Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Novel Insights into the Oncogenic Role and Clinical Significance of ORC6L in Breast Cancer

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Purpose: ORC6L has been implicated in various malignancies, and our preliminary bioinformatic analysis revealed its significant overexpression in breast cancer specimens. This study aims to investigate the biological functions and clinicopathological significance of ORC6L in breast cancer, and to evaluate its potential as a prognostic biomarker and therapeutic target.

Methods: ORC6L protein expression in breast cancer tissues was assessed via immunohistochemistry, followed by statistical analysis to determine its diagnostic and prognostic significance. To investigate the biological functions of ORC6L, a series of in vitro experiments were conducted in breast cancer cell lines. Cell proliferation was measured using colony formation, CCK-8, and EdU assays. Apoptosis was evaluated by flow cytometry. Cell migration and invasion were assessed via wound-healing and transwell assays, respectively.

Results: ORC6L expression was markedly elevated in breast cancer tissues compared with adjacent non-tumor tissues. This elevated expression demonstrated high diagnostic value and was positively correlated with higher pathological grade. Moreover, ORC6L emerged as an independent predictor of poor survival outcomes in breast cancer patients. In vitro functional assays further revealed that ORC6L overexpression promoted proliferation, suppressed apoptosis, and enhanced migration, whereas ORC6L knockdown effectively inhibited proliferation, induced apoptosis, and attenuated migration.

Conclusion: ORC6L serves as a key driver of breast cancer progression, supporting its potential as a therapeutic target, though its independent prognostic value requires further validation.

Keywords: ORC6L, breast cancer, cell proliferation, apoptosis

Introduction

Among women worldwide, breast cancer is the second most commonly diagnosed malignancy and the fourth leading cause of cancer mortality.[1] In 2022 alone, this disease accounted for approximately 2.3 million new cases and 670,000 deaths, highlighting a substantial global burden with marked regional disparities. Higher-income countries report higher incidence but lower mortality due to advanced screening and care, whereas lower-income countries experience lower incidence yet higher mortality driven by healthcare inequities. If current trends persist, the global burden is projected to rise sharply by 2050—a 38% increase in cases and a 68% increase in deaths—disproportionately affecting low- and middle-income countries.[2] As a highly heterogeneous disease, breast cancer develops through a complex interplay of genetic, hormonal, reproductive, behavioral, and lifestyle factors, along with dysregulated signaling pathways and related receptors.[3] Yet a systematic understanding of the key drivers underlying distinct molecular subtypes, the unrecognized biomarkers of early progression, and therapeutically actionable vulnerabilities remains lacking. This knowledge gap narrows prevention opportunities, contributes to variable treatment responses, and perpetuates drug resistance and disease relapse.[4] Therefore, deciphering core mechanisms of breast cancer pathogenesis, identifying novel biological signatures, and translating these insights into more precise and accessible therapies has become urgent priorities for advancing global breast cancer control and reducing health inequities.

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The Origin Recognition Complex (ORC) is a six-subunit DNA-binding assembly that licenses origins and initiates DNA replication once per cell cycle.[5–7] During mitosis, the smallest subunit ORC6L of human ORC is located on the centromere and the reticular structure at the cell edge, where it regulates chromosome segregation, DNA replication, and cytokinesis. Prolonged ORC6L deficiency leads to reduced cell proliferation and increased cell death, highlighting its critical role in cell cycle regulation.[8]

Beyond its essential role in normal cell cycle progression, accumulating evidence has implicated ORC6L in tumor malignancy through diverse mechanisms. Sang et al demonstrated that ORC6L drives cell cycle progression by upregulating cyclins A2, B1, and D1, enhances DNA replication, suppresses apoptosis, and facilitates migration and invasion.[9] Zhang et al further showed that in non-small cell lung cancer, ETV4 interacts with ORC1L and ORC6L subunits of the origin recognition complex, facilitating ORC assembly at specific replication origins and enhancing DNA replication initiation, and thereby enhancing cell proliferation.[10] In addition to its role in replication, ORC6L contributes to genome maintenance. Lin et al reported that ORC6L localizes to replication forks and acts as an accessory factor in the mismatch repair complex, facilitating its assembly and activation under oxidative stress to ensure genomic stability.[11] Post-replication, proteasome-dependent dissociation of ORC6L prevents aberrant MCM reloading and tetraploidization.[12]

Consistent with these mechanistic insights, ORC6L overexpression has been linked to aggressive phenotypes across cancer types, including enhanced proliferation, migration, and invasion in hepatocellular carcinoma.[13] Pan-cancer profiling revealed ORC6L mRNA to be broadly up-regulated in 29 tumor types, except acute myeloid leukemia.[14] Zhu et al subsequently confirmed consistent ORC6L overexpression in glioma and other solid malignancies.[15] Consistently, Deng et al identified ORC6 as a prognostic gene in hepatocellular carcinoma, with its high expression associated with poor patient prognosis and contributing to tumor cell proliferation, migration, and invasion.[16] Elevated levels independently predict poor prognosis in colorectal and gastric cancer,[17,18] whereas in glioma, they correlate with wild-type IDH, higher tumor grade, and shortened overall survival (OS).[19] Collectively, these findings establish ORC6L as a multifunctional regulator of DNA replication, repair, and cell cycle progression, whose dysregulation significantly contributes to cancer pathogenesis. Thus, targeting ORC6L represents a promising therapeutic strategy for disrupting replication fidelity and genome stability in cancers such as breast cancer.

Whether these pan-cancer observations extend to breast cancer remains insufficiently explored. Building on our previous bioinformatic identification of ORC6L as a significantly upregulated and adverse prognostic factor in breast cancer,[20] the present study aimed to validate its oncogenic role through comprehensive expression analysis in clinical specimens and functional assays in breast cancer cells, thereby establishing ORC6L as a therapeutically actionable vulnerability in this disease.

