

Clinical implications of NRF3 mRNA and protein expression in ovarian cancer

Mahdi Hosseinzadeh^{1,2}, Zahra Madjd^{3,4,5}, Mohsen Rashid¹, Elham Kalantari³, Behrooz Shokouhi⁶, Manizheh Sayyah-Melli^{7,8}, Mohammad Reza Sadeghi^{1,9*}

¹Department of Molecular Medicine, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

²Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran

³Oncopathology Research Center, Iran University of Medical Sciences (IUMS), Tehran, Iran

⁴Department of Pathology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

⁵Department of Molecular Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran

⁶Department of Pathology, Tabriz University of Medical Sciences, Tabriz, Iran

⁷Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

⁸Fellowship of Gynecologic Oncology, Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

⁹Research Center for Pharmaceutical Nanotechnology, Biomedicine institute, Tabriz University of Medical Sciences, Tabriz, Iran

Article Info



Article Type:

Original Article

Article History:

Received: 12 Apr. 2026

Revised: 13 Sep. 2026

Accepted: 3 Oct. 2026

ePublished: 27 Oct. 2026

Keywords:

Ovarian cancer

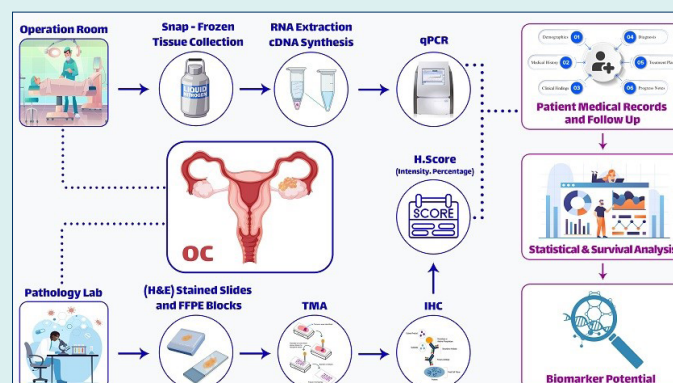
NRF3

Prognosis

Biomarker

Protein expression

Survival analysis



Abstract

Introduction: The clinical role of the transcription factor NRF3 in ovarian cancer (OC) remains unclear, particularly regarding the relationship between its mRNA and protein levels. This study investigates NRF3 expression as a potential prognostic indicator in OC.

Methods: This multicenter study analyzed 171 archival FFPE ovarian cancer specimens (2015–2023) alongside 29 prospectively collected, snap-frozen cases (2021–2024). Samples included diverse histologic subtypes predominantly high-grade serous carcinoma with comprehensive FIGO staging and Silverberg grading. We quantified NRF3 expression via tissue microarrays and qPCR, correlating the results with clinicopathological data. Diagnostic and prognostic performance were assessed using ROC curves, Kaplan-Meier survival analysis, and Cox regression.

Results: NRF3 was significantly upregulated in OC, as evidenced by both increased mRNA levels ($P = 0.021$) and elevated IHC H-scores in tumor cores ($P < 0.001$). These high expression levels correlated with aggressive clinical markers, such as advanced FIGO stage, metastasis and recurrence ($P < 0.001$), and were predictive of poor overall and progression-free survival ($P = 0.001$). The prognostic value was further supported by ROC analysis, which yielded an AUC of 0.779 for relapse prediction.

Conclusion: NRF3 is consistently upregulated at both the mRNA and protein levels in ovarian cancer, with its accumulation strongly linked to advanced disease and poor survival, pointing to its role in stress-driven tumor invasiveness. As NRF3 expression lost independent significance in our multivariate analysis, we identify it primarily as a molecular marker of aggressive disease burden rather than an independent prognostic factor. Future work should focus on validating its potential utility within broader diagnostic panels.



*Corresponding author: Mohammad Reza Sadeghi, Email: sadeghimr@tbzmed.ac.ir



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Introduction

Ovarian cancer (OC) is one of the most lethal gynecologic malignancies, presenting a significant challenge to global women's health. Although it is the eighth most common cancer in women, its impact is disproportionately severe, accounting for nearly 4.7% of all cancer-related deaths worldwide.¹ With roughly 240,000 new diagnoses annually, the disease primarily affects women between 55 and 70 years of age.² Unfortunately, the clinical reality is grim: because early symptoms are often vague or entirely absent, roughly 70% of patients present with advanced disease (FIGO stage III–IV). Consequently, the survival outcomes remain poor, with five-year survival rates struggling to exceed 30–40%.²

OC is anything but monolithic; it displays profound heterogeneity, most notably within its most prevalent form, epithelial ovarian cancer (EOC), which itself accounts for approximately 90% of all diagnosed cases.³ Current classification divides EOC into five distinct histotypes, a division necessitated by their unique molecular signatures and divergent clinical behaviors. Within this landscape, high-grade serous ovarian carcinoma (HGSOC) stands out as the most clinically significant subtype; it not only constitutes 70–80% of all EOC cases but is also characterized by an exceptionally aggressive clinical course.³

The global burden of OC is characterized by significant geographic variation, driven largely by underlying regional disparities. While improved preventive strategies have successfully tempered incidence rates across several developed nations, regions in Asia and Eastern Europe continue to confront disproportionately high prevalence levels.⁴ The scale of this challenge is immense: over 900,000 women have been diagnosed with OC in just the last five years, and forecasts suggest that total mortality could reach eight million by 2050 in the absence of enhanced prevention and control protocols.⁵ Nevertheless, the clinical landscape is shifting. Breakthroughs in molecular oncology, specifically the expanding understanding of the BRCA pathway and the integration of PARP inhibitors, are fundamentally altering survival trajectories for patients with homologous recombination defects.²

Survival in ovarian cancer is inextricably linked to early detection, yet current clinical standards, specifically serum CA125 and transvaginal ultrasound, routinely fail to detect the disease in its earliest stages. This inherent limitation has driven a necessary departure from reliance on single-marker tests. Instead, we are seeing a shift toward multimodal diagnostics, in which liquid biopsy techniques (ctDNA, miRNA) are synthesized with AI-supported multiomic platforms.^{6,7} Advances in nanotechnology are further pushing this frontier, enabling more granular, high-sensitivity risk stratification.⁸ Against this backdrop, we explore NRF3 as an underexplored molecular correlate that could extend the capabilities of current diagnostic platforms.

