



OPEN Low KIF4A expression is associated with gastric cancer progression and aggressive phenotypes

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Kinesin family member 4A (KIF4A) regulates chromosome condensation and segregation during mitosis and is upregulated as an oncogene in various malignancies. However, its role in gastric cancer (GC) remains unclear. Therefore, this study aims to evaluate the clinicopathological and prognostic significance of KIF4A expression in GC. KIF4A protein expression was assessed via immunohistochemistry and quantified using QuPath-based digital image analysis. KIF4A expression was analyzed for its associations with clinicopathological and molecular characteristics, as well as its prognostic significance. Additionally, bioinformatics tools were used to confirm the prognostic value of KIF4A at the mRNA level and perform enrichment and comprehensive immune analyses. Low KIF4A expression was significantly associated with adverse clinicopathological features and inversely correlated with HER2 amplification. Survival analysis revealed that patients with low KIF4A expression had poor clinical outcomes, consistent with mRNA-level analyses from publicly available databases. Univariate Cox regression revealed low KIF4A expression as a significant prognostic factor. However, low KIF4A expression was no longer significant in multivariate analysis. In enrichment analysis, low KIF4A expression is associated with EMT activation, whereas high expression correlates with E2F-mediated proliferation. Although high KIF4A expression suggests an immune-inflamed phenotype, its predictive ability was limited in an independent validation cohort.

Keywords Gastric cancer, Prognosis, Immunohistochemistry, Digital pathology, Kinesin family member 4A, KIF4A 4

Gastric cancer (GC) is a common digestive system malignancy, ranking fifth in global incidence and mortality, based on the 2022 Global Cancer Statistics (GLOBOCAN 2022)¹. GC is anatomically classified as cardia and non-cardia subtypes, with distinct epidemiological and etiological features. *Helicobacter pylori* infection is the main cause of non-cardia GC, while obesity and gastroesophageal reflux disease are key risk factors for cardia GC². Furthermore, genetic predisposition has recently gained recognition³.

Gastric adenocarcinoma, arising from the mucosa, is the predominant histologic type of GC and exhibits substantial histologic and molecular heterogeneity. Histologically, gastric adenocarcinoma is classified as intestinal or diffuse types based on the Lauren classification system⁴, while the World Health Organization (WHO) classification system provides detailed morphological subtypes⁵. At the molecular level, the Cancer Genome Atlas (TCGA) Research Network identified four distinct subtypes: Epstein-Barr virus (EBV)-positive, microsatellite instability (MSI), genomically stable (GS), and chromosomal instability (CIN)⁶.

Surgical resection is the main curative treatment for early and locally advanced GC, while adjuvant chemotherapy is recommended to improve survival in advanced GC. Recently, the treatment landscape has expanded to include immunotherapies and agents targeting HER2 and CLDN18.2^{7–9}. However, the prognosis for patients with advanced or metastatic disease remains poor. Therefore, identifying clinically useful predictive and prognostic biomarkers is essential for improving patient management.

In recent years, studies on GC have highlighted cellular heterogeneity, microenvironmental adaptation, and epithelial-mesenchymal transition (EMT) as key contributors to tumor progression. Within a single

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tumor, subpopulations of cells with distinct metabolic states coexist, representing intratumoral metabolic heterogeneity¹⁰. At the functional level, different GC cell lines undergo divergent forms of regulated cell death in response to the same cytotoxic stimulus, with cell fates determined by intrinsic molecular profiles¹¹. Furthermore, primary tumors and their paired metastatic lymph nodes display marked heterogeneity in both cancer cells and the tumor microenvironment, reflecting the spatiotemporal evolution of GC¹².

GC cells evade immune surveillance through PD-L1-mediated immunosuppression and resistance to immune-induced ferroptosis¹³. Under chemotherapeutic stress, GC cells upregulate the interferon-stimulated immune regulator OASL to escape therapy-induced immunogenic cell death and shape an immunosuppressive microenvironment, thereby reducing chemosensitivity¹⁴. Additionally, an EMT-associated transcription factor is epigenetically upregulated by promoter hypomethylation in GC, driving tumor cell invasion and metastasis via NGFR–ERK1/2 signaling¹⁵. EMT is progressively activated from primary tumors to metastatic lymph nodes, where declining local immunity accompanies an immune-“cold” niche¹². While these processes are increasingly recognized in GC, our understanding of the precise mechanisms integrating them is still limited.

Kinesin family member 4A (KIF4A), a chromokinesin, is essential for chromosome condensation and segregation during mitosis^{16,17}. Bioinformatics analysis of TCGA RNA-seq data reveals that KIF4A is upregulated in most tumor tissues and may function as an oncogene in tumorigenesis^{18,19}. Additionally, KIF4A overexpression is associated with poor clinical outcomes in major malignancies—lung, hepatocellular carcinoma, breast, colorectal, and renal cell carcinoma—highlighting its potential as a novel prognostic biomarker and therapeutic target^{20–24}. However, Jie Wu et al. report that high KIF4A expression inhibits the proliferation and migration of cervical cancer cells in vitro, and bioinformatics analysis using TCGA data reveals that patients with cervical cancer who have high KIF4A expression had better prognoses²⁵.

The role of KIF4A in GC remains unclear. Jie Gao et al. report that low KIF4A expression correlates with poor differentiation, while overexpression suppresses cell proliferation and tumor formation²⁶. In this study, we performed immunohistochemical (IHC) staining on a large series of resected specimens to assess the association of KIF4A expression with clinicopathological and molecular characteristics in GC. IHC staining was performed using QuPath, a digital pathology platform, to ensure objective and reproducible results.