Materials and Methods

Immunohistochemistry

Human breast cancer tissue microarrays (comprising 280 cancer specimens, with 238 available for analysis) and adjacent non-tumor tissue microarrays (containing 77 specimens, with 64 available) were procured from Outdo Biotech Co., Ltd. (Shanghai, China). The final analysis included 237 breast cancer specimens with complete overall survival data. Clinicopathological and prognostic data were also obtained for these cases, including age, gender, tissue type, pathological grade, tumor stage, survival status, survival duration, ER status, PR status, HER-2 status, and Ki-67 expression index, with data availability varying by factor. The tissue collection and usage were approved by the Ethics Committee of Shanghai Outdo Biotech Co., Ltd. in 2016 and 2019 (Approval Nos. SHYJS-CP-1607001, SHYJS-CP-1607006, and SHYJS-CP-1901002), with written informed consent secured from all participants. This study was also approved by the Ethics Committee of the Affiliated Hospital of Guilin Medical University (Approval No. 201900147). For immunohistochemical staining, tissue microarrays were subjected to overnight incubation at 4°C using a mouse polyclonal anti-ORC6L antibody (Abcam, UK; Cat. # ab88686, diluted 1:400). Following washing, arrays underwent 60-minute exposure to HRP-conjugated anti-mouse IgG (1:1000 dilution) at room temperature. Immunoreactive signals were visualized utilizing 3,3′-diaminobenzidine (DAB) chromogen, followed by nuclear counterstaining employing hematoxylin. Immunohistochemical (IHC) evaluation was performed based on two parameters: (1) staining intensity, graded as 0

(absent), 1 (mild), 2 (moderate), or 3 (intense); and (2) the percentage of immunoreactive cells, categorized as 0 (0%), 1 (1 ~ 25%), 2 (26 ~ 50%), 3 (51 ~ 75%), or 4 (76 ~ 100%). The final immunohistochemical score (range 0 ~ 2) was derived by multiplying the intensity and percentage values.[21]

Cell Culture and Transfection

MDA-MB-231, MDA-MB-436, MCF7, and T47D breast cancer cell lines purchased from the Cell Bank of Chinese Academy of Sciences in Shanghai. Cells were cultured in RPMI 1640 medium (T47D and MDA-MB-436) or DMEM medium (MDA-MB-231 and MCF7) (Corning, USA), both supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA) and 1% penicillin-streptomycin (Solarbio, China). Cultures were incubated at 37°C with 5% CO_2. siRNA sequences targeting ORC6L, along with their negative controls, were commissioned from and synthesized by GenePharma (China). The siRNA target sequences were as follows: si-ORC6L, sense 5′-CAGCAUGCAAGAUUCUAAATT-3′, antisense 5′-UUUAGAAUCUUGCAUGCUGTT-3′; negative control, sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense 5′-ACGUGACACGUUCGGAGAATT-3′. The GV657-ORC6L overexpression plasmid (oe-ORC6L) was constructed and sequence-verified by WZ Biosciences Inc. (Shandong, China). The empty GV657 vector (oe-NC) from the same manufacturer was used as the negative control. Cells were transfected using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's guidelines.

Reverse Transcription and qRT-PCR

Total RNA was extracted from four breast cancer cell lines (MDA-MB-231/436, MCF7, T47D) using Trizol® (Invitrogen, USA) according to the manufacturer's instructions. Subsequently, First-strand cDNA synthesis from 2 μg of total RNA was carried out with the RevertAid cDNA Synthesis Kit (Thermo Fisher Scientific, USA), which includes a DNase I treatment step. For quantitative analysis, qRT-PCR was conducted using TB Green® Premix Ex Taq™ (Tli RNaseH Plus; TaKaRa, Japan) on a StepOnePlus system™ (Applied Biosystems, USA), following the standard thermal cycling protocol. The relative expression of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as the endogenous reference gene for normalization. The primer sequences used were as follows: ORC6L-F: GTCCAGTCTTCCCCAGACAC and ORL6L-R: CCATTTCCTTTGCTGGGGCT; and GAPDH-F: AACGGATTTGGTCGTATTGG and GAPDH-R: TTGATTTTGGAGGGATCTCG.

Western Blotting

Cellular samples were harvested and lysed using RIPA lysis buffer supplemented with protease inhibitors (Roche, Switzerland). For immunoblotting analysis, protein concentrations were determined with a BCA assay kit.(Thermo Fisher Scientific, USA) Samples (30 μg) were separated by 12% SDS-PAGE (CWBIO, China) and transferred to PVDF membranes (Millipore, USA). Which were subsequently incubated with primary antibodies (4°C, overnight) followed by appropriate secondary antibodies (37°C, 1 h).Protein bands were detected using an enhanced chemiluminescence (ECL) substrate (Monad, China) and visualized with a gel documentation system for image capture and quantification.

Colony Formation Assay

Following 24-hour transfection protocols—ORC6L siRNA for T47D cells and ORC6L overexpression plasmid for MDA-MB-231 cells—the treated cells underwent trypsinization, collection, and plating in 6-well plates at 500 cells per well for colony formation assays. Culture maintenance utilized RPMI 1640 (T47D) or DMEM (MDA-MB-231), both containing 10% FBS with 1% antibiotic cocktail (penicillin-streptomycin), maintained at standard incubation: 37°C and 5% CO_2. After 12 days of incubation, medium was aspirated followed by triple PBS washes. Subsequent processing involved 60-minute fixation using 4% paraformaldehyde and 8-minute staining with crystal violet solution. To ensure clear imaging, the plates were air-dried using a blower.