As a member of the CNC-bZIP family, the transcription factor NRF3 (NFE2L3) has emerged as a significant, albeit nuanced, component in oncogenesis. Evidence

suggests that it functions as both a tumor suppressor and a promoter, a duality dictated largely by cellular context. Under homeostatic conditions, NRF3 remains sequestered within the endoplasmic reticulum. Upon cellular stress, however, it translocates to the nucleus to orchestrate the expression of genes governing metabolic pathways, proliferation, and differentiation.⁹ High NRF3 expression correlates with adverse prognosis and diminished survival across several malignancies, underscoring its utility as a diagnostic and prognostic marker.^{10–12} Given the aggressive phenotype and diagnostic challenges characteristic of OC, NRF3 represents a compelling candidate for therapeutic targeting. Deciphering its precise role in this pathophysiology is therefore a critical requirement for advancing future treatment paradigms.⁹

Data from single-cell RNA sequencing reveal a striking transcriptional overlap between ovarian clear cell carcinoma (CCC) and endometriosis-associated epithelia. The presence of robust NFE2L3 regulon activity within M2 macrophages suggests that NRF3 acts as a primary architect in modulating the tumor microenvironment.¹³ Loss of NFE2L3 attenuates inflammation associated with colorectal cancer,¹² highlighting its role in the complex crosstalk within the tumor-microenvironment, which warrants further mechanistic investigation.

The oncogenic potential of NRF3 is not limited to ovarian malignancies; evidence across other cancer types demonstrates a consistent capacity to drive aggressive phenotypes. In pancreatic cancer, for example, NRF3 expression is tightly linked to nodal metastasis and the orchestration of angiogenesis via VEGFA modulation.¹⁴ Similarly, gastric cancer studies describe a mechanism through which NRF3 facilitates the epithelial-mesenchymal transition (EMT) while simultaneously altering T-cell infiltration profiles.¹⁵ Comprehensive multi-cancer analyses reveal that NRF3 is consistently upregulated and correlates with poor survival outcomes and unfavorable prognostic indicators, including in patients with OC, thereby establishing it as a critical pan-cancer biomarker.¹²

Unlike its family counterparts NRF1 and NRF2, NRF3 operates through a highly specialized transcriptional program, primarily directing the expression of genes involved in cellular stress responses and extracellular matrix architecture.¹⁶ This distinct regulatory profile, particularly concerning antioxidant-responsive genes, likely accounts for the specific contributions of NRF3 to metastatic progression, providing a strong rationale for pursuing it as a viable therapeutic target. Clinical observations further support this potential, as the consistent upregulation of NRF3 across digestive system malignancies underscores its growing value as a high-fidelity diagnostic and prognostic marker.¹⁷

Although NRF3 deregulation is a known indicator of poor outcomes in various malignancies, the specific mechanisms underlying its role in ovarian tumorigenesis remain poorly defined. Much of this ambiguity stems from a clear dissociation between mRNA abundance and actual protein effector activity. Because NRF3 stability is

heavily dictated by post-transcriptional and translational events, we assessed the expression of this factor at both the mRNA and protein levels using a combination of OC tissues and public clinical datasets. By integrating these multidimensional findings with clinicopathologic features and patient survival data, our work establishes a more granular prognostic profile for NRF3.

Materials and methods

Patient's characteristics and sample collection

This cross-sectional study utilized two sets of tissue specimens. The first phase comprised 29 prospectively collected, snap-frozen pairs of tumoral and adjacent non-tumoral margin (control) tissues, acquired during surgical procedures at Al-Zahra Hospital (Tabriz, Iran) between 2021 and 2024. All samples were immediately preserved by snap-freezing in liquid nitrogen (-196°C). Patients had not undergone prior radiotherapy, chemotherapy, or immunotherapy. Pathological characteristics were confirmed by an experienced pathologist. The second phase involved the collection of 171 formalin-fixed, paraffin-embedded (FFPE) tissue specimens from patients diagnosed with primary OC. These samples, collected between 2015 and 2023, were sourced from Kowsar Hospital (Urmia), Al-Zahra Referral Hospital (Tabriz), and the biobank of Iran University of Medical Sciences (Tehran, Iran) (Table S1).

To gain a comprehensive understanding of the clinical and pathological characteristics, we obtained hematoxylin and eosin (H&E)-stained slides alongside archival medical records. This allowed us to extract key parameters, including patient demographics, such as age, and clinical details such as maximum tumor diameter, histological type and subtype, histological grade according to the Silverberg grading system, tumor stage according to the International Federation of Gynecology and Obstetrics (FIGO) system, presence of distant metastasis, tumor recurrence, lymph node status, lymphovascular space invasion (LVSI), and other metastatic and invasive characteristics related to tumor involvement and spread. All patients received chemotherapy after surgery according to standard guidelines; therefore, treatment modalities were not analyzed as a variable. The detailed histological distribution of the study series is presented in a graphical summary available in Fig. S1 (Supplementary file 1).

RNA isolation and qRT-PCR

Total RNA was extracted using the RiboEX reagent (GeneAll, Korea). Purity and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) based on the A260/A280 and A260/A230 ratios, and RNA integrity was confirmed by 2% agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized using the SMOBIO cDNA Synthesis Kit (Taiwan) in a Bio-Rad thermal cycler.

Quantitative PCR for NRF3 expression was performed in triplicate on a Roche LightCycler[®]96 instrument (Germany) using SYBR Green Master Mix (AMPLIQON, Denmark). The specificity and efficiency of PCR amplification were

confirmed through analysis of amplification plots and melt curves, indicating minimal primer-dimer formation and nonspecific amplification at the optimized annealing temperature of 60°C . Gene expression was normalized to GAPDH as the endogenous reference gene (Table S2). Relative expression was quantified using the $2^{-\Delta\Delta\text{CT}}$ method,¹⁸ comparing tumor samples with control tissues.

Tissue microarray construction

To construct the OC tissue microarrays (TMAs), we followed established protocols,^{18,19} utilizing an expert pathologist to identify three representative tumor regions on donor blocks that correlated with H&E slides. Core samples were then extracted from these regions using a Tissue Arrayer Minicore instrument (ALPHELYS, Plaisir, France) and arrayed into recipient paraffin blocks. We sectioned the completed TMAs at $4\text{ }\mu\text{m}$ and mounted them onto adhesive slides. To account for tumor heterogeneity, we utilized triplicate blocks for each specimen, a method supported by validation studies showing that dual-core analysis captures over 95% of whole-tissue staining patterns, while single-core analysis remains over 90% accurate.^{20,21} Here the final score for each sample was derived from the average of these three cores.

Immunohistochemistry

We performed IHC on the TMA sections using an Envision polymer-conjugated system.^{22,23} After deparaffinizing and rehydrating the slides, we conducted antigen retrieval via autoclaving in a pH 6.0 citrate buffer for 10 minutes, followed by quenching endogenous peroxidase with 3% H_2O_2 .

The primary antibody, rabbit anti-NFE2L3 (Sigma, Cat. No. HPA055889), was applied overnight at 4°C (1:50 dilution); rabbit IgG served as the isotype control. After HRP-polymer detection, visualization was achieved using 3,3'-diaminobenzidine (DAB, Dako, Glostrup, Denmark), followed by hematoxylin counterstaining. Positive controls included BLCA,²⁴ PaC,¹⁴ and CRC tissues,²⁵ with TBS used as a negative control.