Results

Clinicopathological characteristics of patients with gastric cancer

The median age of patients diagnosed with GC was 65 years (range: 28–90 years), including 149 females (29.4%) and 358 males (70.6%). Tumors were located in the proximal stomach (cardia, fundus, body, angle) in 206 cases (40.6%) and the distal stomach (antrum, pylorus) in 301 cases (59.4%). Early GC occurred in 268 cases (52.9%) and advanced GC in 239 cases (47.1%). Lymph node metastasis occurred in 212 patients (41.8%), lymphovascular invasion (LVI) in 259 cases (51.1%), perineural invasion (PNI) in 196 cases (38.7%), and 234 patients (46.2%) underwent adjuvant chemotherapy. No patients in this cohort underwent targeted therapy. Supplementary Table S1 shows the detailed clinicopathological characteristics of the 507 patients with GC.

Kinesin family member 4A expression and clinicopathological characteristics

Low KIF4A expression was significantly associated with tumor progression, including larger tumor size, higher pT category, nodal metastasis, and advanced stage (all $p < 0.001$). Additionally, low KIF4A expression was associated with adverse histopathological features, including poor differentiation (diffuse/mixed Lauren classification), LVI, and PNI (all $p < 0.001$). However, no significant associations were observed with age, sex, or tumor location. Table 1 summarizes the relationship between KIF4A expression and clinicopathological features.

Kinesin family member 4A expression and molecular characteristics

According to the TCGA molecular classification, 33 cases (6.5%) were classified as EBV subtype and 43 cases (8.5%) as MSI subtype, while the remaining 431 cases (85.0%) were classified as GS/CIN subtype. KIF4A expression was not significantly associated with TCGA molecular subtype. HER2 amplification occurred in 26 cases (5.1%), all EBV-negative and MSS. High KIF4A expression was significantly associated with HER2 amplification ($p = 0.028$). Table 2 summarizes its association with molecular characteristics.

Prognostic implication of Kinesin family member 4A expression

Kaplan–Meier analysis revealed that patients with low KIF4A expression exhibited significantly shorter overall survival (OS) ($p = 0.001$) and recurrence-free survival (RFS) ($p < 0.001$) than those with high KIF4A expression (Fig. 1a, b). Kaplan–Meier Plotter was used to analyze mRNA expression in 459 cases without distant metastasis. Survival curves were reconstructed using raw plot data exported from the Kaplan–Meier Plotter database. Low KIF4A expression was significantly associated with shorter OS ($p = 0.009$; Fig. 1c), and with shorter progression-free survival (PFS), though this was not statistically significant ($p = 0.061$; Fig. 1d).

In univariate analysis, significant predictors of shorter OS included low KIF4A expression ($p = 0.001$), older age ($p < 0.001$), diffuse/ mixed Lauren classification ($p = 0.040$), higher pT category ($p < 0.001$), nodal metastasis ($p < 0.001$), advanced stage ($p < 0.001$), LVI ($p < 0.001$), and PNI ($p < 0.001$). Shorter RFS was significantly associated with low KIF4A expression ($p < 0.001$), poor differentiation ($p < 0.001$), diffuse/mixed Lauren classification ($p < 0.001$), higher pT category ($p < 0.001$), nodal metastasis ($p < 0.001$), advanced stage ($p < 0.001$), LVI ($p < 0.001$), and PNI ($p < 0.001$). Multivariate analysis revealed older age ($p < 0.001$), advanced stage ($p < 0.001$), and PNI ($p = 0.015$) as independent prognostic factors for shorter OS. Advanced stage ($p < 0.001$) and PNI ($p = 0.047$) independently predicted shorter RFS. However, low KIF4A expression was not statistically significant. Table 3 summarizes the results of univariate and multivariate analyses.

Variables	KIF4A expression		P-value
	Low expression (%)	High expression (%)	
Age			0.951
< 65 years	147 (48.0)	159 (52.0)	
≥ 65 years	96 (47.8)	105 (52.2)	
Sex			0.614
Female	74 (49.7)	75 (50.3)	
Male	169 (47.2)	189 (52.8)	
Tumor size			< 0.001
< 4 cm	105 (37.8)	173 (62.2)	
≥ 4 cm	138 (60.3)	91 (39.7)	
Location			0.220
Proximal	106 (51.2)	101 (48.8)	
Distal	137 (45.7)	163 (54.3)	
Histologic type*			< 0.001
Differentiated	72 (36.2)	127 (63.8)	
Undifferentiated and others	171 (55.5)	137 (44.5)	
Lauren			< 0.001
Intestinal	73 (34.8)	137 (65.2)	
Diffuse and mixed	170 (57.2)	127 (42.8)	
pT category			< 0.001
pT1 and pT2	81 (30.2)	187 (69.8)	
pT3 and pT4	162 (67.8)	77 (32.2)	
Nodal status			< 0.001
Negative	107 (36.3)	188 (63.7)	
Positive	136 (64.2)	76 (35.8)	
Stage			< 0.001
I	92 (32.9)	188 (67.1)	
II and III	151 (66.5)	76 (33.5)	
LVI			< 0.001
Not identified	81 (32.7)	167 (67.3)	
Identified	162 (62.5)	97 (37.5)	
PNI			< 0.001
Not identified	113 (36.3)	198 (63.7)	
Identified	130 (66.3)	66 (33.7)	

Table 1. Correlation between KIF4A expression and clinicopathological characteristics in patients with GC (n = 507). *Differentiated, well-differentiated tubular adenocarcinoma, moderately differentiated tubular adenocarcinoma, papillary adenocarcinoma; Undifferentiated, poorly differentiated tubular adenocarcinoma, poorly cohesive carcinoma; Others, mucinous adenocarcinoma, other histologic subtypes; Stage, AJCC 8th edition. KIF4A, Kinesin family member 4A; PNI, perineural invasion; LVI, lymphovascular invasion; GC, Gastric cancer.