CCK-8 Assay

After transfection of T47D cells with ORC6L siRNA and MDA-MB-231 cells with the ORC6L overexpression plasmid for whole day, Following trypsinization, cells were harvested and seeded in 96-well plates at 1500 cells per well in

100 μL of complete medium. Cell proliferation was assessed by measuring absorbance at multiple intervals over a 0- to 4-day period. Specifically, at each indicated time point, 10 μL of CCK-8 solution (BIOSS, China) was added to each well followed by gentle mixing. A 2-hour incubation at 37°C in a 5% CO_2 atmosphere was conducted to enhance cellular metabolic processes. The optical density was subsequently measured at 450 nm using a microplate reader.

EdU Cell Proliferation Assay

After T47D cells were transfected with ORC6L siRNA and MDA-MB-231 cells were transfected with the ORC6L overexpression plasmid for 24 hours, cell proliferation was assessed using the BeyoClick™ EdU Proliferation Kit (Alexa Fluor 488; Beyotime, China). Following the manufacturer's protocol, cells in 24-well plates were incubated for 2 hours with 200 μL of pre-warmed (37°C) 20 μM EdU solution mixed with an equal volume of culture medium. Following medium aspiration, samples were fixed with 4% paraformaldehyde for 30 minutes room temperature and thoroughly rinsed with PBS. Cell permeabilization was then carried out using a 15-minute treatment with 0.3% Triton X-100, after which the samples were washed again with PBS.The click reaction was initiated by adding 100 μL of the reaction cocktail, Subsequently, incubate room temperature in the dark for 30 minutes. After PBS washing, nuclear counter-staining was performed using Hoechst 33342. Finally, the samples were rinsed three times with PBS and examined under a fluorescence microscope.

APC Annexin V/7-AAD Staining Assay for Cell Apoptosis

Following 48-hour transfection of T47D cells with ORC6L siRNA and MDA-MB-231 cells with the ORC6L over-expression plasmid, a suspension of 1×106 breast cancer cells was prepared in 500 μL of Annexin V Binding Buffer. A staining solution containing Annexin V-APC and 7-AAD (5 μL each) was applied to the cell samples, followed by incubation at room temperature in the dark for 20 minutes. Assessment of apoptosis was conducted on a BD FACSCanto II flow cytometer. (BD Biosciences, USA).

Wound-Healing Assay

T47D and MDA-MB-231 cells were seeded in 6-well plates and cultured until reaching 50% confluence. For ORC6L knockdown, T47D cells were transfected with ORC6L-specific siRNA for 48 hours; for ORC6L overexpression, MDA-MB-231 cells were transfected with an ORC6L plasmid for the same duration. After transfection, cells were cultured until they reached 90%~100% confluence. A uniform scratch was created in each well using a sterile 10 μL pipette tip, and the wells were washed 2–3 times with PBS to remove detached cells and debris. To minimize proliferation interference, cells were maintained in low-serum medium (RPMI 1640 or DMEM with 1% FBS). Wound healing was monitored and imaged at 0, 12, 24, and 48 hours using an inverted microscope (4 × objective). Wound closure was quantified using ImageJ software (National Institutes of Health, USA).

Transwell Assay

Cell invasion capabilities were evaluated utilizing 6.5-mm diameter Transwell®chambers with 8-μm pores (Corning, USA). Serum-free cell suspensions (12,000 cells in 100 μL) were added to the upper compartments, while the lower chambers contained 600 μL medium supplemented with 10% FBS as chemoattractant. Post whole day culture (37°C, 5% CO_2), non-invaded cells on the membrane's upper side were wiped away with gentle swabbing with cotton tips. Invaded cells on the underside of the membrane were fixed with 4% paraformaldehyde for 30 minutes. Subsequently, they were stained with 0.1% crystal violet solution for 10 minutes room temperature to visualize the cells. Subsequently, they were stained with 0.1% crystal violet solution for 10 minutes room temperature to visualize the cells. Following three PBS washes, the membranes were dissected and mounted on glass slides for bright-field microscopy. (Nikon Eclipse Ti, 100× magnification).

Statistical Analyses

All statistical analyses were conducted in GraphPad Prism 10. For comparisons between two groups, an unpaired Student's *t*-test was used. For comparisons involving three or more groups, one-way ANOVA followed by Tukey's post hoc test was applied. Results are expressed as mean ± standard deviation. Statistical significance was set at $P < 0.05$, with

significance thresholds defined as: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ***$P < 0.0001$. R software (version 4.3.0) was used for initial data organization and descriptive statistics.

Results

ORC6L Expression and Its Clinicopathological Relevance in Breast Cancer

To investigate ORC6L expression in clinical samples, IHC staining was performed on invasive breast cancer and adjacent non-tumor tissues. ORC6L was predominantly localized in the cytoplasm in both breast cancer and benign adjacent tissues. Significant upregulation of ORC6L was observed in breast cancer samples compared with benign mammary epithelium, with mean IHC scores of 9.5 ± 2.6 in cancer tissues versus 6.1 ± 2.4 in adjacent non-tumor tissues ($P < 0.001$; Figure 1A).

ROC curve analysis indicated the diagnostic value of ORC6L, yielding an area under the curve (AUC) of 0.739 (95% CI: 0.682–0.796), which supports its moderate discriminative ability for breast cancer detection (Figure 1B). Survival analysis revealed that high ORC6L expression was significantly associated with shorter OS in breast cancer patients (Log rank test, HR = 1.71, 95% CI: 1.04–2.82, $P = 0.035$; Figure 1C). Additional analyses showed a correlation between ORC6L expression and tumor pathological grade. Specifically, ORC6L expression was notably elevated in advanced (grade III) breast cancer tissues compared with early-stage (grade I) disease ($P<0.05$); however, no statistically significant differences were observed between adjacent grades (grade I vs II, grade II vs III, both $P > 0.05$; Figure 1D). When assessed across molecular subtypes, Kruskal–Wallis analysis revealed notable differences in ORC6L expression (H = 7.886, $P = 0.048$). However, Dunn's post hoc test did not identify any statistically significant differences between individual subtype pairs (all adjusted $P > 0.05$), which may be attributed to the uneven sample size distribution and the correction for multiple comparisons. Furthermore, ORC6L expression levels did not differ significantly across age groups ($P > 0.05$), suggesting that its oncogenic role may be independent of both age and molecular subtype.