Immunostaining assessment

NRF3 expression in OC TMAs was scored independently by two experienced pathologists blinded to clinical data, with discrepancies resolved by consensus. Scoring criteria included staining intensity and the proportion of positive tumor cells, which were combined to calculate the Histochemical score (H-score), ranging from 10 to 300.²⁶ Intensity was graded on a 4-point scale (0.1 for negative/non-staining, 1 for weak, 2 for moderate, 3 for strong). Cases were dichotomized into low ($\leq$ median) and high ($>$ median) expression groups.

Bioinformatic and regulatory network analysis

Protein-protein interaction (PPI) analysis for *Homo sapiens* NRF3 (NFE2L3) was performed using the STRING database (v12.0; <https://string-db.org>), retaining only interactions with a confidence score ≥ 0.700 and excluding

disconnected nodes. The resulting network was assessed for enrichment in Gene Ontology (GO) terms and KEGG/Reactome pathways using STRING's statistical framework, with *P* values adjusted by the false discovery rate (FDR).

Complementary transcription factor (TF) enrichment of the NRF3 interactors was performed using Enrichr (<https://maayanlab.cloud/Enrichr/>). Specifically, the ChEA 2022, ARCHS4 TF co-expression, and TRRUST 2019 databases were interrogated. Enrichr employed Fisher's exact test, providing raw and Benjamini–Hochberg corrected *P* values, odds ratios, and combined scores. Results from STRING and Enrichr were integrated to contextualize NRF3 within regulatory networks relevant to stress response, immune signaling, and OC biology.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA), with all experimental values expressed as mean $\pm$ SD or SEM. To ensure appropriate test selection, we first assessed data normality using the Shapiro–Wilk test; accordingly, non-parametric methods were employed for any datasets that failed to meet normal distribution criteria. Differences between multiple groups were evaluated using the Kruskal–Wallis test. For subsequent pairwise comparisons, Mann–Whitney U tests were performed with a Bonferroni correction applied, resulting in an adjusted significance level of (adjusted $\alpha = 0.017$).

For association analyses between NRF3 expression and clinicopathological features, Pearson's Chi-Square (χ^2) test was employed using IBM SPSS Statistics (version 25.0; IBM Corp., Armonk, NY, USA). The prognostic performance of NRF3 expression level for differentiating disease states was assessed using receiver operating characteristic (ROC) curve analysis, and the area under the curve (AUC) was calculated, also within SPSS.

Survival outcomes—including progression-free survival (PFS), overall survival (OS), and disease-specific survival (DSS)—were analyzed using the Kaplan–Meier method. Differences between survival curves were compared using the log-rank (Mantel–Cox) test. To determine the independent prognostic impact of variables, univariate and multivariable Cox proportional hazards regression models were utilized. Variables that showed a hazard ratio (HR) (> 1.0) with a *P* value (< 0.05) in the univariate analysis were included in the subsequent multivariable model. Throughout all analyses, a two-tailed *P* value (< 0.05) was considered statistically significant.

Results

NRF3 mRNA expression by qPCR

Quantitative PCR analysis demonstrated lower NRF3 mRNA expression in benign lesions (cystic follicle and cystic teratoma) relative to normal ovarian tissue. Conversely, EOC subtypes exhibited increased expression, with high-grade serous carcinoma (HGSC) showing the maximal elevation among all analyzed subtypes (Fig. 1).

Relative NRF3 mRNA expression was significantly lower in normal ovarian tissue (median = 0.36) compared to both low-stage (median = 1.87) and high-stage tumors (median = 1.50) (Kruskal–Wallis $H = 7.743$, $df = 2$, $P = 0.021$). Post-hoc analysis confirmed that NRF3 levels were significantly elevated in both low-stage ($P_{adj} = 0.011$) and high-stage tumors ($P_{adj} = 0.013$) compared to normal tissue (Mann–Whitney U tests, Bonferroni corrected). Interestingly, this upregulation remained consistent regardless of tumor stage, as no significant variation was observed between the low- and high-stage groups ($P = 0.526$) (Table 1).

NRF3 protein expression in ovarian cancer

NRF3 protein expression, quantified by H-score via IHC in 171 archival OC tissue samples, was significantly upregulated in tumor cores ($N = 116$; mean = 173.22 ± 65.99) compared with combined normal/benign tissues ($N = 55$; mean = 64.96 ± 78.60) ($t(91.34) = 8.84$, $P < 0.001$). Positive staining was predominantly localized in the cytoplasm with partial nuclear presence in cancer cells (Fig. 2).

Bioinformatic analysis of NRF3 network and regulatory enrichment

Through STRING analysis, we mapped an NRF3 (NFE2L3) interactome characterized by a highly interconnected cluster ($P = 6.55E-5$, 20 edges). Functional enrichment highlighted integrated stress response signaling, RNA Pol II transcriptional regulation, and AP-1 complex activity as the top pathways, underscoring NRF3's role in stress adaptation and transcription (Fig. 3, Table S3).

Transcription factor enrichment via Enrichr (ChEA 2022, ARCHS4 TF co-expression, TRRUST 2019) identified factors linked to NRF3 interactors. ChEA 2022 implicated EGR1, BRD4, EP300, HIF1A, and GATA family members (adj. $P < 0.01$). ARCHS4 revealed co-expression with stress-responsive and oncogenic regulators (e.g., ZFP36L1, NR4A1, JUND, KLF4, ATF3, JUNB). TRRUST 2019 confirmed enrichment for HDAC3, FOSL1, JUN, ESR2, ARNT, and RB1. Collectively, these results indicate an NRF3-associated network governing stress response, chromatin remodeling, and hormone-related signaling (Table S4).

Association between NRF3 expression and clinicopathological parameters in OC

NRF3 expression correlated significantly with multiple clinicopathological parameters in OC (Table 2). Elevated NRF3 expression was associated with older age ($P = 0.018$), larger tumor size ($P = 0.001$), higher histological grade ($P < 0.001$), and advanced FIGO stage ($P < 0.001$). Strong associations were observed with lymph node metastasis, LVSI, multi-organ invasion, distant metastasis, and recurrence (all $P < 0.001$), indicating that NRF3 linked to aggressive disease and dissemination. No significant correlations were observed with tumor laterality or histological subtype.

