Heliyon 9 (2023) e21062

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

EFHD1 expression is correlated with tumor-infiltrating neutrophils and predicts prognosis in gastric cancer

Bin Zhao [a,1], Shanshan Wang [b,1], Li Xue [a], Qingqing Wang [c], Yushan Liu [a], Qiang Xu [a], Qiu Xue [d,*]

[a] Department of Pathology, Nantong Tumor Hospital, Nantong Fifth People's Hospital, Affiliated Tumor Hospital of Nantong University, Jiangsu, 226361, China
[b] Department of General Surgery, The Affiliated Suqian Hospital of Xuzhou Medical University and Nanjing Drum Tower Hospital Group Suqian Hospital, Suqian, 223800, China
[c] Department of General Surgery, Affiliated Hospital of Nantong University & Medical School of Nantong University, Jiangsu, 226001, China
[d] Department of General Surgery, Nantong Tumor Hospital, Nantong Fifth People's Hospital, Affiliated Tumor Hospital of Nantong University, Jiangsu, 226361, China

ARTICLE INFO

Keywords:
EFHD1
Tumor-infiltrating neutrophils
Gastric cancer
Tumor microenvironment
Multiplex immunohistochemistry

ABSTRACT

Background: Gastric cancer (GC) ranks third in terms of mortality worldwide. The tumor microenvironment is critical for the progression of gastric cancer. This study investigated the association between EF-hand domain containing 1 (EFHD1) expression and its clinical significance in the tumor microenvironment (TME) of gastric cancer.
Methods: We used bioinformatic analyses to assess the relevance of EFHD1 mRNA in the TME of gastric carcinoma tissues and its relationship with clinical features. Therefore, we performed multiplex immunohistochemistry analyses to determine the potential role of the EFHD1 protein in the TME of gastric cancer.
Results: EFHD1 expression increased dramatically in gastric cancer tissues compared to levels in non-cancerous tissue samples ($t = 6.246$, $P < 0.001$). The EFHD1 protein presentation was associated with invasion depth ($\chi^2 = 19.120$, $P < 0.001$) and TNM stages ($\chi^2 = 14.468$, $P = 0.002$). Notably, EFHD1 protein expression was significantly related to CD66b + neutrophil infiltration of the intratumoral ($r = 0.420$, $P < 0.001$) and stromal ($r = 0.367$, $P < 0.001$) TME in gastric cancer. Additionally, Cox regression analysis revealed that EFHD1 was an independent prognostic predictor (hazard ratio [HR] = 2.262, $P < 0.001$) in patients with gastric cancer.
Conclusions: Our study revealed the pattern of EFHD1 overexpression in the TME of patients with gastric cancer and demonstrated its utility as a biomarker for unfavorable clinical outcomes, thereby providing a potential immunotherapy target.

1. Introduction

Gastric cancer is the most common malignancy of the digestive tract and ranks fifth in incidence and third in cancer-related mortality rates globally [1,2]. Despite advancements in gastric cancer treatment, the survival rate of patients remains low due to

* Corresponding author.
E-mail address: xueqiu@ntu.edu.cn (Q. Xue).
[1] These authors contributed equally to this work and share first authorship.

https://doi.org/10.1016/j.heliyon.2023.e21062
Received 16 July 2023; Received in revised form 9 October 2023; Accepted 13 October 2023
Available online 14 October 2023

advanced-stage diagnosis and therapeutic resistance [3,4]. Therefore, the development of effective predictive biomarkers and treatment targets is essential to prolong the survival of patients with GC [5].

The significance of TME in the initiation and progression of cancer has garnered increasing attention in recent years [6]. Furthermore, tumor-infiltrating immune cell (TIIC) dysfunction in the TME of gastric cancer is vital for its development and progression [7]. TIICs such as tumor-infiltrating neutrophils (TINs), regulatory T cells, tumor-associated macrophages, and $CD8^+$ T cells have been described as outcome indicators in multiple cancer types, including gastric cancer [5,8–11]. Targeting of TIICs has exhibited promise in regard to improving current immunotherapies [12,13].

With advances in next-generation sequencing, bioinformatic analysis has recently emerged as a promising method for comprehensively analyzing detailed gene information, thus aiding in biomarker screening and molecular mechanism exploration [14,15].

