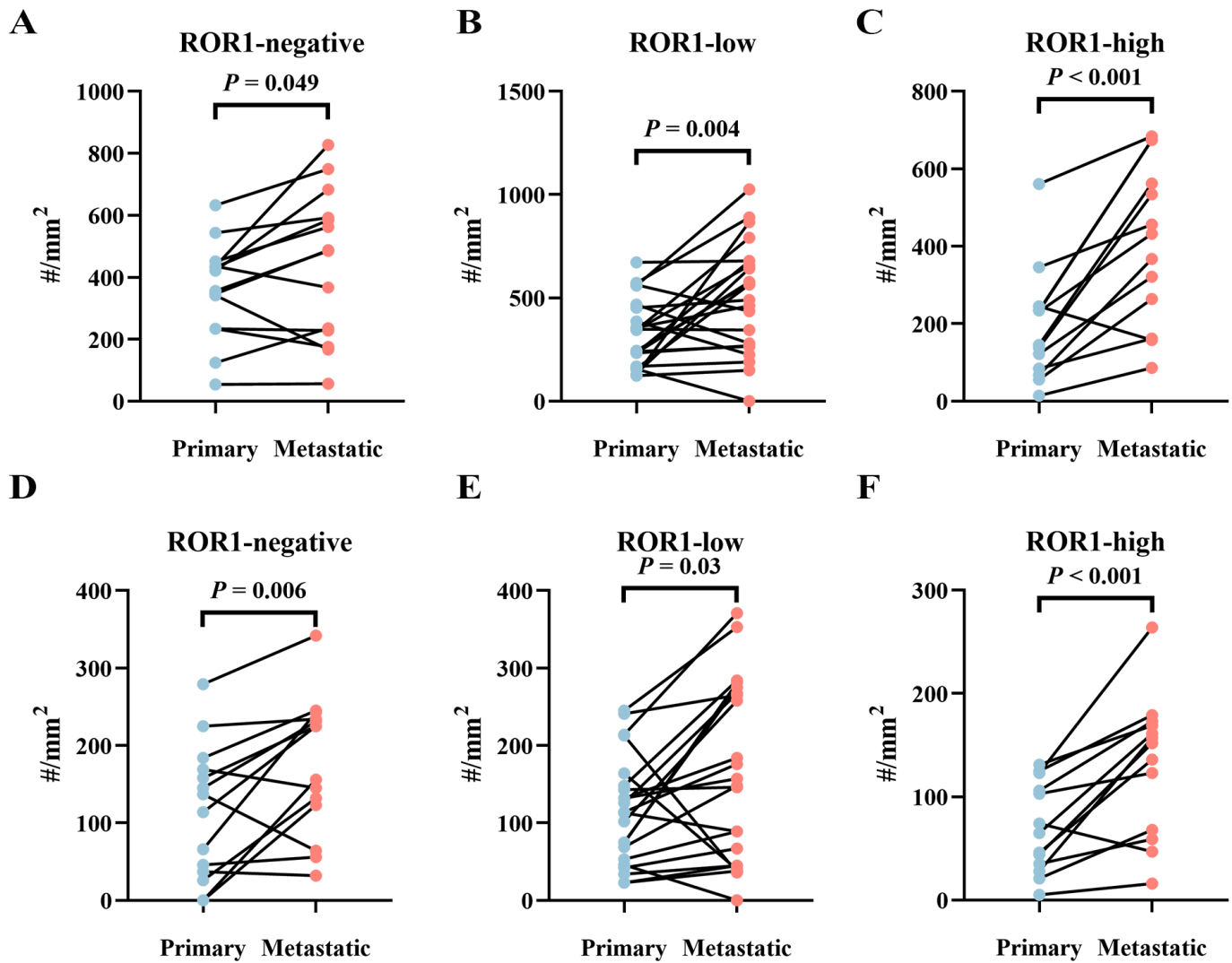


**Figure 4**

Differential expression of immune cells in matched primary tumors and metastatic lesions. CD3 expression in intratumoral areas of the metastatic tissues in the ROR1-negative (**A**), ROR1-low (**B**) and ROR1-high (**C**) groups. CD8 expression in intratumoral areas of the metastatic tissues in the ROR1-negative (**D**), ROR1-low (**E**) and ROR1-high (**F**) groups.