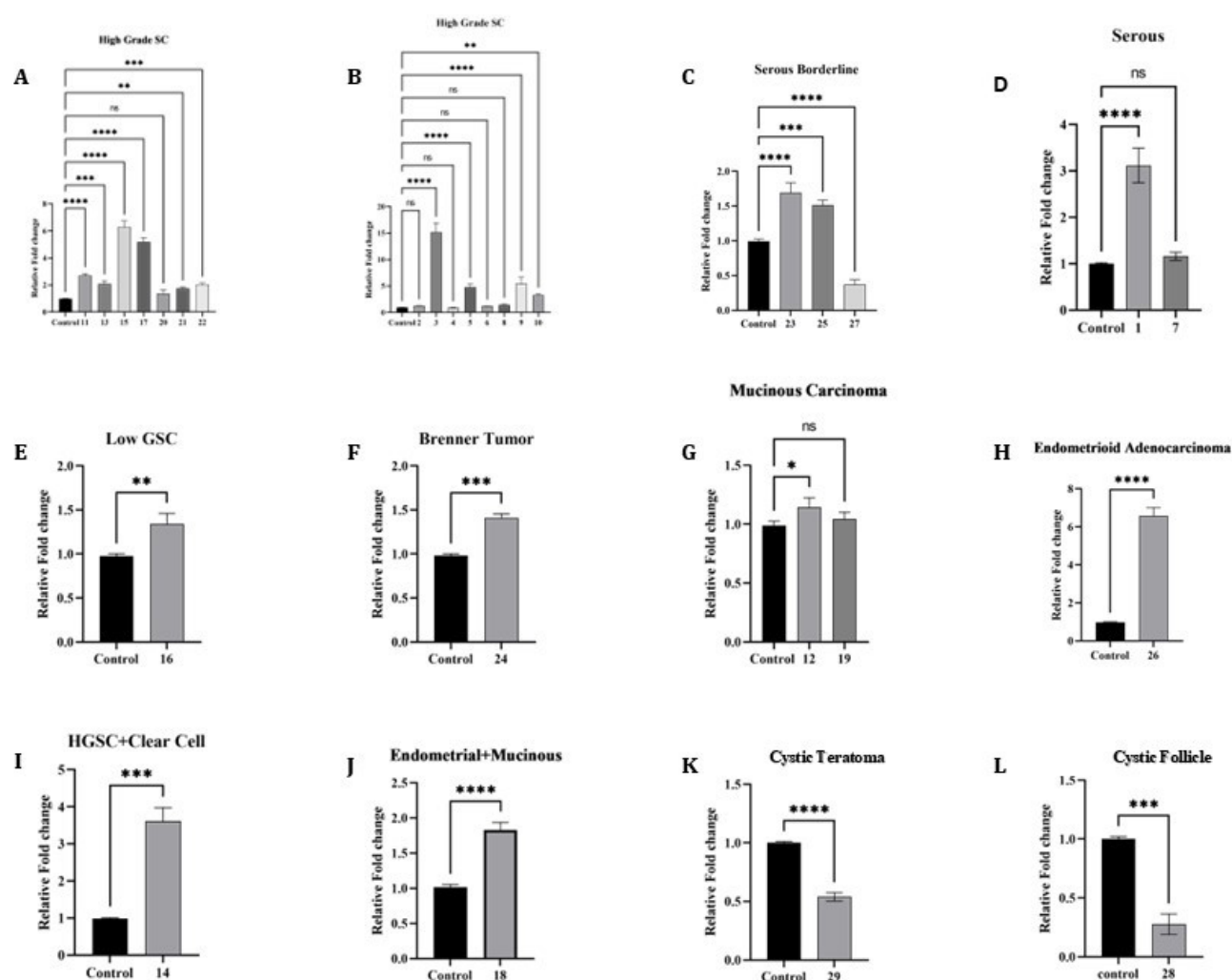


Fig. 1. Differential NRF3 mRNA expression in ovarian tissues via qPCR. NRF3 levels, normalized to GAPDH, were significantly higher in ovarian tumors than normal counterparts ($P < 0.05$). Subtypes are detailed in panels A-L, with sample identification by cDNA tags (1-29).

Table 1. Relative NRF3 mRNA expression in OC and normal ovarian tissues

Group	Median	IQR	Mean Rank	Kruskal-Wallis / Mann-Whitney U results
Normal	0.36	0.84	6.00	vs. High: $U = 9.0$, $Z = -2.488$, $P = 0.013^*$ vs. Low: $U = 6.0$, $Z = -2.530$, $P = 0.011^*$
Low-stage ^a	1.87	3.06	19.58	vs. Normal: $P = 0.011^*$ vs. High: $U = 77.0$, $Z = -0.634$, $P = 0.526$
High-stage ^b	1.50	1.54	17.53	vs. Normal: $P = 0.013^*$ vs. Low: $P = 0.526$
Overall	—	—	—	Kruskal-Wallis $H = 7.743$, $df = 2$, $P = 0.021^*$

IQR, Interquartile range.

^a FIGO I-II.

^b FIGO III-IV.

Prognostic performance of NRF3 expression in OC

ROC analysis showed that NRF3 expression discriminated relapse (AUC=0.779) and death (AUC=0.746) with good accuracy (Fig. 4). Optimal cut-offs (~115–120 IHC score) achieved high sensitivity (≈ 0.90 – 0.93) and moderate specificity (≈ 0.46 – 0.55) according to Youden's index. Patients exceeding this threshold exhibited markedly higher risks of recurrence and mortality.

Survival studies

Among 171 OC cases, 94 were evaluable for PFS and 57 for OS within the Stage III–IV subgroup. Early-stage

patients showed minimal events, consistent with favorable prognosis. Kaplan-Meier analysis demonstrated that high NRF3 expression was significantly associated with worse survival outcomes. Mean OS was 35.5 months (95% CI: 31–40) versus 74.6 months (95% CI: 65–84) for low expression ($\chi^2 = 10.284$, $P = 0.001$ for OS and DSS). Similarly, mean PFS for high-expression patients was 20.4 months (95% CI: 16–25) compared with 57.3 months (95% CI: 44–70) for low-expression patients ($\chi^2 = 11.966$, $P = 0.001$). Survival curves were clearly stratified by FIGO stage (Fig. 5).

Univariate Cox regression identified NRF3 expression, FIGO stage, grade, nodal involvement, and peritoneal

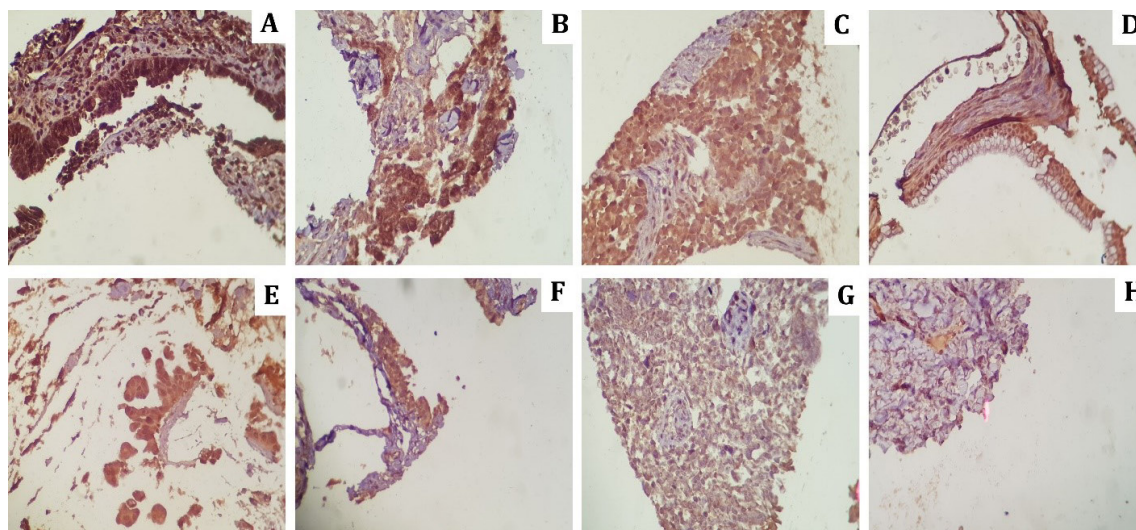


Fig. 2. Representative Immunohistochemical Staining Patterns of NRF3 in OC Tissue Microarray (TMA) Cores (40× magnification). The figure illustrates variable NRF3 expression across different tumor grades and histological types. Strong staining intensity was generally highest in High-Grade Serous Carcinoma (HGSC) cases (A) and lower in Mucinous Cystadenocarcinoma (F) and another HGSC sample (G). Panel H shows a representative tumor-less core (negative control)