Biological functions and pathways associated with Kinesin family member 4A expression

We performed functional enrichment using 13,530 unique Entrez Gene IDs. Of these, 3,697 genes were mapped to Hallmark gene sets, 6,036 to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and between 7,993 and 10,732 to each of the three Gene Ontology (GO) categories: biological process (BP), molecular function (MF), and cellular component (CC). At a false discovery rate (FDR) threshold of < 0.1, five Hallmark gene sets showed significant enrichment. Notably, EMT and E2F targets (a hallmark of cell cycle progression) were identified as the most representative pathways based on normalized enrichment scores (NES). EMT was significantly enriched in the KIF4A low expression group (NES = −1.620, FDR = 0.041), whereas E2F targets were enriched in the high expression group (NES = 1.627, FDR = 0.051). Additionally, two terms within the GO CC category and one KEGG pathway also showed significant enrichment. Figure 2 illustrates representative Hallmark gene set results, and Supplementary Table S2 lists the top 20 enriched gene sets and pathways across the three databases.

Kinesin family member 4A expression and immune features

Immune infiltration analysis revealed that KIF4A expression was negatively correlated with tumor-stroma ratio (TSR; stromal score: $\rho = -0.367$, $p < 0.001$), as well as infiltration of cancer-associated fibroblasts (CAFs; xCell: $\rho = -0.416$, $p < 0.001$), M2 macrophages (TIDE: $\rho = -0.208$, $p < 0.001$), and the T-cell dysfunction score (TIDE:

Variables	KIF4A expression		P-value
	Low expression (%)	High expression (%)	
TCGA classification			
GS/CIN	208 (48.3)	223 (51.7)	0.876
MSI	19 (44.2)	24 (55.8)	
EBV-positive	16 (48.5)	17 (51.5)	
HER2 status			
No amplification	236 (49.1)	245 (50.9)	0.028
Amplification	7 (26.9)	19 (73.1)	

Table 2. Correlation between KIF4A expression and molecular characteristics in patients with GC (n = 507). MSI, microsatellite instability; EBV, Epstein–Barr virus; HER2, human epidermal growth factor receptor-2; KIF4A, Kinesin family member 4A; GS, genomically stable; CIN, chromosomal instability; TCGA, the Cancer Genome Atlas; GC, Gastric cancer.