EFHD1 is a Ca^{2+}-binding protein that plays essential roles in neuronal differentiation and acts as a mitochondrial regulator in certain diseases [16,17]. Numerous studies have reported that mitochondrial dysfunction is essential for malignancies [18], and recent studies have suggested that mitochondrial regulatory agents possess the potential to kill cancer cells [19]. When targeting the field of cancer, the expression of EFHD1 has been evaluated in breast and colorectal cancers in the context of genomic studies [20,21]. Moreover, EFHD1 regulates clear cell renal cell carcinoma (ccRCC) metastasis, acts as a potential therapeutic target, and serves as a diagnostic biomarker [22]. To date, the clinical significance of EFHD1 in stomach cancer has not been explored.

In this study, we evaluated EFHD1 mRNA expression in gastric tumors using the Gene Expression Omnibus (GEO) database [23]. We also analyzed the significance of EFHD1 mRNA expression in gastric carcinoma. Due to the nonlinear relationship between the transcriptome and proteome, particularly the changes between post-transcription and post-translation [24], we used multiplex immunohistochemistry (mIHC) with tissue microarray (TMA) to validate the relationship between EFHD1 protein expression in the TME and its clinical features.

2. Materials and methods

2.1. Bioinformatics analyses

GEO (https://www.ncbi.nlm.nih.gov/geo/) is a database that stores substantial publicly available gene expression data [23]. To analyze differences in EFHD1 mRNA expression, we downloaded the GEO dataset (GSE54129) that contained 132 human gastric tissue samples (111 gastric cancer and 22 normal samples). The Kaplan–Meier plotter database [25] (https://kmplot.com/analysis/) was used to study the impact of EFHD1 mRNA on overall survival (OS) in gastric cancer.

The Tumor Immune Estimation Resource (TIMER; https://cistrome.shinyapps.io/timer/) was utilized to examine immune cell infiltration levels in various cancer types [26]. Using this gene module, we evaluated the relevance of EFHD1 mRNA expression and gastric cancer TIICs.

2.2. Patient characteristics and TMA construction

A total of 310 patients with gastric cancer underwent radical surgical therapy at Nantong Tumor Hospital between 2010 and 2015. Patients were diagnosed histologically and received no chemotherapy, radiotherapy, or biological immunotherapy prior to surgery. Clinicopathological features were obtained from the medical records. We constructed a paraffin-embedded TMA containing 310 gastric cancer tissues and 68 non-cancerous gastric tissues. Each core was 2 mm in diameter and represented a tissue sample in the TMA using a Quick-Ray (UT06, UNITMA, Korea). The Human Research Ethics Committee of the local hospital approved this study.

2.3. Fluorescence-based mIHC and multispectral imaging

The TMA sections were deparaffinized and rehydrated, and this was followed by microwave treatment in AR6 buffer (AR600; AKOYA, USA). Blocking buffer (ARD1001EA, AKOYA, USA) was used to block the tissues. After an overnight incubation with primary antibodies, the slides were treated with secondary antibodies. Next, mIHC staining was performed using the PerkinElmer Opal 7-Color Technology Kit (NEL81001KT, USA). The staining steps were repeated continuously, and each multiplex staining process was completed along with the microwave treatment step at the end of each cycle. Finally, images were obtained using the Vectra 3.0 automated quantitative pathology imaging system (PerkinElmer, Akoya, USA). The immune scores were quantified using Form Cell Analysis software (PerkinElmer, USA). The score was then multiplied by summing the percentages of stained positive cells and ranged from 0 to 100.

The primary antibodies that were used included EFHD1 antibody (orb688403, Biorbyt, UK), CK antibody (orb69073, Biorbyt, UK), CD8a antibody (85336s, Cell Signaling Technology, USA), CD3 antibody (85061s, Cell Signaling Technology, USA), CD4 antibody (NBP2-52663, Novus, USA), CD68 antibody (76437s, Cell Signaling Technology, USA), CD86 antibody (orb388891, Biorbyt, UK), CD163 antibody (93498s, Cell Signaling Technology, USA), CD66b antibody (ARG66287, Arigo. biolaboratories, China), CD20 antibody (ab78237, Abcam, UK), and CD208 antibody (ab271053, Abcam, UK).