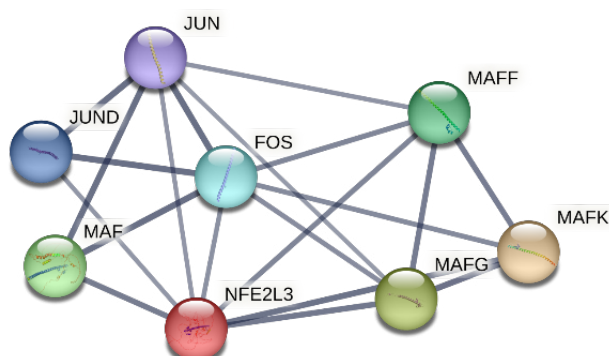


Fig. 3. NRF3 (NFE2L3) Protein-Protein Interaction Network. The network of 8 interacting proteins demonstrates significant connectivity (PPI enrichment $P=6.55 \times 10^{-5}$)

involvement as significant factors for OS and DSS (Table 3 and Table 4). In multivariable models, FIGO stage ($HR \approx 3.4$, $P=0.041$) and peritoneal involvement ($HR \approx 2.4$, $P=0.028$) remained independent predictors of OS, while only FIGO stage ($HR \approx 2.0$, $P=0.023$) independently predicted PFS. NRF3 expression showed strong univariate associations ($HR \approx 4.5$ for OS/DSS; $HR \approx 2.8$ for PFS) but lost significance upon adjustment. Stratified analysis indicated a borderline association between NRF3 and progression risk ($HR = 1.85$, 95% CI 0.91–3.74, $P=0.087$) but not with OS or DSS (Table S5). Subgroup analysis in Stage III–IV patients showed non-significant trends toward worse OS ($HR = 1.88$, $P=0.241$) and PFS ($HR = 1.70$, $P=0.182$) in the high NRF3 group. DSS analysis in Stage I–II was not feasible due to the absence of events (Fig. 6). We confirmed that the proportional hazards assumption was tested for all variables in the model using Schoenfeld residuals, and that the assumption was met.

Discussion

NRF3 mRNA expression was observed to be upregulated in OC tissues relative to normal controls, substantiating its purported role in malignant transformation. The uniform

elevation across low- and high-stage tumors indicates that NRF3 upregulation likely occurs early in disease progression, suggesting utility as a diagnostic, rather than a staging, biomarker.

Variation in NRF3 transcript levels across epithelial subtypes highlights the molecular diversity of ovarian carcinoma, pointing toward distinct pathological drivers for each histology. We also observed reduced NRF3 expression in benign lesions, a pattern that hints at a highly contingent regulatory mechanism that deserves closer investigation regarding early inflammatory or premalignant changes.

Discordance between mRNA and protein expression in low vs. high stage OC

A key observation in this study is the divergence between NRF3 transcript and protein levels across tumor stages. While NRF3 mRNA levels remained elevated in both low- and high-stage cancers compared with normal tissue, with no significant inter-stage difference, IHC analysis showed a marked increase in protein intensity within advanced-stage tumors.

This mismatch points to post-transcriptional and post-translational mechanisms, such as microRNA-mediated control or altered protein turnover, that govern functional NRF3 abundance.^{27,28} Beyond these regulatory factors, tumor heterogeneity likely facilitates significant protein accumulation despite stable mRNA profiles.²⁹ Discrepancies between these findings also reflect the fundamental difference between assays: qPCR captures bulk RNA and is sensitive to sample quality and size,³⁰ whereas TMA/IHC provides context-specific visualization of the functional protein effector within the tissue architecture, often revealing clinically relevant, stage-dependent variations.³¹ Consequently, protein expression may serve as a more accurate proxy for the functional effectors driving tumor biology than transcript-based assays alone.^{32,33}

These findings suggest that NRF3 transcriptional activity

Table 2. Association between expression of NRF3 and the clinicopathological parameters of the OC samples

Patients and tumor characteristics	Total samples N (%)	H-score (cutoff = 140), N (%)		P (χ^2)	Sig.
		Low (≤ 140)	High (> 140)		
Mean age, years (Range)	48 (16-86)			0.018	s
$\leq$ Mean age	84 (49.1)	50 (58.1)	34 (40.0)		
$>$ Mean age	87 (50.9)	36 (41.9)	51 (60.0)		
Ovarian Involvement				0.118	ns
Bilateral	81 (47.4)	35 (40.7)	46 (54.1)		
Right ovary	37 (21.6)	19 (22.1)	18 (21.2)		
Left ovary	46 (26.9)	26 (30.2)	20 (23.5)		
Not identified	7 (4.1)	6 (7.0)	1 (1.2)		
Mean Tumor Size (cm)	8.0			0.001	s*
$\leq$ Mean size	106 (62.0)	50 (58.1)	56 (65.9)		
$>$ Mean size	45 (26.3)	18 (20.9)	27 (31.8)		
Not identified	20 (11.7)	18 (20.9)	2 (2.4)		
Histological Grade				< 0.001	s*
Grade 1	62 (36.3)	44 (71.0)	18 (29.0)		
Grade 2	28 (16.4)	17 (60.7)	11 (39.3)		
Grade 3	81 (47.4)	25 (30.9)	56 (69.1)		
FIGO stage				< 0.001	s*
Stage I	70 (40.9)	51 (72.9)	19 (27.1)		
Stage II	33 (19.3)	17 (51.5)	16 (48.5)		
Stage III	44 (25.7)	13 (29.5)	31 (70.5)		
Stage IV	24 (14.0)	5 (20.8)	19 (79.2)		
Lymph Node Metastasis				< 0.001	s*
Absent	98 (58.0)	66 (67.3)	32 (32.7)		
Present	69 (40.8)	20 (29.0)	49 (71.0)		
Not identified	2 (1.2)	0 (0.0)	2 (100.0)		
Lymphovascular Space Invasion				< 0.001	s*
None	105 (61.4)	58 (55.2)	47 (44.8)		
Seen	38 (22.2)	6 (15.8)	32 (84.2)		
Not identified	28 (16.4)	22 (78.6)	6 (21.4)		
Colon Involved	33 (19.3)	7 (21.2)	26 (78.8)	< 0.001	s*
None	108 (63.2)	57 (52.8)	51 (47.2)		
Not identified	30 (17.5)	22 (73.3)	8 (26.7)		
Small intestine Involved	19 (11.1)	3 (15.8)	16 (84.2)	0.004	s
None	128 (74.9)	68 (53.1)	60 (46.9)		
Not identified	24 (14.0)	15 (62.5)	9 (37.5)		
Fallopian tube Involved	82 (48.0)	28 (34.1)	54 (65.9)	< 0.001	s*
None	85 (49.7)	54 (63.5)	31 (36.5)		
Not identified	4 (2.3)	4 (100.0)	0 (0.0)		
Cervix Involved	14 (8.2)	4 (28.6)	10 (71.4)	0.009	s
None	127 (74.3)	60 (47.2)	67 (52.8)		
Not identified	30 (17.5)	22 (73.3)	8 (26.7)		
Endometrium Involved	32 (18.7)	3 (9.4)	29 (90.6)	< 0.001	s*
None	107 (62.6)	61 (57.0)	46 (43.0)		
Not identified	32 (18.7)	22 (68.8)	10 (31.3)		
Myometrium Involved	10 (5.8)	4 (40.0)	6 (60.0)	0.001	s*
None	43 (25.1)	32 (74.4)	11 (25.6)		
Not identified	118 (69.0)	50 (42.4)	68 (57.6)		
Peritoneum Involved	59 (34.5)	20 (33.9)	39 (66.1)	0.001	s*