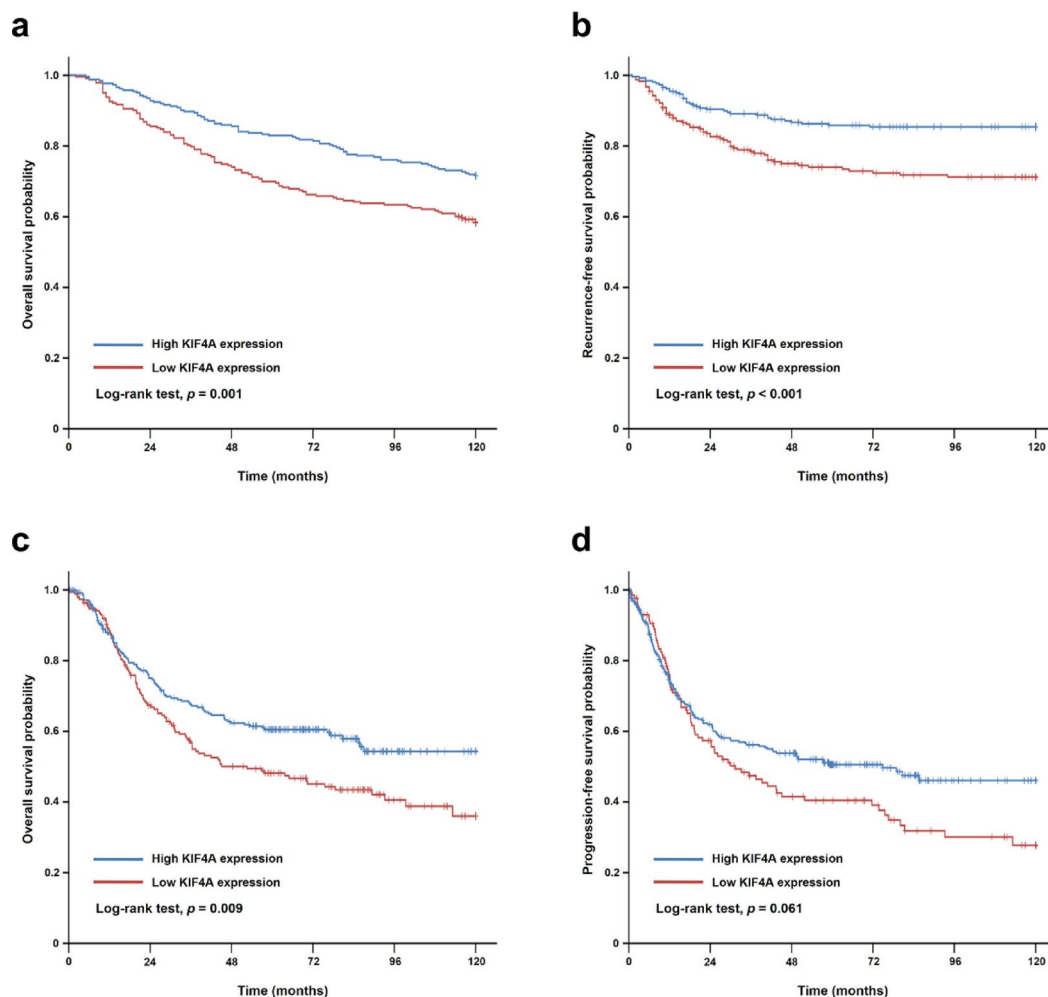


Fig. 1. Prognostic significance of KIF4A expression. Kaplan–Meier survival curves indicate that KIF4A expression was associated with (a) OS ($p = 0.001$) and (b) RFS ($p < 0.001$). Web-based analysis using Kaplan–Meier plotter revealed that low KIF4A mRNA expression was linked to (c) shorter OS ($p = 0.009$) but not with (d) PFS ($p = 0.061$). Abbreviations: KIF4A, Kinesin family member 4A; OS, overall survival; RFS, recurrence-free survival; PFS, progression-free survival.

$\rho = -0.452$, $p < 0.001$). In contrast, high KIF4A expression was characterized by T-cell exhaustion (ImmuCellAI: $\rho = 0.407$, $p < 0.001$), NK-cell inactivation (CIBERSORT-ABS: $\rho = 0.290$, $p < 0.001$), and the upregulation of CD274 (PD-L1; TIDE: $\rho = 0.309$, $p < 0.001$). A full list of correlations between KIF4A expression and the 175 immune features is presented in Supplementary Table S3. CTLA4 showed statistically significant association

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
<i>Overall survival</i>						
KIF4A expression* (low vs. high)	1.651	1.224–2.226	0.001	1.077	0.786–1.478	0.643
Age group (≥ 65 vs. < 65)	1.888	1.404–2.539	< 0.001	1.949	1.445–2.628	< 0.001
Histologic type [†]	1.259	0.926–1.712	0.142			
Lauren classification [‡]	1.378	1.014–1.872	0.040	1.106	0.792–1.543	0.556
pT category (T3-4 vs. T1-2)	3.393	2.461–4.679	< 0.001			
Nodal status (positive vs. negative)	3.509	2.576–4.782	< 0.001			
Stage (II, III vs. I)	3.817	2.772–5.256	< 0.001	2.494	1.493–4.165	< 0.001
LVI (identified vs. not identified)	2.928	2.121–4.044	< 0.001	1.057	0.643–1.739	0.827
PNI (identified vs. not identified)	3.443	2.541–4.664	< 0.001	2.797	1.119–2.797	0.015
<i>Recurrence-free survival</i>						
KIF4A expression* (low vs. high)	2.132	1.421–3.196	< 0.001	1.021	0.672–1.553	0.921
Age group (≥ 65 vs. < 65)	0.858	0.571–1.289	0.461			
Histologic type [†]	3.711	2.203–6.253	< 0.001			
Lauren classification [‡]	3.580	2.172–5.899	< 0.001	1.587	0.938–2.684	0.085
pT category (T3-4 vs. T1-2)	16.242	8.176–32.266	< 0.001			
Nodal status (positive vs. negative)	5.680	3.607–8.946	< 0.001			
Stage (II, III vs. I)	11.917	6.642–21.383	< 0.001	4.820	2.072–11.216	< 0.001
LVI (identified vs. not identified)	8.531	4.758–15.294	< 0.001	1.591	0.669–3.619	0.269
PNI (identified vs. not identified)	7.690	4.816–12.279	< 0.001	1.845	1.009–3.371	0.047

Table 3. Univariate and multivariate Cox regression analyses in patients with GC (n = 507). *The high KIF4A expression group was used as the reference; [†]Histologic type: undifferentiated and others vs. differentiated; [‡]Lauren classification, diffuse and mixed vs. intestinal; Stage, AJCC 8th edition. Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval; LVI, lymphovascular invasion; PNI, perineural invasion; KIF4A, Kinesin family member 4A; GC, Gastric cancer.