We further conducted univariate and multivariate Cox regression analyses to evaluate the impact of age, tumor stage, molecular subtype, and ORC6L protein expression level on patient outcomes. Univariate Cox regression analysis identified both high ORC6L expression (HR = 1.71, 95% CI: 1.04–2.82, $P = 0.035$) and advanced tumor stage (HR = 2.83, 95% CI: 1.71–4.68, $P < 0.001$) as significant predictors of poor OS. However, in the multivariate model, ORC6L expression did not retain independent prognostic significance (HR = 1.42, 95% CI: 0.85–2.36, $P = 0.184$; Figure 1E and F).

ORC6L Promotes the Proliferation of Breast Cancer Cells and Inhibits Cell Apoptosis

Our previous GSEA analysis revealed activation of the cell-cycle-checkpoints signaling pathway in breast cancer tissues with high ORC6L expression.[20] To further investigate whether ORC6L is involved in breast cancer cell proliferation, we first examined ORC6L expression levels in four human breast cancer cell lines (MDA-MB-231, MDA-MB-436, MCF7, and T47D) using qRT-PCR. The results indicated that ORC6L mRNA expression was relatively low in MDA-MB-231 and MDA-MB-436, but significantly higher in T47D ($P < 0.001$; Figure 2A). Based on these findings, we selected MDA-MB-231—which showed low ORC6L expression along with high proliferative and invasive capacity—for ORC6L overexpression experiments, and T47D, which exhibits high endogenous ORC6L expression, for knockdown experiments. After transient overexpression of ORC6L in MDA-MB-231 cells and siRNA-mediated knockdown using three different siRNA sequences in T47D cells, qRT-PCR and Western blot analyses showed that ORC6L expression was significantly upregulated in the overexpression group compared to the control group ($P < 0.001$; Figure 2B and C). Among the siRNA-treated groups, only si-ORC6L-1 effectively knocked down ORC6L expression, reducing the efficiency by approximately 75%, while the other siRNAs did not exhibit significant inhibitory effects (Figure 2B and C). si-ORC6L-1 was selected for further studies due to its high efficiency.

Comprehensive functional analyses demonstrated that ORC6L expression levels directly regulate the proliferative capacity of breast cancer cells. This conclusion was consistently supported by three independent functional assays. Colony formation assays revealed that ORC6L overexpression significantly enhanced clonogenic potential ($P < 0.001$), whereas siRNA-mediated knockdown markedly reduced colony formation ($P < 0.05$; Figure 3A). Further supporting these results, EdU incorporation assays indicated that ORC6L-overexpressing cells exhibited accelerated DNA synthesis ($P < 0.0001$), while ORC6L silencing led to a significant decrease in proliferation ($P < 0.001$; Figure 3B). Additionally,