Table 2. Continued

Patients and tumor characteristics	Total samples N (%)	H-score (cutoff = 140), N (%)		P (χ^2)	Sig.
		Low (≤ 140)	High (> 140)		
None	109 (63.7)	66 (60.6)	43 (39.4)		
Not identified	3 (1.8)	0 (0.0)	3 (100.0)		
Omentum Involved	60 (35.1)	17 (28.3)	43 (71.7)	< 0.001	s*
None	109 (63.7)	69 (63.3)	40 (36.7)		
Not identified	2 (1.2)	0 (0.0)	2		
Distant Metastasis	171 (100.0)			< 0.001	s*
Absent	141 (82.5)	78 (55.3)	63 (44.7)		
Present	26 (15.2)	4 (15.4)	22 (84.6)		
Not identified	4 (2.3)	4 (100.0)	0 (0.0)		
Relapse (Tumor Recurrence)				< 0.001	s*
No	115 (67.3)	73 (63.5)	42 (36.5)		
Yes	56(32.7)	13 (23.2)	43 (76.8)		
Histological Type				0.335	ns
Epithelial ovarian cancer	146 (85.4)	70 (47.9)	76 (52.1)		
Germ cell tumors	11 (6.4)	7 (63.6)	4 (36.4)		
Sex-cord stromal tumors	12 (7.0)	7 (58.3)	5 (41.7)		
Not identified	2 (1.2)	2 (100.0)	0 (0.0)		
Total	171 (100.0)	86 (50.3)	85 (49.7)		

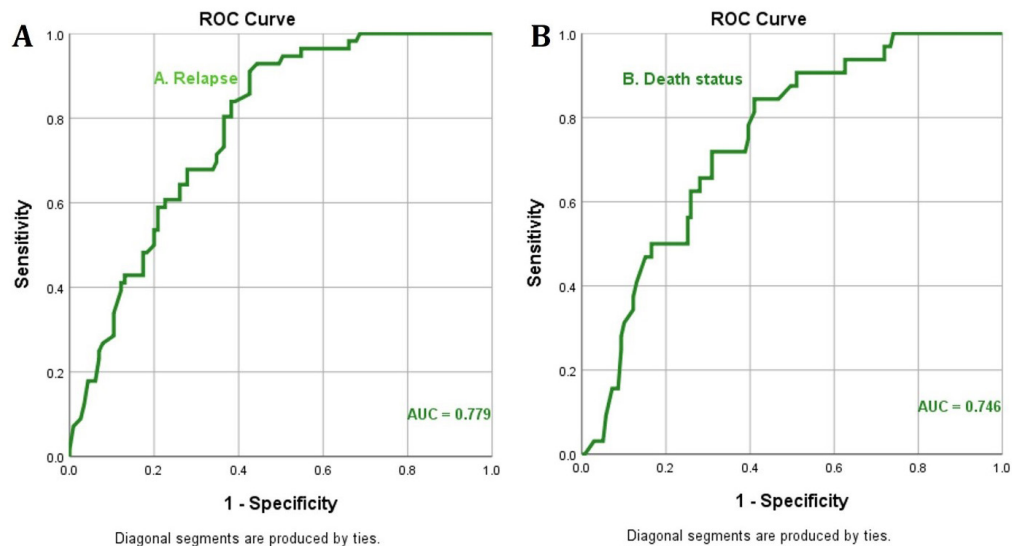


Fig. 4. Receiver Operating Characteristic (ROC) curve analysis of NRF3 IHC scoring for prediction of tumor A. relapse and B. death status in OC.

is an early event in ovarian carcinogenesis, although significant protein accumulation occurs only in advanced disease. Consequently, effective biomarker assessment relies on integrating both transcriptomic and proteomic data to provide a reliable clinical picture.

Functional and regulatory context of NRF3 in OC

Combined bioinformatic analyses (STRING and Enrichr) mapped NRF3 within a dense protein-protein interaction network tied to stress response, transcriptional regulation, and immune signaling. The recurrence of stress-responsive factors such as EGR1, ATF3, and AP-1 family members (JUN/FOS) highlights the protein’s potential role in cellular adaptation.

Furthermore, the enrichment of epigenetic regulators

(HDAC3/4, BRD4), alongside signaling partners such as ESR2 (Estrogen Receptor Beta) and ARNT (Hypoxia Signaling), integrates NRF3 into established mechanisms of tumor progression and therapeutic resistance in OC. This configuration suggests that NRF3 acts as a central node governing stress adaptation and hormone-driven malignancy. These in silico findings define the potential NRF3 landscape and serve as a foundation for future mechanistic studies, such as ChIP-seq or co-immunoprecipitation, to validate these associations in ovarian malignancy.