2.4. Statistical analysis

The Student's t-test was used to assess EFHD1 expression in gastric cancer and non-cancerous samples. We stratified patients into different EFHD1 expression groups (high vs. low) with the cutoff point set at 8.50 that was obtained using the X-tile software. Pearson's

χ^2 test and Spearman's rank correlation were respectively applied to examine the relationship of EFHD1 presentation with clinicopathologic parameters and TIIC abundances. Additionally, we used Cox regression analysis to determine the risk factors for OS. All data were analyzed using GraphPad Prism version 6 (La Jolla, CA, USA) and IBM SPSS version 23 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. EFHD1 mRNA expression and its prognostic value in gastric cancer

To explore EFHD1 as a potential biomarker for gastric carcinoma, we investigated EFHD1 mRNA expression in gastric cancer using the GEO dataset GSE54129. EFHD1 mRNA was overexpressed in gastric cancer samples ($t = 6.460$, $P < 0.001$) (Fig. 1A). Moreover, the Kaplan–Meier plotter database revealed a correlation between high EFHD1 mRNA expression and short-term OS in patients with gastric cancer (HR = 2.05, $P < 0.001$) (Fig. 1B).

3.2. Association between EFHD1 mRNA and TIICs

Considering the importance of the TME in gastric cancer progression, we investigated the association between EFHD1 expression and TIICs. We observed that EFHD1 mRNA expression was strongly associated with macrophages (Fig. 2A), moderately associated with $CD4^+$ T cells and dendritic cells (Fig. 2B and C), and weakly associated with $CD8^+$ T cells and neutrophils (Fig. 2D and E).

3.3. Clinical characteristics and prognostic features of EFHD1 protein expression in gastric cancer

Considering the non-linear relationship between transcriptomic data and the proteome [27], we performed mIHC to verify EFHD1 protein expression in gastric cancer tissues. Our investigation revealed EFHD1 protein overexpression in gastric cancer samples compared to that in benign gastric cancer samples ($t = 6.246$, $P < 0.001$) (Fig. 3A and B), and this was consistent with the mRNA expression data acquired from the GEO database. We analyzed the relevance of EFHD1 protein levels to clinical characteristics. The high EFHD1 expression group exhibited an increased tumor size ($\chi^2 = 19.120$, $P < 0.001$) and high TNM staging ($\chi^2 = 14.468$, $P = 0.002$). However, no relationship was observed between EFHD1 expression and sex, age, tumor differentiation, lymph node metastasis, or distant metastasis (Table 1).

Subsequently, we performed Cox regression analysis to determine the clinical outcome of EFHD1 expression in patients with gastric cancer. The result indicated that EFHD1 expression, tumor size (T), lymph node metastasis (N), and distant metastasis (M) were associated with OS ($P < 0.05$) (Table 2). Multivariate analysis indicated that increased EFHD1 expression (HR = 2.262, $P < 0.001$) and high TNM stage (HR = 2.167, $P < 0.001$) predicted poor survival (Table 2).

3.4. Relationship between EFHD1 protein expression and immune infiltrating cell densities in gastric cancer

Gastric cancer tissues possess varying degrees of TIICs in the intra-and peritumor regions. Therefore, EFHD1 protein expression coupled with intratumoral and stromal immune cell densities was analyzed in gastric cancer tissue samples. High EFHD1 expression in