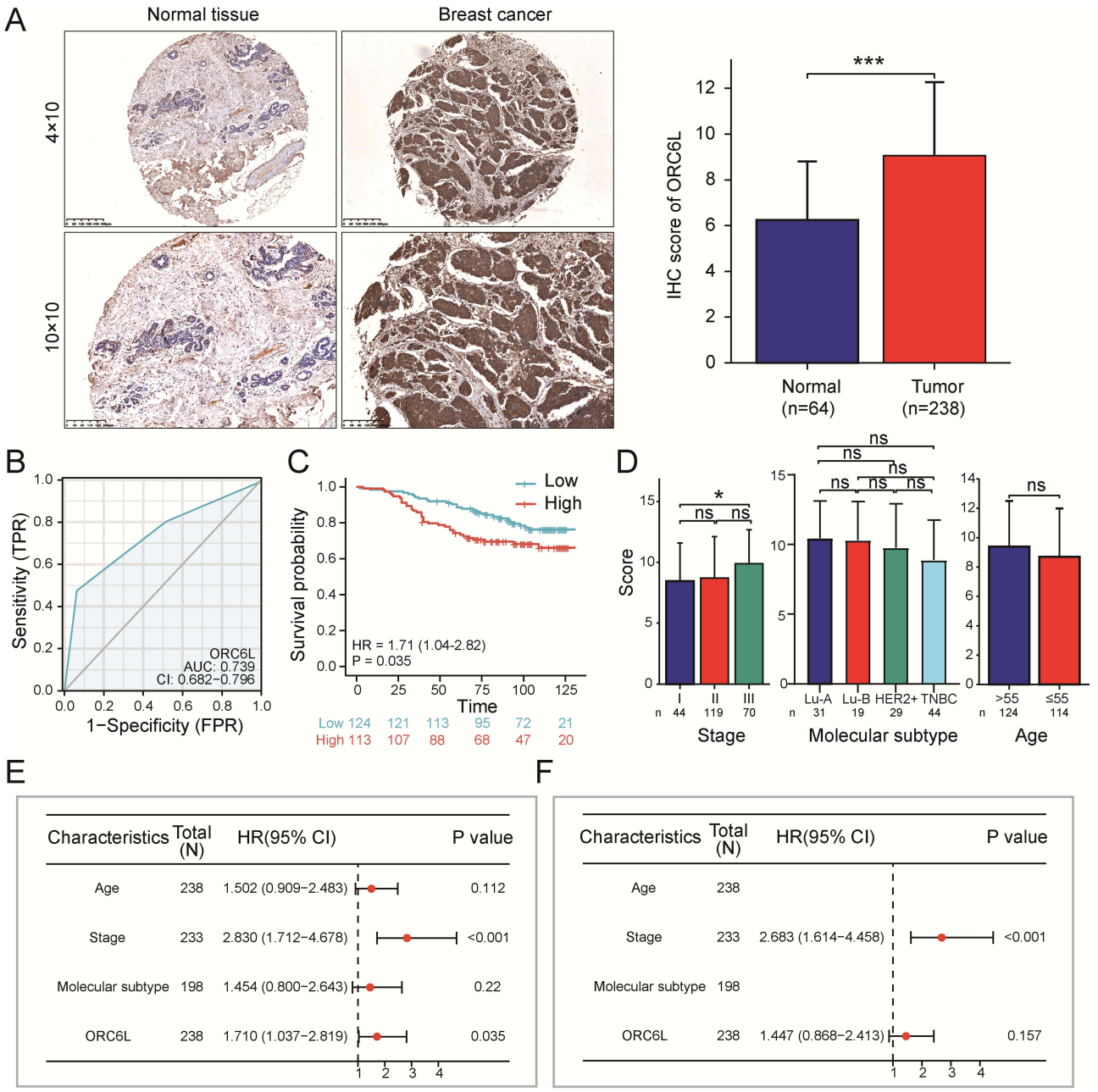

Figure 1 ORC6L Expression and Its Clinicopathological Relevance in Breast Cancer. (**A**) Left: Representative IHC images of ORC6L in breast cancer and adjacent non-tumor tissues (40× and 100×). Right: IHC scores of ORC6L in 238 breast cancer and 64 non-tumor tissues were analyzed. (**B**) Diagnostic performance of ORC6L for breast cancer evaluated by ROC curve analysis. (**C**) Survival analysis comparing OS between breast cancer patients with high ORC6L expression and those with low expression. (**D**) ORC6L expression levels were compared across subgroups based on pathological stage, molecular subtype, and age. (**E**) Univariate Cox regression analysis of ORC6L expression and clinicopathological factors for OS. (**F**) Multivariate Cox regression analysis of ORC6L expression and clinicopathological factors for OS. Data were analyzed using unpaired *t*-test for comparisons between two groups, and one-way ANOVA for comparisons among multiple groups. *$P < 0.05$, ***$P < 0.001$.
Abbreviation: ns, not significant.

CCK-8 viability assays confirmed that ORC6L overexpression increased cell viability ($P < 0.0001$), and its knockdown resulted in reduced viability ($P < 0.0001$; Figure 3C). Given the pronounced role of ORC6L in promoting proliferation, we next investigated its effect on apoptosis in MDA-MB-231 and T47D cell lines. Flow cytometric analysis showed that ORC6L overexpression significantly suppressed apoptosis in MDA-MB-231 cells ($P < 0.01$), whereas ORC6L knockdown markedly promoted apoptosis in T47D cells ($P < 0.001$; Figure 3D).

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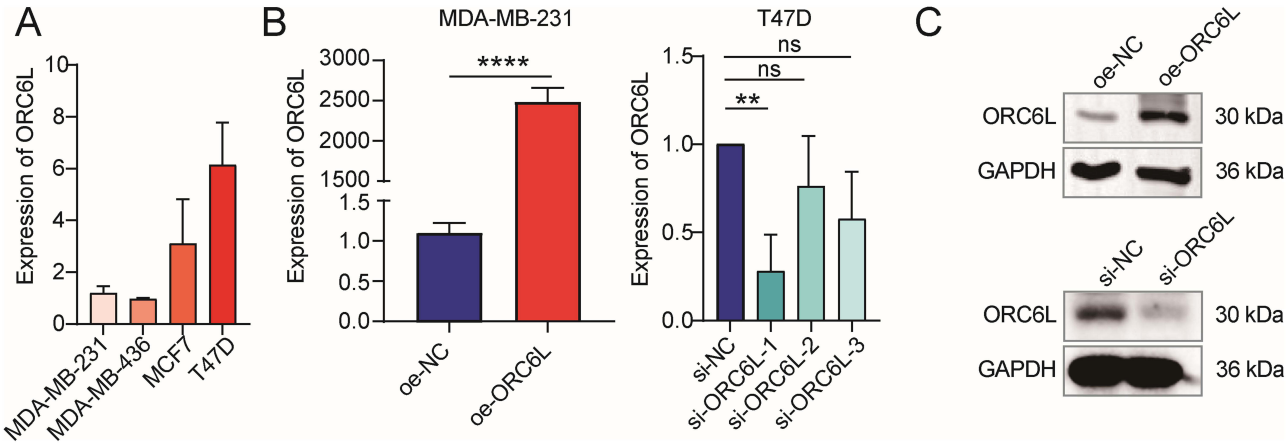

Figure 2 Expression of ORC6L mRNA in human breast cancer cells and validation of its overexpression and knockdown. (**A**) ORC6L mRNA expression levels in human breast cancer cell lines (MDA-MB-231, MDA-MB-436, MCF7, and T47D) were detected by qRT-PCR. (**B**) qRT-PCR analysis of ORC6L mRNA after transient transfection with oe-NC or oe-ORC6L in MDA-MB-231, and siRNA knockdown in T47D. (**C**) Western blot analysis of ORC6L protein expression in MDA-MB-231 cells transfected with oe-NC or oe-ORC6L, and in T47D cells transfected with siRNA. Data were analyzed using unpaired *t*-test. ***P* <0.01, *****P* < 0.0001.
Abbreviation: ns, not significant.