NRF3 association with clinicopathological parameters

Our data show that high NRF3 levels were associated with clinical markers of tumor aggressiveness, specifically large

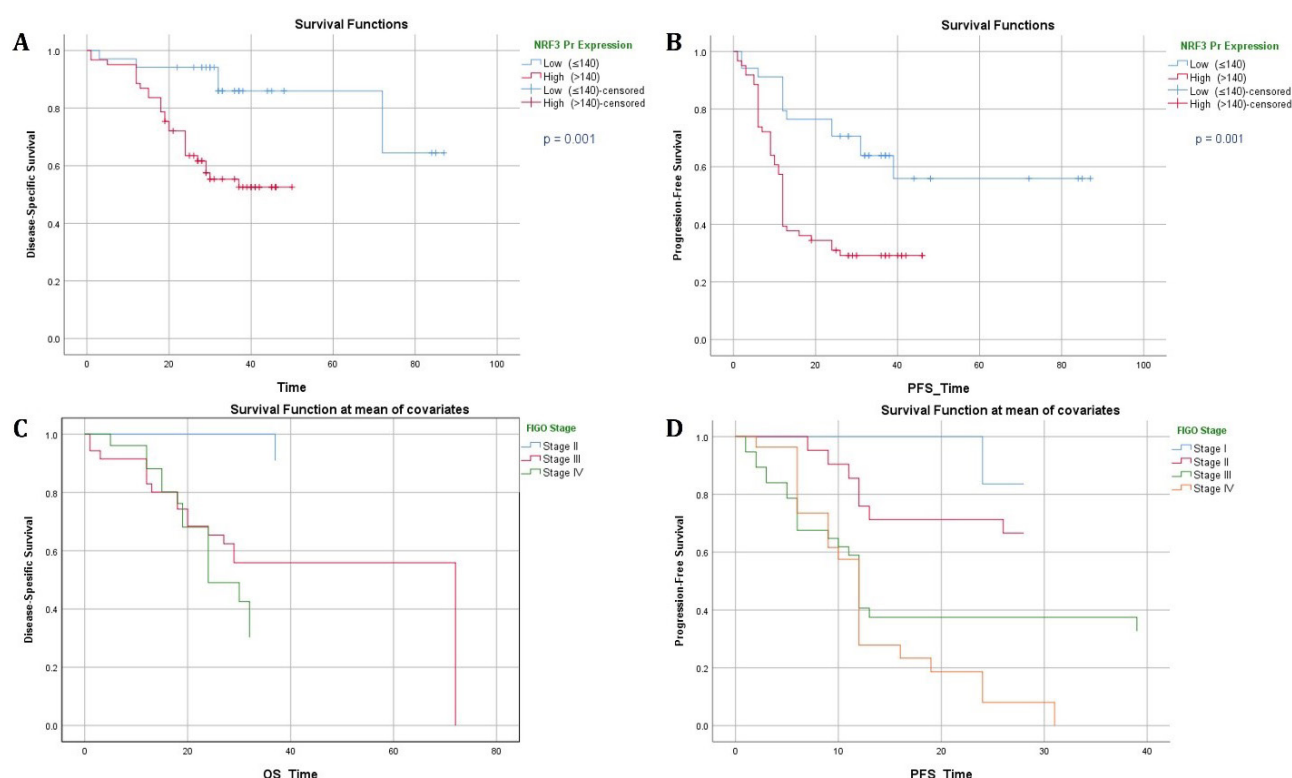


Fig. 5. Kaplan–Meier plots for NRF3 expression A. Disease-Specific Survival (DSS), B. Progression-Free Survival (PFS), and K–M plots stratified by FIGO stage for C. DSS and D. PFS.

tumor size, high histological grade, and advanced FIGO stage. We also found that elevated NRF3 expression was common in cases with a history of lymph node or distant metastasis and frequent recurrence. Interestingly, NRF3 status was not associated with ovarian laterality or the specific histological subtype of the tumor. This pattern indicates that NRF3 expression is likely a signature of the tumor’s fundamental biological state rather than a byproduct of its anatomical location or classification.

Prognostic significance of NRF3

Our ROC analysis indicates that NRF3 overexpression is associated with adverse clinical outcomes in OC, yielding AUC values of 0.779 for relapse and 0.746 for death. However, this prognostic signal fades in multivariate models once we account for standard clinical factors such as FIGO stage. This implies that while NRF3 marks an aggressive tumor phenotype, it is not an independent prognostic driver. Rather than relying on the current data

Table 3. Univariate and multivariate Cox regression analyses of potential prognostic factors for overall survival and disease-specific survival in patients with ovarian cancer

Covariate	Univariate analysis		Multivariate analysis	
	OS and DSS -HR (95% CI)	P value	DSS-HR (95% CI)	P value
NRF3 expression high vs. low	4.692 (1.637 – 13.445) 4.538 (1.580 – 13.040)	0.004 0.005	2.073 (0.672 – 6.391)	0.205
Relapse (Tumor recurrence)	30.590 (4.159 – 224.999) 65.383 (3.420 – 1250.107)	0.001 0.005	215,118.736 (0 – 3.095E + 123)	0.930
Distant metastasis	4.445(2.171 – 9.100) 4.642 (2.239 – 9.624)	0.001 < 0.001	0.386 (0.102 – 1.461)	0.161
FIGO stage	3.054(1.895 – 4.923) 3.207 (1.943 – 5.291)	< 0.001 < 0.001	3.397 (1.051 – 10.978)	0.041*
Histopathological grade	2.533 (1.072 – 5.985) 2.927 (1.097 – 7.814)	0.034 0.032	0.876 (0.288 – 2.662)	0.815
Lymph node metastasis	2.308 (1.083 – 4.917) 2.999 (1.339 – 6.718)	0.030 0.008	0.685 (0.184 – 2.550)	0.573
Peritoneum involvement	4.949 (2.369 – 10.337) 4.506 (2.124 – 9.562)	< 0.001 < 0.001	2.425 (1.100 – 5.347)	0.028*

* Statistically significant.

The variables with *P* value less than 0.05 and HR more than 1.0 were included in multivariable analyses.

As univariate results were consistent for DSS and OS, only DSS was used in multivariable analyses.

HR, hazard ratio; CI, Confidence interval; OS, overall survival; DSS, disease-specific survival.

Table 4. Univariate and multivariate Cox regression analyses of potential prognostic factors for progression-free survival in patients with ovarian cancer

Covariate	Univariate analysis		Multivariate analysis	
	PFS-HR (95% CI)	P value	PFS -HR (95% CI)	P value
NRF3 expression high vs. low	2.752 (1.471 – 5.149)	0.002	1.579 (0.821 – 3.039)	0.171
Distant metastasis	3.254 (1.885 – 5.615)	< 0.001	0.762 (0.316 – 1.839)	0.545
FIGO stage	2.285 (1.685 – 3.099)	< 0.001	2.000 (1.098 – 3.644)	0.023*
Histopathological grade	2.438 (1.343 – 4.426)	0.003	1.031 (0.488 – 2.175)	0.937
Lymph node metastasis	3.296 (1.788 – 6.077)	< 0.001	1.725 (0.782 – 3.805)	0.177
Peritoneum involvement	2.338 (1.444 – 3.786)	0.001	1.424 (0.852 – 2.379)	0.177

* Statistically significant.
The variables with *P* value less than 0.05 and HR more than 1.0 were included in multivariable analyses.
HR: hazard ratio, CI: confidence interval.