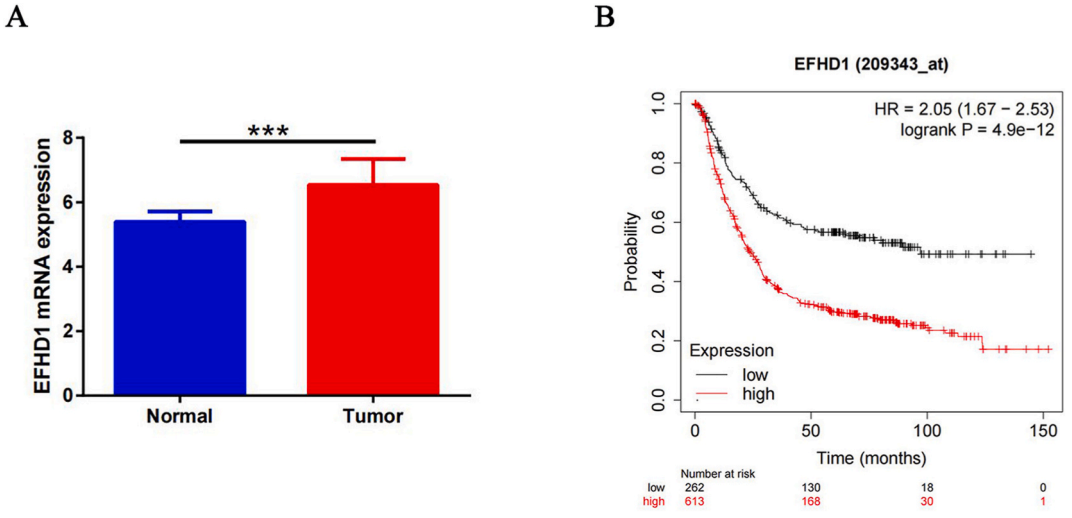

Fig. 1. Relationship between EFHD1 mRNA representation and overall survival (OS) in gastric cancer. **(A).** EFHD1 mRNA expression in gastric cancer and normal/benign samples. **(B).** High EFHD1 mRNA expression was related to inferior prognosis. ***$P < 0.001$.

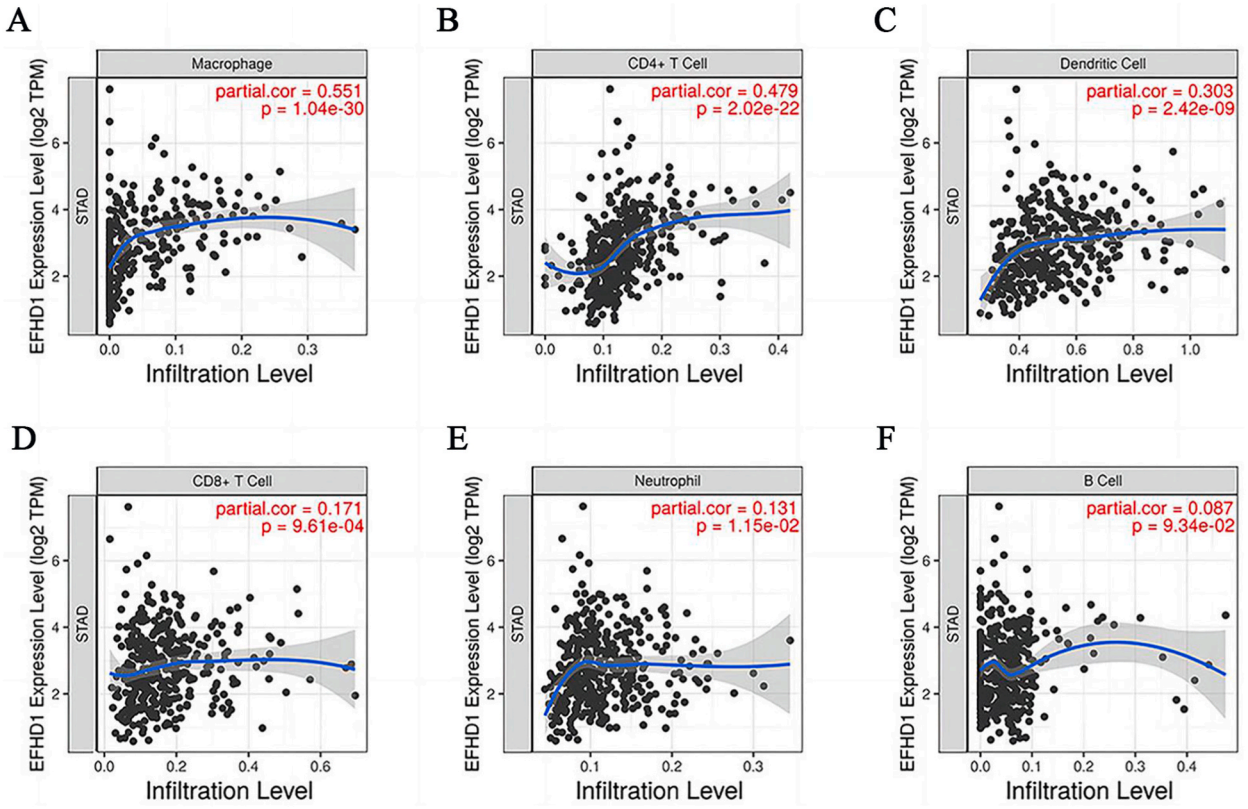

Fig. 2. Relevance of EFHD1 mRNA and TIICs in gastric cancer samples. **(A–E).** EFHD1 mRNA exhibits a significant correlation with macrophages ($r = 0.551$, $P < 0.001$), $CD4^+$ T cells ($r = 0.479$, $P < 0.001$), dendritic cells ($r = 0.303$, $P < 0.001$), $CD8^+$ T cells ($r = 0.171$, $P < 0.001$), and neutrophils ($r = 0.131$, $P < 0.05$). **(F).** EFHD1 mRNA expression is not related to B cell infiltration ($P > 0.05$).