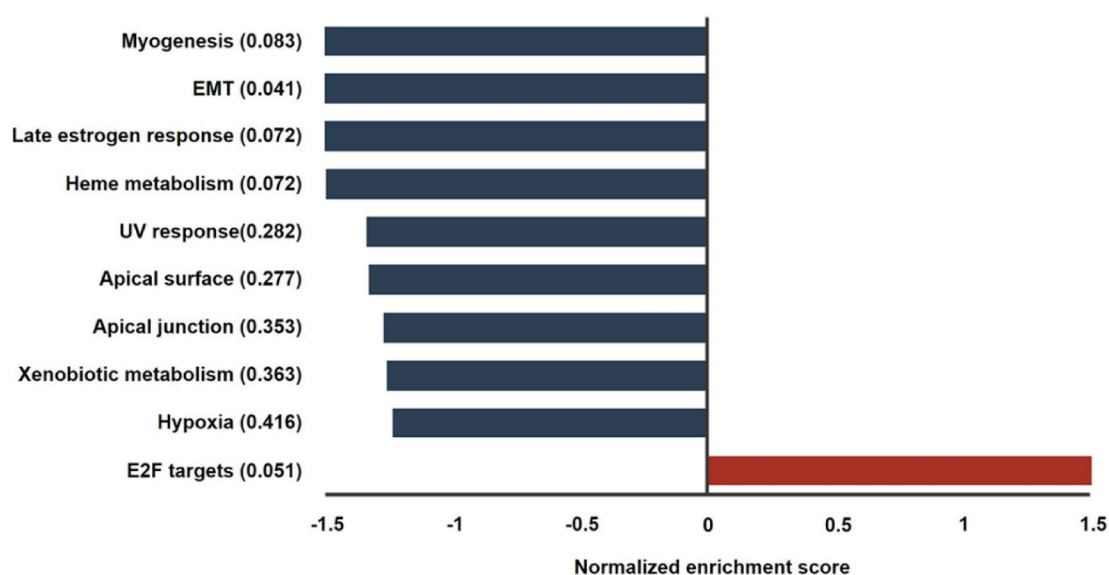


Fig. 2. Hallmark gene set enrichment analysis. Bar charts illustrate 10 key gene sets enriched in the KIF4A-high or -low expression groups, with FDR values in parentheses. Abbreviations: EMT, epithelial-mesenchymal transition; KIF4A, Kinesin family member 4A; FDR, false discovery rate.

with high KIF4A expression ($p=0.188$, $p<0.001$), whereas LAG3 showed a consistent positive trend ($p=0.114$, $p=0.062$). In the PRJEB25780 GC cohort²⁷, treated with anti-PD-1 immunotherapy ($n=45$), KIF4A expression showed suboptimal predictive performance for immunotherapy response (AUC=0.576).

Discussion

In this study, we evaluated KIF4A expression in GC using IHC and digital pathology analysis with QuPath software. Low KIF4A expression was significantly linked to advanced stage and aggressive histopathological features. Survival analyses revealed that patients with low KIF4A expression had significantly poorer outcomes, consistent with Kaplan–Meier Plotter results. While KIF4A expression showed prognostic significance in the univariate analysis, it was not identified as an independent predictor in the multivariate analysis, indicating that it is not a standalone prognostic biomarker in GC. This likely reflects the attenuation of its prognostic effect after adjustment for established clinicopathological covariates. Although KIF4A expression was not an independent factor in the multivariate analysis, its strong association with tumor stage, as well as aggressive phenotypes such as LVI and PNI, suggests that it reflects both tumor progression and aggressive behavior in gastric cancer.

Functional enrichment analysis revealed a prominent association between KIF4A expression and the ‘E2F targets’ gene set, suggesting that KIF4A is a core component of E2F-mediated proliferation. Interestingly, we identified an inverse correlation between KIF4A expression and the ‘EMT’ gene set. In our cohort, low KIF4A expression was significantly associated with tumor progression, and *in silico* pathway analysis demonstrated enrichment of EMT-related signatures, suggesting a hypothetical link to tumor invasion and migration in GC.

Low KIF4A expression was associated with the enrichment of M2 macrophages and an elevated T-cell dysfunction score. While CAF infiltration was high, the exclusion scores showed no significant association. These findings suggest that cytotoxic T cell dysfunction is the dominant mechanism of immune evasion in the KIF4A-low group²⁸. In contrast, high KIF4A expression in GC correlated with an elevated T-cell exhaustion score. Specifically, both CTLA4 and LAG3, key immune checkpoint genes used in the ImmuCellAI method²⁹, displayed positive correlations with KIF4A expression, though the correlation for LAG3 was not significant. In combination with high PD-L1 expression, these results suggest that the KIF4A-high group may exhibit an immune-inflamed phenotype. However, in the PRJEB25780 GC cohort, the predictive value of KIF4A expression was suboptimal. This may be partly explained by the specific characteristics of this validation dataset, where clinical response was primarily associated with MSI and EBV-positive subtypes, the cohort was small and limited to a single institution and ethnicity, and intratumoral heterogeneity potentially limited how representative a single biopsy was of KIF4A expression. These *in silico* findings suggest that KIF4A alone may be insufficient to predict immunotherapy responses in the complex GC microenvironment, where diverse immune-related factors influence therapeutic outcomes. In addition, residual confounding by tumor purity remains a potential limitation, despite the application of purity adjustment. Therefore, further comprehensive studies integrating various immune components are necessary.

Although the exact mechanism of KIF4A is unclear, most studies report its oncogenic roles in various cancer types. Studies on hepatocellular carcinoma and colorectal cancer show that KIF4A inhibits tumor suppressor pathways and promotes cell proliferation, while its knockdown reduces tumor cell migration and invasion^{21,23}. Furthermore, Hui Zhang et al. report that KIF4A is upregulated in glioma cells compared to adjacent normal brain tissue, facilitating cytoskeletal remodeling and cell cycle progression via the Rac1/Cdc42 pathway³⁰. Yeon-Jee Kahm et al. report that KIF4A sustains cancer stem cell traits, promotes mesenchymal phenotype and migration in lung cancer and glioma cells, and cross-regulates plasminogen activator inhibitor-1³¹. Wumin Jin et al. show that KIF4A drives ovarian cancer progression by upregulating BUB1³².