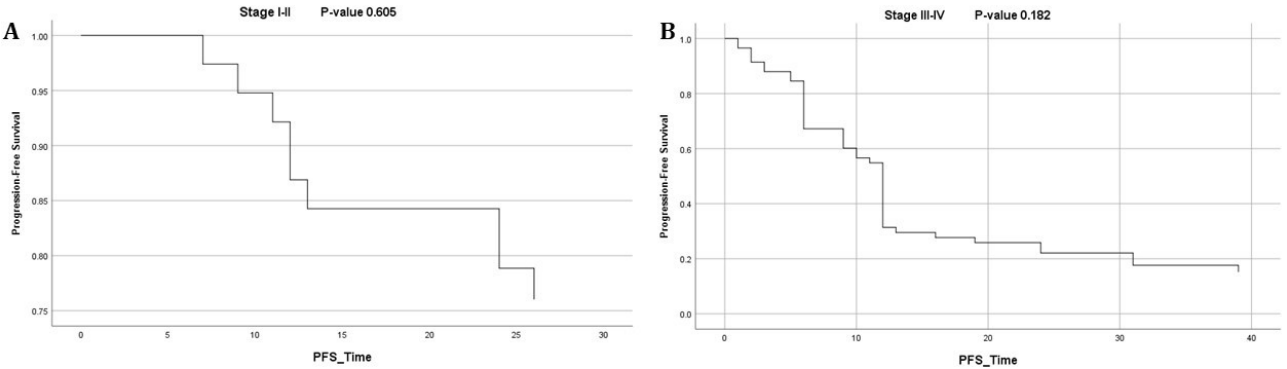


Fig. 6. Kaplan–Meier survival curves for Progression-Free survival in A. Stage I–II, and B. Stage III–IV OC patients, stratified by NRF3 expression.

to support clinical utility, we believe future large-scale prospective studies—utilizing decision-curve analysis—are needed to determine whether NRF3 actually adds value beyond standard clinicopathologic staging.

NRF3 thresholds in OC

In our study, a ROC-derived cut-off of 120 was established, whereas prior melanoma research identified 110 as optimal for NRF3, underscoring context-dependent variability in biomarker stratification.³⁴ To assess diagnostic performance, optimal thresholds were determined via ROC analysis using Youden’s index. For comparative clinicopathological and survival analyses, patients were dichotomized by the median IHC score of 140. This higher threshold is particularly appropriate given that transcriptomic evidence confirms NRF3 (NFE2L3) expression in normal ovarian tissues.³⁵ Utilizing a median-based approach in this context helps differentiate significant pathological overexpression from the higher physiological baseline characteristic of female reproductive organs. Furthermore, this dichotomization ensures a balanced distribution between groups, providing a more stable baseline for Kaplan-Meier and Cox regression analyses.³⁶

Survival studies

Patient survival was heavily dependent on disease extent. Advanced FIGO stage reliably predicted poorer OS, DSS, and PFS, while peritoneal spread independently increased the risk for OS. Although NRF3 expression correlated with adverse outcomes in univariate analysis, its effect

was not independent of stage, suggesting that it may act as a surrogate marker of disease burden rather than a standalone prognostic factor. The clinical dominance of stage and peritoneal status simply eclipsed other variables. However, stratified analyses revealed that NRF3 maintained prognostic relevance within specific stage-defined subgroups.

Limitations

While our study spans a wide spectrum of ovarian cancers, the predominance of high-grade serous carcinoma limits our ability to draw strong conclusions for rarer subtypes. Furthermore, given the exploratory nature of our work and the absence of formal adjustment for multiple comparisons across diverse endpoints, these findings warrant further validation in future targeted studies. Our ROC data suggest a prognostic signal for NRF3, yet we advise against treating it as a standalone clinical tool at this stage. Ultimately, prospective validation is essential to determine whether NRF3 truly adds incremental predictive value beyond the standard models offered by current clinicopathologic staging.

Conclusion

We observed that NRF3 is consistently upregulated in ovarian cancer; however, yet the divergence between mRNA and protein levels in advanced disease highlights the dominant role of post-transcriptional and post-translational regulation. This protein accumulation that likely drives the malignant behavior of these tumors.

Integrated analysis revealed that high NRF3 protein expression is associated with aggressive clinicopathological features—specifically advanced FIGO stage, metastasis, and poorer survival outcomes (OS, DSS, and PFS).

While our ROC analyses suggest that an IHC cut-off of approximately 115–120 could help identify patients at higher risk of relapse, its clinical application requires nuance. NRF3 did not maintain independent predictive value in our multivariate models, in which FIGO staging remained the primary determinant of prognosis. Therefore, we interpret NRF3 not as a standalone prognostic factor, but as a robust molecular correlate of aggressive tumor burden.

We see the greatest potential for NRF3 as a component of composite biomarker panels. Future prospective validation should focus on whether adding this protein to current staging criteria provides the incremental clinical utility needed to refine decision-making for patients with advanced ovarian cancer.

Acknowledgments

We are grateful to Dr. Siamak Naji Hadadi and Dr. Reza Salahlou for their support in collecting pathology samples from the hospital archives. Finally, we thank the patients who generously participated in this study, as this work would not have been possible without them.

Authors' Contribution

Conceptualization: Mohammad Reza Sadeghi, Mahdi Hosseinzadeh

Data curation: Elham Kalantari, Mohsen Rashid

Formal analysis: Mahdi Hosseinzadeh, Mohsen Rashid, Mohammad Reza Sadeghi

Funding acquisition: Mohammad Reza Sadeghi

Investigation: Mahdi Hosseinzadeh, Elham Kalantari, Behrooz Shokouhi

Methodology: Zahra Madjd, Mohsen Rashid

Project administration: Mohammad Reza Sadeghi

Resources: Mohammad Reza Sadeghi, Zahra Madjd

Supervision: Mohammad Reza Sadeghi

Validation: Behrooz Shokouhi, Zahra Madjd

Visualization: Mahdi Hosseinzadeh, Mohsen Rashid

Writing—original draft: Mahdi Hosseinzadeh

Writing—review and editing: Mahdi Hosseinzadeh, Zahra Madjd, Mohsen Rashid, Elham Kalantari, Behrooz Shokouhi, Manizheh Sayyah-Melli, Mohammad Reza Sadeghi

Data Availability Statement

Study data supporting our findings are available from the corresponding author upon request.

Declaration of AI-assisted Tools in the Writing Procedure

Microsoft Copilot was used only for linguistic polishing, and the authors performed all experimental work and data analyses independently. Additionally, no AI tools were involved in creating our figures or artwork, as all visual content is original to our research team.

Ethical Approval

The Ethical Committee of Tabriz University of Medical Sciences approved this study (IR.TBZMED.REC.1400.911) in accordance with the Declaration of Helsinki. All participants provided informed consent prior to inclusion.

Funding

We acknowledge funding from the Molecular Medicine Department at Tabriz University of Medical Sciences (Grant No. 68393). This work forms part of the requirements for a PhD degree.

Supplementary files

Supplementary file 1 contains Tables S1–S5 and Fig. S1.

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