ORC6L Promotes Migration and Invasion in Breast Cancer Cells

To investigate the functional role of ORC6L in cellular migration, Wound-healing assay were performed. Quantitative analysis demonstrated that ORC6L-overexpressing MDA-MB-231 cells exhibited significantly accelerated wound closure ($P < 0.001$). In contrast, ORC6L-silenced T47D cells displayed markedly delayed Wound closure ($P < 0.01$; Figure 4A). For the invasion assays, cells were transfected with either overexpression constructs or specific siRNAs before being seeded into Transwell chambers. Overexpression of ORC6L strongly enhanced the invasive capacity of MDA-MB-231 cells ($P < 0.001$), whereas ORC6L knockdown significantly suppressed invasion in T47D cells ($P < 0.0001$; Figure 4B).

Discussion

This study represents the first systematic validation of ORC6L expression and biological function in breast cancer at the protein level, integrating immunohistochemical analysis of clinical specimens with comprehensive in vitro functional assays. Our data confirm significant upregulation of ORC6L in breast cancer tissues and demonstrate its oncogenic roles in promoting tumor cell proliferation, migration, and survival while suppressing apoptosis, thereby supporting its potential as a therapeutic target. However, in contrast to our previous bioinformatics analysis based on the TCGA database, the prognostic value of ORC6L was not validated as an independent predictor in the current clinical cohort.[20] This discrepancy raises critical questions regarding the translation of bioinformatics-derived biomarkers into clinical practice and underscores the necessity of rigorous experimental validation.

Aberrant ORC6L expression has been documented across multiple malignancies. In hepatocellular carcinoma, ORC6L was identified as a prognostic biomarker with elevated expression associated with shortened patient survival.[16] Similarly, in lung adenocarcinoma, ORC6L is not only significantly overexpressed but also shows a strong positive correlation with poor prognosis.[22] In clear cell renal cell carcinoma, high ORC6L expression predicts shorter OS and serves as an independent prognostic factor.[23] In the present study, IHC analysis revealed significantly higher ORC6L protein levels in breast cancer tissues compared with adjacent non-tumor tissues, with an AUC of 0.739 indicating moderate diagnostic accuracy. This finding aligns with the trend observed in our previous bioinformatics analysis (AUC = 0.907), although the diagnostic performance was attenuated at the protein level. This divergence likely reflects differences in detection sensitivity between mRNA sequencing and immunohistochemistry, as well as potential post-transcriptional regulatory mechanisms. Collectively, these findings extend the aberrant ORC6L expression profile to breast cancer and support its potential as a diagnostic and prognostic biomarker across diverse tumor types.

Importantly, ORC6L expression correlated positively with pathological grade, with significantly elevated levels observed in grade III compared with grade I tumors. This pattern is biologically plausible, as higher-grade, rapidly proliferating tumors have