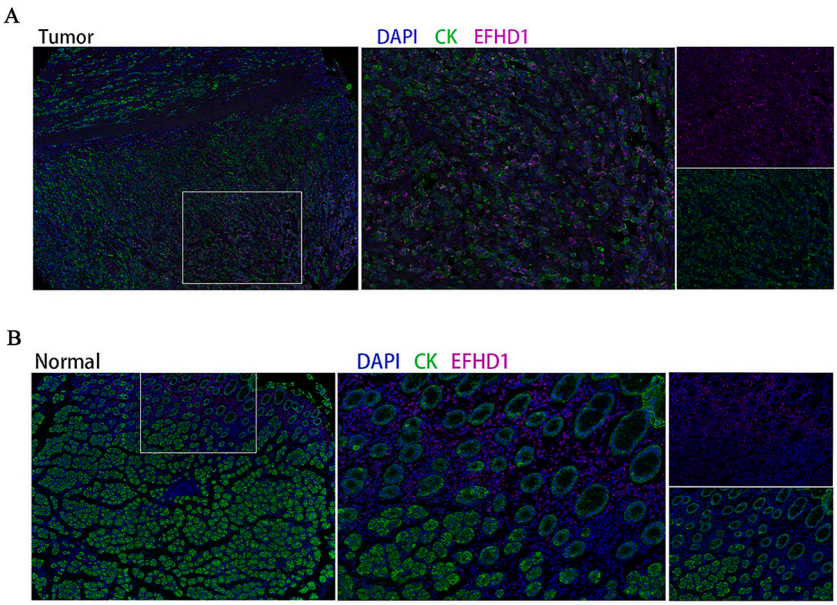

Fig. 3. EFHD1 protein expression in gastric cancer samples compared to that in non-cancerous samples. **(A–B).** Fluorescence-based mIHC revealed that the EFHD1 protein was overexpressed in gastric carcinoma tissues compared to levels in benign gastric tissues. Cytokeratin (CK) was used to identify tumor tissue epithelial cells and defined intra-tumor and peri-tumor regions.

Table 1
Relationship of EFHD1 protein expression with clinicopathological factors in gastric cancer.

Characteristic	Total	Low Expression (%)	High Expression (%)	Pearson χ^2	*P*
Total	310	111(35.80)	199(64.20)		
Sex				0.029	0.865
Male	216	78(36.10)	138(63.90)		
Female	94	33(35.10)	61(64.90)		
Age				0.5	0.479
≤60	117	39(33.30)	78(66.70)		
>60	193	72(37.30)	121(62.70)		
Differentiation				2.297	0.371
Well	4	0(0.00)	4(100.00)		
Middle	93	33(35.50)	60(64.50)		
Poor	213	78(36.60)	135(63.40)		
T				19.120	< 0.001*
Tis + T1	56	34(60.70)	22(39.30)		
T2	45	16(35.60)	29(64.40)		
T3	50	15(30.00)	35(70.00)		
T4	159	46(28.90)	113(71.10)		
N				6.650	0.084
N0	98	44(44.90)	54(55.10)		
N1	49	17(34.70)	32(65.30)		
N2	61	22(36.10)	39(63.90)		
N3	102	28(27.50)	74(72.50)		
M				1.443	0.230
M0	303	110(36.30)	193(63.70)		
M1	7	1(14.30)	6(85.70)		
TNM				14.468	0.002*
I	72	39(54.20)	33(45.80)		
II	50	17(34.00)	33(66.00)		
III	179	54(30.20)	125(69.80)		
IV	7	1(14.30)	6(85.70)		

*$P < 0.05$. M, distant metastasis; N, lymph node metastasis; T, tumor size.

Table 2
Univariate and multivariable analysis of prognostic features for OS in gastric cancer patients.