Contrary to its oncogenic role in other malignancies, Wu et al. report that high KIF4A expression in cervical cancer is linked to better survival. Additionally, KIF4A inhibited cervical cancer cell proliferation and migration, suggesting a tumor suppressor role. Supporting this observation, this study showed that low KIF4A expression was linked to frequent chromosomal aberrations, particularly deletions, and an immune escape phenotype, while high KIF4A expression increased sensitivity to cisplatin and gemcitabine²⁵. Gao et al. show that KIF4A is downregulated in GC compared to adjacent tissues and correlates with poor tumor differentiation. They report that KIF4A overexpression suppresses cell proliferation *in vitro* and tumor growth *in vivo*²⁶.

KIF4A is essential for chromosome condensation and segregation, contributing to the maintenance of genomic stability. Consequently, low KIF4A expression may be associated with the development of the CIN subtype in GC. To investigate this relationship, we categorized the molecular subtypes into GS/CIN, MSI, and EBV for our study; however, no significant correlation was observed between KIF4A expression and these molecular subtypes. This negative finding does not definitively exclude the hypothesis; rather, it suggests that further investigations with proper CIN-profiling techniques are required. While KIF4A is widely reported to play an oncogenic role in various cancer types, our findings indicate that low KIF4A expression contributes to tumor progression in GC, potentially associated with EMT activation and T-cell dysfunction-related immune evasion. The biological effect of KIF4A may be context-dependent, varying with the signaling pathways within each tumor type, although this hypothesis remains to be further investigated.

The TGF- β /Smad2/3 signaling pathway is a well-established regulator of EMT and contributes to tumor progression in cancer³³. A potential mechanistic link has recently been reported in glioma, where KIF4A activates this signaling to promote EMT³⁴. Whereas KIF4A overexpression promotes EMT in glioma, EMT in our GC cohort was associated with low KIF4A expression; this contrasting trend is consistent with the context-dependent functions of KIF4A across tumor types. Although a putative link between KIF4A and the TGF- β /Smad2/3 signaling pathway in GC remains to be established, this pathway is prominently implicated in EMT. We therefore speculate that the EMT phenotype associated with low KIF4A expression may, at least in part, reflect enhanced activity of this signaling. MMP1 has been reported to promote EMT and contribute to the establishment of an immunosuppressive microenvironment by enhancing macrophage infiltration and

impairing CD8⁺ T cell function³⁵. This pattern parallels the EMT- and immune-related features observed in our KIF4A-low group. Tumor microenvironment alterations, including ECM remodeling³⁶ and pre-metastatic niche formation³⁷, are increasingly recognized as key contributors to metastatic progression. While the functional involvement of KIF4A remains to be experimentally verified, our findings suggest a potential association between low KIF4A expression and a permissive tumor microenvironment in GC.

Although the functional interaction between EMT and the immune landscape in GC has not yet been fully understood, recent studies offer insights into their potential coordination. For instance, in 5-FU-resistant esophageal squamous cell carcinoma (ESCC) with EMT-like features, upregulated ADAM10 mediates PD-L1 shedding and restricts CD8⁺ T cell infiltration³⁸. Although the mechanistic link between EMT activation and ADAM10-mediated PD-L1 shedding remains unexplored in GC, both ADAM10 overexpression³⁹ and elevated sPD-L1⁴⁰ have been associated with poor prognosis. Additionally, MCT4 has been shown to promote tumor cell proliferation and migration while correlating with EMT activation and contributing to an immunosuppressive microenvironment in GC⁴¹. In this study, high MCT4 expression in GC was associated with reduced CD4⁺ and CD8⁺ T cell infiltration, decreased neutrophil recruitment, and disrupted M2 macrophage polarization. In contrast, existing literature on ESCC has demonstrated that MCT4 expression in the epithelial compartment was associated with the co-infiltration of CD8⁺ T cells, M2 macrophages, and neutrophils, and was paradoxically correlated with improved overall survival⁴². These contrasting findings reflect the complexity of MCT4-mediated immune responses across various cancer types. Taken together, ADAM10-mediated PD-L1 shedding and the MCT4-induced acidic microenvironment might serve as potential pathways underlying the interplay between EMT activation and T-cell dysfunction in GC, and further studies are warranted to confirm the precise mechanisms involved.

KIF4A expression was assessed using QuPath digital image analysis software (version 0.6.0), an open-source platform designed for biomarker quantification. Automated tissue microarray (TMA) analysis tools within digital image analysis platforms enable high-throughput screening to identify novel therapeutic targets and candidate prognostic or predictive biomarkers⁴³. Moreover, digital image analysis reduces observer bias and offers greater reproducibility and objectivity than manual assessment^{44–46}. QuPath offers distinct advantages, including fast processing and high agreement with manual scoring^{47,48}. However, ensuring reproducible analysis requires controlling factors that affect pixel values, including variations in tissue processing, staining protocols, and scanner choice across institutions⁴⁹. Furthermore, image processing and annotation are time-consuming and technically demanding, requiring precise cell segmentation and manual artifact correction⁴⁶.

This study has some limitations. First, the retrospective, single-institution design may introduce selection bias, limiting the generalizability of our findings. Second, KIF4A expression was assessed solely by IHC; therefore, complementary transcriptomic studies are needed to examine potential mRNA–protein discrepancies. Furthermore, the lack of an independent protein-level validation cohort remains a limitation. Third, due to the restriction of our analysis to representative regions of the tumor, potential sampling bias associated with intratumoral heterogeneity cannot be excluded. Fourth, the mechanisms underlying KIF4A's tumor-suppressive role were suggested only by *in silico* analyses without experimental validation. Further experimental studies are required to confirm these hypothetical mechanisms.