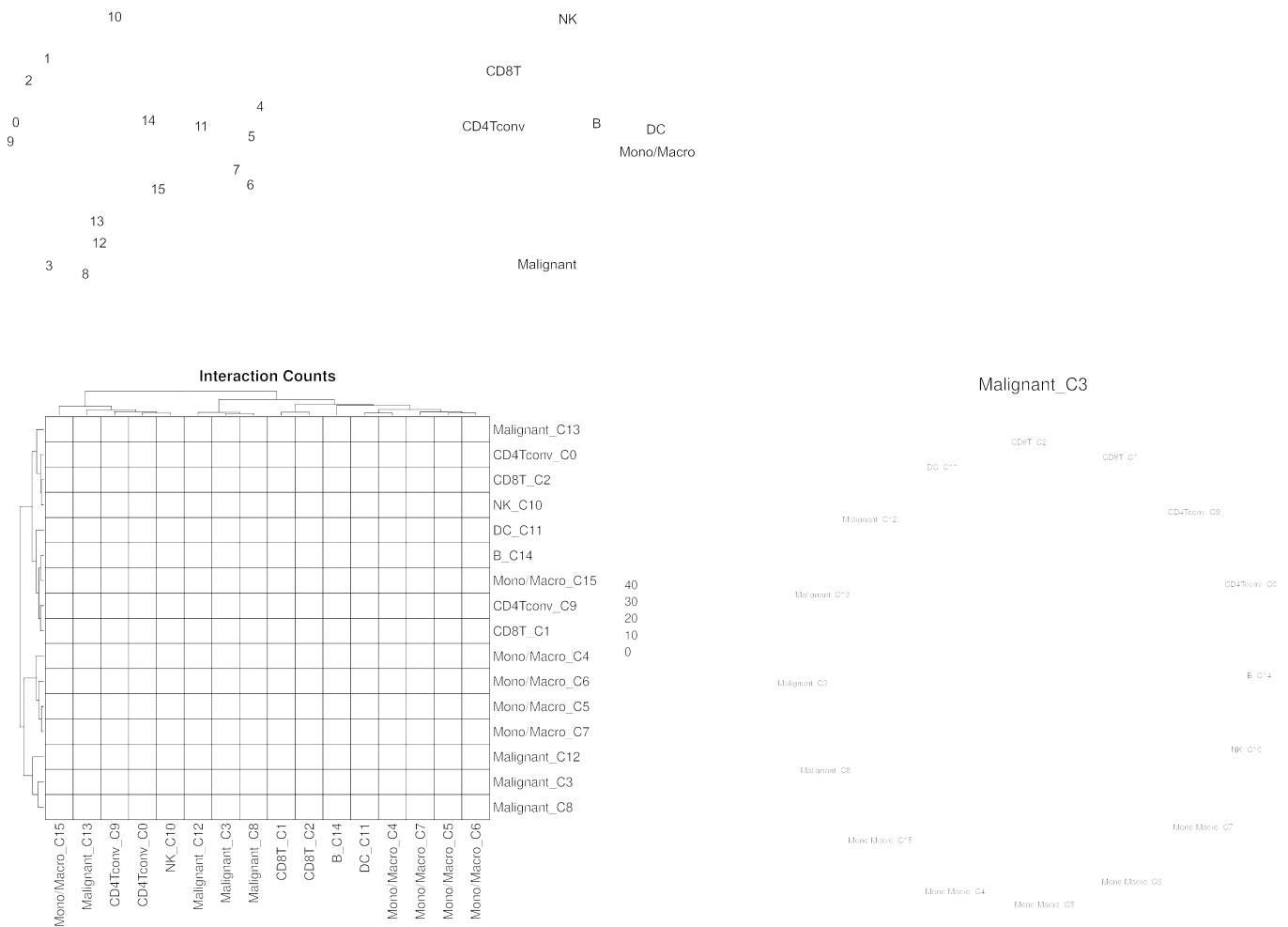


**Figure 5**

ROR1<sup>+</sup> cells in breast cancer tissues were identified in the GSE150660 dataset. The identified cell clusters **(A)** and cell types **(B)** and the expression level of ROR1 in the identified cell types **(C)** in the GSE150660 dataset. **(D)**. Heatmap demonstrating the interactions of all the identified cell clusters. **(E)**. Interactions between ROR<sup>+</sup> malignant cells and other cells.

## Supplementary Files

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