	Univariate analysis				Multivariate analysis			
	HR	*P* >|z|	95 % CI		HR	*P* >|z|	95 % CI	
EFHD1 expression	2.666	<0.001*	1.745	4.072	2.262	<0.001*	1.478	3.463
Age (y)	1.202	0.314	0.840	1.720				
≤60 vs. > 60								
Sex	0.948	0.776	0.654	1.374				
Male vs Female								
Differentiation	1.433	0.057	0.990	2.074				
Well vs. Middle vs Poor								
T	1.690	<0.001*	1.403	2.035				
Tis + T1 vs T2 vs T3 vs T4								
N	2.073	<0.001*	1.751	2.455				
N0 vs NI vs N2 vs N3								
M	4.085	0.001*	1.794	9.304				
M0 vs M1								
TNM	2.273	<0.001*	1.747	2.959	2.167	<0.001*	1.656	2.837
I vs II vs III vs IV								

*$P < 0.05$. CI, confidence interval; HR, hazard ratio; M, distant metastasis; N, lymph node metastasis; T, tumor size.

tumors correlated with a high number of infiltrating CD66b + neutrophils, whereas the CD66b + neutrophil count decreased with low EFHD1 expression (Fig. 4A and B). Moreover, intratumoral CD66b + neutrophils ($r = 0.420$, $P < 0.001$) and stromal CD66b + neutrophils ($r = 0.367$, $P < 0.001$) were significantly associated with EFHD1 expression. Additionally, EFHD1 expression was associated with intratumoral and stromal $CD68^+CD86^+$ M1 macrophages ($r = 0.209$, $P < 0.001$; $r = 0.191$, $P = 0.001$), $CD68^+CD163$+ M2 macrophages ($r = 0.176$, $P = 0.002$; $r = 0.198$, $P = 0.001$, respectively), and LAMP3+ dendritic cells ($r = 0.187$, $P = 0.001$; $r = 0.116$, $P = 0.042$, respectively). $CD4^+$ T, B, and $CD8^+$ T cells infiltrated at higher levels with increased EFHD1 protein expression ($r = 0.125$, $P = 0.040$; $r = 0.161$, $P = 0.004$; $r = 0.181$, $P = 0.003$).

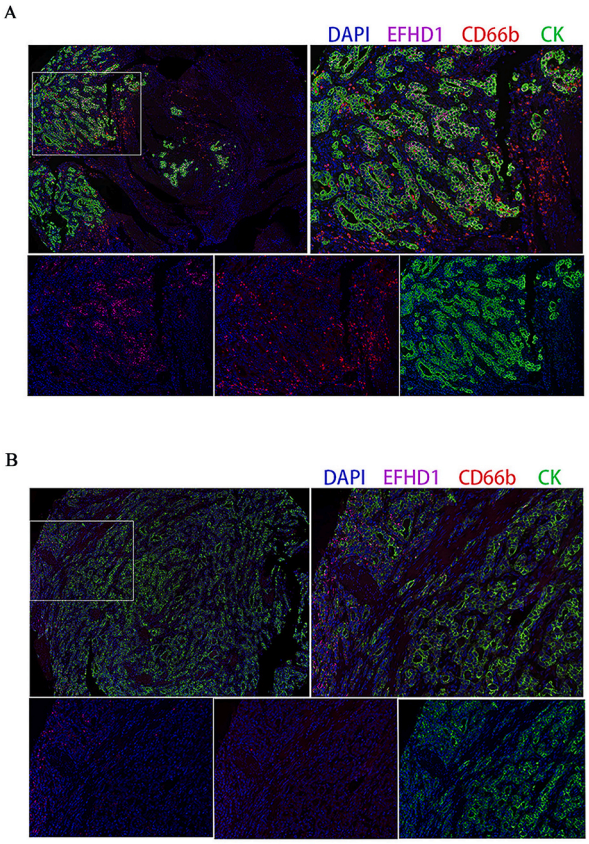

Fig. 4. Association between EFHD1 protein expression and CD66b + neutrophils in the gastric cancer tumor microenvironment. **(A).** Increased EFHD1 protein expression is related to high CD66b + neutrophil infiltration. **(B).** CD66b + neutrophils infiltrated to a lesser degree with reduced EFHD1 expression.

4. Discussion

Regarding the complexity and diversity of the TME, emerging evidence has uncovered its critical roles in tumor progression, immune escape, and therapy resistance [28,29]. Predicting the TME immune cell infiltration levels is crucial for developing novel therapeutic strategies [30,31]. Targeting immune cells in gastric cancer TME provides new directions for immunotherapy, and preclinical studies have confirmed its antitumor immune function with some treatment methods entering the clinical trial stage [32]. Our study revealed the relationship between EFHD1 expression and the prognosis and TME of gastric cancer using bioinformatics analyses and mIHC staining, respectively, and this aids in identifying novel diagnostic and treatment targets.