In conclusion, we visualized KIF4A protein expression in GC tissues via IHC and quantified it with QuPath to ensure reproducibility and objectivity. While not identified as an independent prognostic factor, low KIF4A expression correlates with tumor progression and aggressive phenotypes based on comprehensive clinicopathological data. Bioinformatic analysis revealed that while low KIF4A expression correlates with EMT activation, high expression reflects E2F-mediated proliferation and a potential immune-inflamed phenotype.

Methods

Patient selection and collection of clinicopathological data

This retrospective study included 547 patients with GC who underwent curative surgery at Hanyang University Hospital (Seoul, Republic of Korea) between February 2005 and August 2010. We excluded two patients who underwent neoadjuvant chemotherapy and two who died from postoperative complications within 1 month after surgery. After excluding samples lost during TMA construction, 507 cases were included in the final analysis. The Institutional Review Board of Hanyang University Hospital approved this study (IRB file No. HYUH 2019-04-032-001, approval date: 22 May 2019) and waived the requirement for informed consent.

In this study, clinical data were extracted from electronic medical records, including patient age, sex, follow-up duration, survival, and recurrence status. All hematoxylin and eosin (H&E)-stained diagnostic slides were reviewed to assess the following pathologic features: tumor size, location, gross type, histologic type, histologic grade, pathologic TNM stage, LVI, and PNI. Tumors were classified histologically based on the Lauren classification and the 5th edition of the WHO classification system (2019). The pathological stage was determined based on the 8th edition of the American Joint Committee on Cancer system.

Tissue microarray construction and immunohistochemical staining

TMA construction was performed using tissue cores from representative tumor areas to allow efficient IHC analysis of a large cohort. Representative viable tumor areas were identified on H&E-stained sections, and 3.0-mm diameter cylindrical tissue cores were punched from the corresponding formalin-fixed, paraffin-embedded blocks (donor blocks) and transferred to recipient blocks using a TMA instrument (Unitma Co. Ltd., Seoul, Republic of Korea).

IHC staining for KIF4A was performed on 4-μm-thick sections from TMA blocks using the Benchmark XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA) according to the protocol of the manufacturer. The primary antibody used was rabbit polyclonal anti-KIF4A (ab12227, Abcam, Cambridge, UK) at a 1:100 dilution.

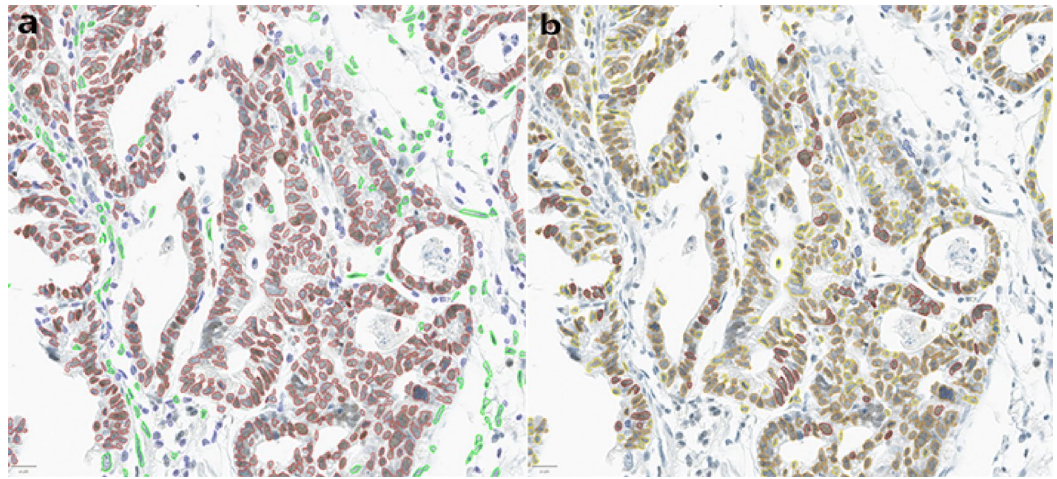


Fig. 3. Representative images of cell classification in QuPath. **(a)** Detected cells were classified as tumor (red), inflammatory (blue), and stromal (green) cells based on supervised cell phenotype classification. **(b)** Threshold-based classification according to nuclear staining intensity: tumor cell nuclei were classified as negative (blue), 1+ (yellow), 2+ (orange), or 3+ (red) based on mean nuclear OD. Magnification, $\times 42.76$. Abbreviation: OD, optical density.

Assessment of molecular characteristics

Mismatch repair (MMR) protein expression was assessed to determine MSI status. IHC for MLH1 (G168-728), PMS2 (MRQ-28), MSH2 (G219-1129; Cell Marque, CA, USA), and MSH6 (PU29; Leica Biosystems, Nussloch, Germany) was performed on entire sections from representative tumor blocks. Cases with complete loss of nuclear expression in tumor cells were considered negative, while those negative for at least one of the four IHC stains were classified as MSI. Tumors retaining all four MMR proteins were classified as microsatellite stable (MSS). EBV status was assessed using EBV-encoded RNA in situ hybridization. Automated staining was performed using the INFORM EBER Probe (Roche, Basel, Switzerland) according to the instructions of the manufacturer. Cases showing diffuse nuclear staining in tumor cells were classified as EBV-positive. Limited comprehensive genomic data prevented the distinction between GS and CIN subtypes, which were analyzed together.