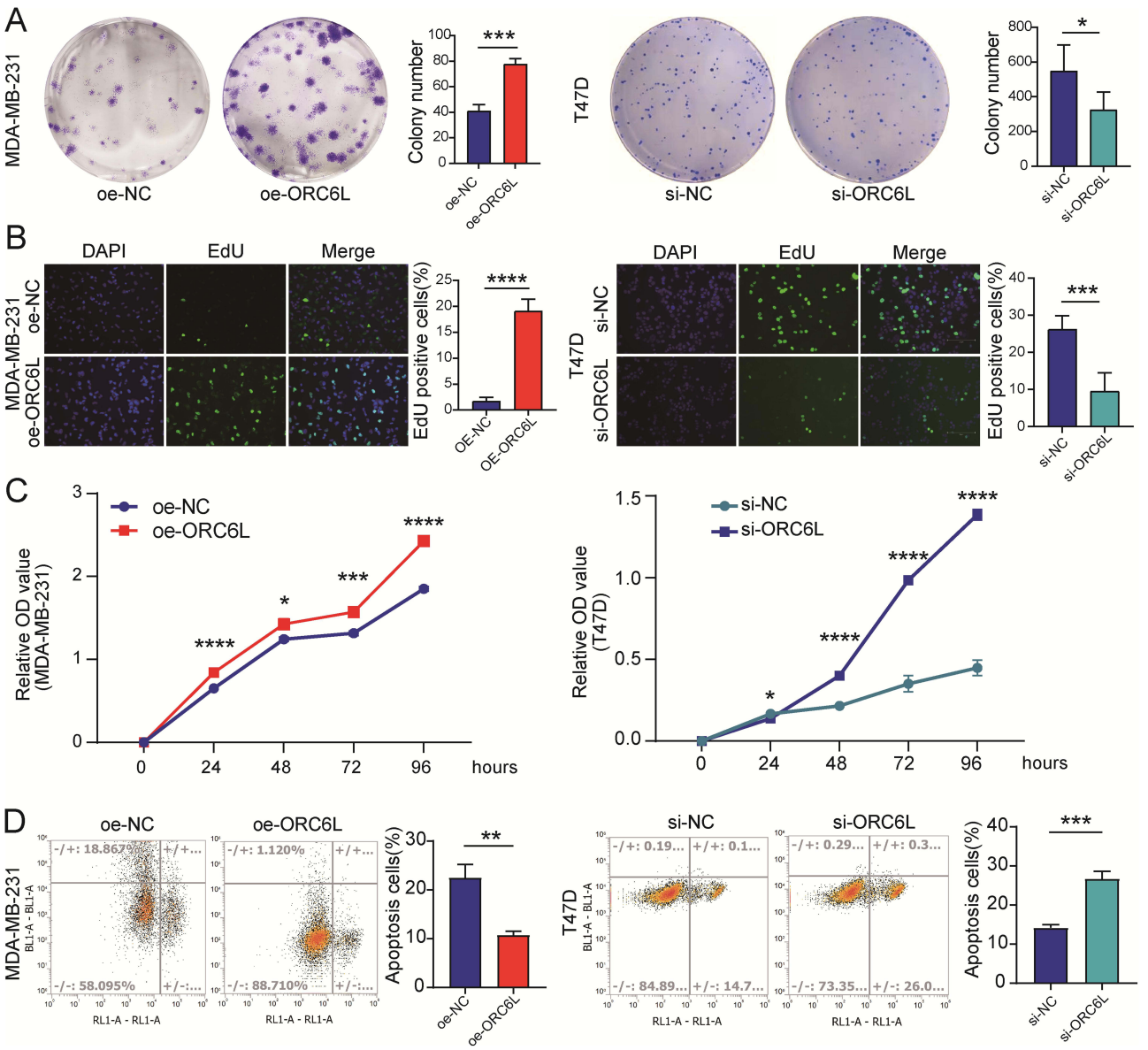

Figure 3 ORC6L promotes Breast Cancer cell proliferation and inhibits apoptosis. (**A**) Assessment of ORC6L-mediated proliferative effects by colony formation assay. (**B**) Quantitative analysis of ORC6L-dependent proliferation dynamics using EdU assay. (**C**) Kinetic monitoring of ORC6L-regulated cell proliferation through CCK-8 assay. (**D**) Quantitative flow cytometric analysis of ORC6L-mediated apoptotic regulation in breast cancer cells. Data were analyzed using unpaired *t*-test. *P <0.05, ** P <0.01, *** P <0.001, and **** P <0.0001.

increased demands for DNA replication. However, in the present cohort, ORC6L expression did not differ significantly across the four molecular subtypes or age groups, suggesting that its oncogenic function may operate independently of conventional molecular classification systems and patient demographics. This finding appears to differ from our previous bioinformatic observations, which identified positive associations between ORC6L expression and HER2-positive subtype as well as patient age.[20] The discrepancy may reflect heterogeneity across different patient populations or differences in analytical approaches. Further validation in larger, well-stratified cohorts is warranted to clarify the relationship between ORC6L expression and molecular subtypes.

A notable observation emerging from this investigation concerns the variable prognostic performance of ORC6L when assessed at distinct biological levels. Our previous TCGA (n = 1082) analysis identified ORC6L as an independent prognostic factor, supporting its incorporation into a predictive nomogram. In the current TMA (n = 237) cohort, however, this association was attenuated at the protein level, suggesting that mRNA-based prognostic signatures may not fully recapitulate

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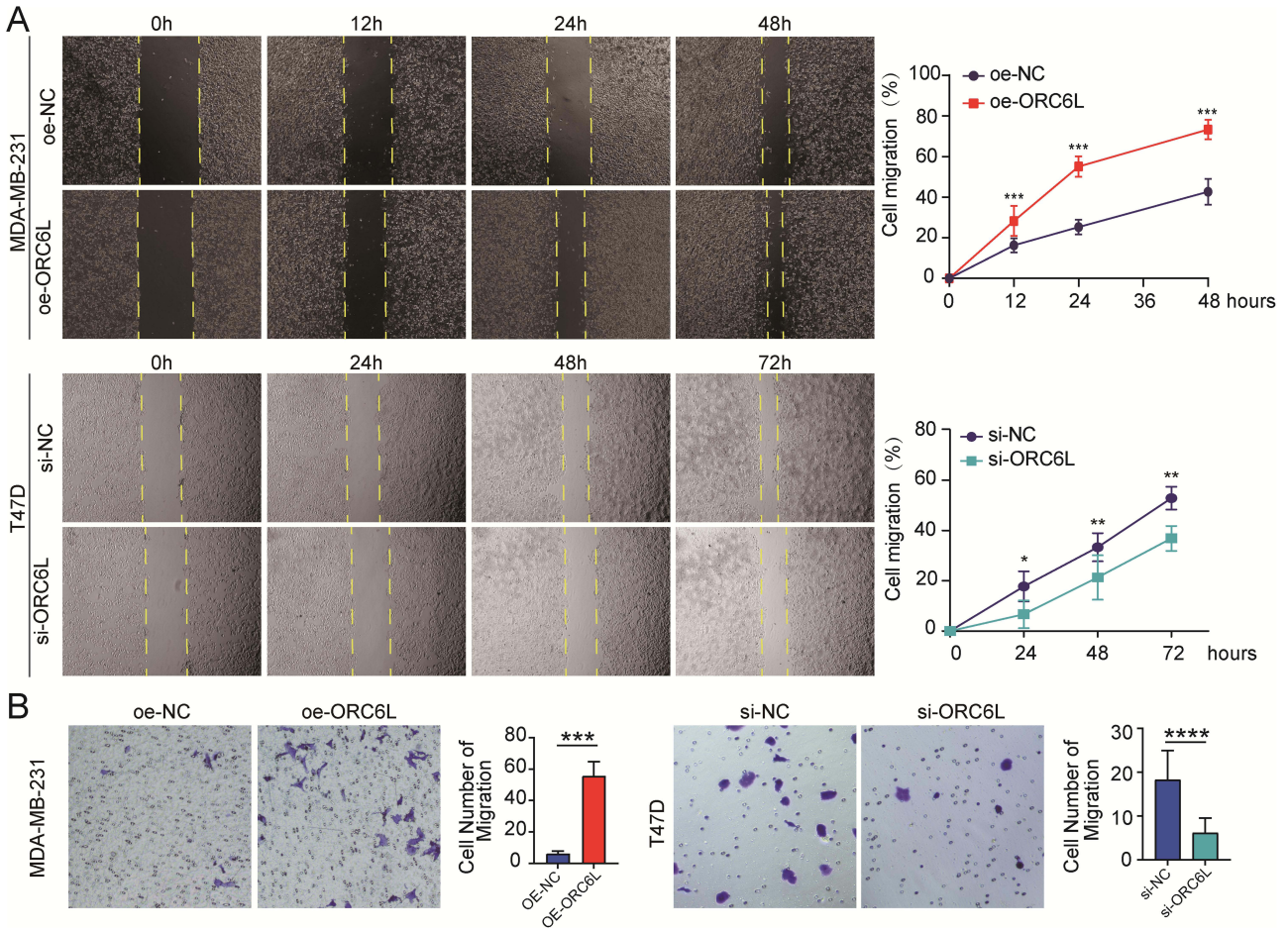