EFHD1 belongs to the EF-hand superfamily proteins and has been reported to participate in various malignancies [21,22,33–35]. However, its role in gastric cancer remains unclear. Our results indicate that EFHD1 expression is increased in gastric cancer tissues, and this is consistent with a previous study focused on breast cancer [34]. Moreover, we revealed that high EFHD1 expression was associated with poor survival rates in patients with gastric cancer, and this is consistent with results that were reported for melanoma [36]. Additionally, EFHD1 expression significantly correlated with TNM stage and tumor size, thus highlighting that EFHD1 plays a role in gastric cancer development. Furthermore, multivariate analysis identified EFHD1 as an independent prognostic predictor of gastric cancer. Thus, these findings indicate that EFHD1 promotes cancer progression and predicts an unfavorable prognosis of gastric cancer. However, the functional roles of EFHD1 in gastric cancer remain unclear.

Various immune cells infiltrate the TME, with neutrophils constituting the most infiltrated type of leukocytes [37,38]. The role of TINs in solid malignancies remains a current topic of interest [39]. Our analysis revealed that intratumoral CD66b + neutrophils and stromal CD66b + neutrophils correlated with EFHD1 expression in gastric tumor tissues. Neutrophil infiltration increases when EFHD1 is overexpressed. Several studies have reported a close connection between increased TINs levels and poor prognosis in patients with gastric cancer [37,40]. TINs can potentially refine risk stratification and help to explore clinical benefits in postoperative chemotherapy patients with gastric cancer, and they can also be incorporated into the TNM staging system [11]. However, TIN-associated biomarkers in gastric cancer require further investigation [41]. Therefore, EFHD1 (a TIN-related biomarker in gastric cancer) may aid in identifying novel therapeutic targets for gastric cancer.

Neutrophil extracellular traps (NETs) are extracellular net-like structures extruded by activated neutrophils that have been implicated extensively in cancer development, metastatic dissemination, and treatment resistance [42]. NETs mediate crosstalk

between the TME and deterioration [43]. The formation of NETs in the TME can drive cancer progression and engage in carcinoma immunotherapy, and they have been demonstrated to inhibit PD-1 blockade [44,45]. Moreover, NETs are formed in the gastric cancer TME, and NET accumulation decreases the tendency of tumors to form paratumor tissue [46]. NETs promote a more invasive mesenchymal phenotype and facilitate gastric cancer progression both in vitro and in vivo [47]. Thus, we speculate that EFHD1 and NETs derived from TINs in the TME synergistically promote gastric cancer progression.

This study possesses certain limitations. First, this was a retrospective study, thus necessitating prospective studies to verify the results. Second, further cytological and animal experiments should be performed to validate our findings. Moreover, the mechanistic role of EFHD1 in gastric cancer and the direct link between EFHD1 and TIICs remain unexplored.

To the best of our knowledge, this is the first study to illustrate the potential predictive value of EFHD1 in gastric cancer. Moreover, EFHD1 overexpression is an independent prognostic factor in gastric cancer. Additionally, our study highlighted the link between EFHD1 expression and TINs. Furthermore, EFHD1 as a TIN-associated biomarker in gastric cancer has implications for the identification of predictive biomarkers and therapeutic targets.

Data availability statement

Data will be made available on request.

Ethics declarations

This study was reviewed and approved by the ethics committee of Nantong Tumor Hospital, with the approval number: 2022–080.
All patients provided informed consent to participate in the study.
All patients provided informed consent for the publication of their anonymised case details and images.

Funding

This study was supported by the Nantong Science and Technology Project (JCZ20134).

CRediT authorship contribution statement

Bin Zhao: Conceptualization, Writing – original draft, Project administration, Resources. **Shanshan Wang:** Methodology, Writing – original draft, Data curation, Formal analysis, Project administration. **Li Xue:** Data curation, Formal analysis, Methodology, Investigation. **Qingqing Wang:** Data curation, Formal analysis, Methodology, Validation. **Yushan Liu:** Data curation, Formal analysis, Software, Visualization. **Qiang Xu:** Data curation, Formal analysis, Software, Visualization. **Qiu Xue:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We sincerely appreciate all lab members and thank the reviewers and editors for their comments.

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