HER2 amplification was evaluated using silver in situ hybridization. Automated staining was performed using the INFORM HER2 Dual ISH DNA probe cocktail (Roche, Basel, Switzerland) following the protocol of the manufacturer. Black (HER2) and red (CEP17) signals were counted in at least 20 cells under a light microscope. GC Cases with a HER2/CEP17 ratio ≥ 2.0 were classified as HER2-positive.

Digital pathology interpretation of immunohistochemical results

All TMA slides were scanned at $40\times$ magnification using the Aperio GT450 DX scanner (Leica Biosystems, Vista, CA, USA) for digital pathology analysis. The scanned images were imported into QuPath v0.6.0 software (<https://qupath.github.io/>), and auto-mated cell detection (watershed-based algorithm) was performed using the optical density (OD) sum parameter. Cell classification was then performed using the Train Object Classifier with the Random Trees machine learning algorithm⁵⁰. Representative areas containing tumor, immune, and other cell areas were manually annotated on randomly selected TMA cores to train the classifier. After training, the classifier was applied to all detected cells (Fig. 3a). For quantitative evaluation, nuclear staining intensity of tumor cells within each TMA core was automatically analyzed, with a median of 4149 cells (range: 604–28,058) per sample. Tumor cell nuclei were classified by staining intensity using the Set Cell Intensity Classification tool. Based on mean nuclear OD measurements, nuclei were classified as negative (OD < 0.05), 1+ (OD 0.05–0.1), 2+ (OD 0.1–0.2), or 3+ (OD ≥ 0.2). H-scores (0–300) were calculated for each TMA core (Fig. 3b).

Receiver operating characteristic curve analysis was performed to determine the optimal KIF4A expression cutoff value for OS, defined as the point maximizing the Youden index⁵¹. Based on this cutoff, cases were stratified into low (H-score < 27.6) and high (H-score ≥ 27.6) KIF4A expression groups for subsequent statistical analyses. Bootstrap internal validation with 1,000 resamples yielded a 95% bias-corrected and accelerated (BCa) confidence interval (CI) of 1.221–2.237 for the hazard ratio (HR), supporting the stability of this cutoff. Two experienced pathologists collaboratively performed digital pathology analysis and determined the optimal cutoff value.

Bioinformatics analyses

The prognostic value of KIF4A was validated using the Kaplan–Meier Plotter⁵² (<https://kmplot.com/analysis/>), an online tool integrating gene expression and clinical data from multiple independent datasets to assess associations between gene expression and survival outcomes. This tool provides gene expression data for 1,065 GC samples derived from Gene Expression Omnibus (GEO) datasets⁵³.

Gene set enrichment analysis was performed to identify biological functions and underlying mechanisms associated with KIF4A. The GSE26253 dataset, from the GEO database, provides gene expression data from a large cohort of 432 GC cases, covering both early and advanced stages. To define the high and low expression groups, the upper and lower quartiles of KIF4A expression ($n = 108$ each) were selected for subsequent analysis. The GEO2R tool (<https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE26253>) was utilized to extract differential expression profiles between these groups. Functional enrichment was performed using WebGestalt⁵⁴ (<https://www.webgestalt.org/>) for Hallmark gene sets⁵⁵, GO terms, and KEGG pathways^{56,57}. Enriched gene sets with an FDR < 0.1 were considered statistically significant.

We used the Tumor Immune Estimation Resource 3 (TIMER3; <https://compbio.cn/timer3/>) platform⁵⁸ to conduct a comprehensive immune infiltration analysis and to predict immunotherapy response based on KIF4A expression. This platform employs TCGA bulk RNA-seq data and various algorithms, such as CIBERSORT and EPIC, to assess immune cell infiltration. The tumor purity adjustment option was applied to account for potential confounding by tumor cellularity. Furthermore, it evaluates the predictive ability of genes for immunotherapy response using public datasets.

Statistical analyses

Statistical analyses were performed using SPSS Statistics version 25.0 (IBM, Armonk, NY, USA). Associations between KIF4A expression and clinicopathological characteristics were assessed using the chi-square or Fisher's exact test, as appropriate. Kaplan–Meier analysis with the log-rank test was used to compare OS and RFS between low and high KIF4A expression groups. Univariate and multivariate Cox proportional hazards regression analyses were performed to identify independent prognostic factors, with P -value < 0.05 considered statistically significant.

Data availability

The datasets generated during and/or analysed during the current study are not publicly available due to patient privacy regulations, but are available from the corresponding author on reasonable request. Publicly available datasets were also used in this study. The Kaplan–Meier Plotter data can be found at [<https://kmplot.com/analysis/>], and the GEO dataset is available under accession number GSE26253 at [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26253>].

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Author contributions

Seongsik Bang: data analysis, drafting of the manuscript, preparation of the illustrations, and preparation of the final text. Seoung Wan Chae: data analysis, preparation of the illustrations, Yeseul Kim, Seungyun Jee: review

of histological material. Hosub Park, Hyunsung Kim, Seung Sam Paik and Bo Gun Jang: revision of the main manuscript text. Jaekyung Myung: elaboration of the research concept, general supervision of the study, and preparation of the final text.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Hanyang University Hospital (IRB file No. HYUH 2019-04-032-001, approval date: 22 May 2019). The requirement for informed consent was waived.

Additional information

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