Figure 4 ORC6L Promotes Migration and Invasion in Breast Cancer Cells. (**A**) The migratory capacity of ORC6L-dependent cells was assessed using wound-healing assay. (**B**) The invasive ability of ORC6L was evaluated by Transwell assay. Data were analyzed using unpaired *t*-test. **P* <0.05, ***P* < 0.01, ****P* <0.001, *****P* <0.0001.

protein-level predictive value. Several methodological factors may account for this attenuation. First, the large TCGA cohort provided sufficient power to detect modest effects, whereas the smaller TMA cohort was likely underpowered, with the wide confidence interval spanning unity indicating imprecision rather than a true null effect. Second, population heterogeneity may contribute to this discrepancy. TCGA encompasses multi-institutional, multi-ethnic populations with diverse treatment histories, while our TMA cohort likely represents a more homogeneous regional population. Third, post-transcriptional regulatory mechanisms—including microRNA-mediated suppression, translational variations, and protein degradation—may decouple mRNA abundance from functional protein levels.[13,24,25] Fourth, database-derived analyses may incorporate longer follow-up or different censoring criteria compared with TMA studies. These observations illustrate the iterative nature of biomarker validation, where initial findings from large-scale databases are progressively refined through targeted experimental studies. The prognostic performance of ORC6L across different analytical contexts warrants continued investigation to establish its optimal clinical application.

Despite equivocal findings from prognostic analyses, our functional assays provide robust evidence for ORC6L oncogenicity. Ectopic overexpression in MDA-MB-231 cells enhanced proliferation, viability, and clonogenic potential, while siRNA knockdown in T47D cells produced converse effects—suppressing growth, inducing apoptosis, and attenuating migration and invasion. Notably, MDA-MB-231 (TNBC) and T47D (non-TNBC) represent distinct molecular subtypes. To avoid confounding by intrinsic biological differences, all functional comparisons were performed within each cell line rather than directly across cell lines. Our interpretation of ORC6L function accordingly considers both its expression variation and the distinct cellular backgrounds. Consistent with our observations, these functional phenotypes align closely with previous mechanistic studies. Sang et al demonstrated that ORC6L drives cell cycle progression through upregulation of Cyclin A2,

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B1, and D1 in non-small cell lung cancer.[9] In pancreatic ductal adenocarcinoma, ORC6L drives proliferation and suppresses apoptosis by inhibiting p53 serine-15 phosphorylation, with its mRNA stability enhanced by METTL3/IGF2BP3 via m^6A modification.[26] Additionally, ORC6L overexpression has been linked to aggressive phenotypes in colorectal and gastric cancers, where it promotes proliferation, migration, and chemotherapy resistance.[17,18] The pro-survival and pro-migratory effects observed in our study are further consistent with hepatocellular carcinoma reports documenting ORC6L-mediated promotion of epithelial-mesenchymal transition and metastasis.[13] Importantly, these experimental findings corroborate our previous Gene Set Enrichment Analysis revealing activation of cell cycle checkpoint, NF-κB, P53, and WNT signaling pathways in ORC6L-high breast cancer specimens.[20] The convergence of bioinformatics prediction and experimental validation at the functional level strengthens the biological rationale supporting ORC6L as a proliferation driver, even as its independent prognostic value remains debated.

Several limitations should be acknowledged. First, the sample size, while sufficient for the primary analyses, may have limited statistical power to detect ORC6L as an independent prognostic factor. Post-hoc power analysis suggests that larger cohorts will be necessary to definitively assess its independent prognostic value. Second, although our functional assays established the phenotypic consequences of ORC6L modulation, the precise downstream effectors—including MCM complex loading dynamics, CDK-RB pathway interactions, and mitochondrial apoptotic regulation—remain to be characterized. Future studies incorporating mechanistic dissection and broader cell line panels will be essential to fully elucidate the oncogenic functions of ORC6L in breast cancer.

Synthesizing current and previous evidence, the clinical utility of ORC6L in breast cancer management requires nuanced evaluation across application contexts. For diagnostic purposes, the moderate discriminatory accuracy of IHC detection (AUC = 0.739), while inferior to mRNA-based prediction, supports potential utility as an adjunctive histopathological marker, particularly in distinguishing high-grade lesions. For prognostic stratification, existing evidence is insufficient to advocate for incorporation into clinical decision-making algorithms; definitive validation in prospective, adequately powered cohorts remains mandatory. For therapeutic targeting, the robust functional evidence for ORC6L-mediated proliferation and survival, combined with its established role in the CDK4/6-RB replication licensing pathway, provides a theoretical foundation for therapeutic targeting.

Overall, this study validates the oncogenic function of ORC6L in breast cancer while providing important empirical evidence to support the clinical translation of bioinformatics-derived prognostic signatures. The biological activity of ORC6L as a proliferation driver is firmly established, yet its independent prognostic value requires further rigorous validation. These findings underscore the indispensable role of experimental validation in precision oncology research and offer methodological insights for the design of future large-scale prospective studies.

Abbreviations

ORC, Origin Recognition Complex; AUC, area under the curve; OS, overall survival; IHC, immunohistochemical.

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Disclosure

The authors report no conflicts of interest in this work.

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