RESEARCH ARTICLE

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Evaluation of *HHLA2* and *CD8* Immunohistochemical Expression in Colorectal Carcinoma and Their Prognostic Significance

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Abstract

Background: Colorectal carcinoma (CRC) is the third most common malignancy worldwide. Human endogenous retrovirus H long terminal repeat-associating protein 2 (*HHLA2*) is a novel immune checkpoint molecule. The association between *HHLA2* expression and clinicopathological features and its prognostic significance in CRC patients are still controversial. The aim of this study is to evaluate the prognostic value of immunohistochemical (IHC) expression of *HHLA2* and *CD8* in CRC. **Material and methods:** This retrospective study included 134 cases diagnosed with primary CRC at the Gastrointestinal Surgery Center (GISC) department, Mansoura Faculty of Medicine, during the period from December 2014 to December 2018. Clinicopathological and survival data were collected. IHC for *HHLA2* and *CD8* was performed, and they were correlated with clinicopathological parameters and patient prognosis. **Results:** Among 134 CRC cases, high *HHLA2* expression was detected in 73 (54.5%). High *HHLA2* expression was significantly related to the depth of invasion (P = 0.005*), lymph node metastasis (P = 0.01*), tumor stage)P = 0.002*), and distant recurrence)P = 0.012*). Multivariate analysis spotted *HHLA2* high expression as an independent prognostic predictor for OS in CRC (P = 0.03*) and DFS (P = 0.008*). *CD8* shows a significant correlation with tumor infiltrating lymphocytes (TILs) (P ≤ 0.001*), absence of metastasis ((P = 0.029*), absence of tumor deposits (P=0.014*). However, *CD8* shows no significant association with survival or *HHLA2*. **Conclusion:** *HHLA2* is an independent prognostic factor for the overall survival and disease free survival of CRC patients and can predict poor prognosis in CRC patients.

Keywords: *HHLA2- CD8-* Colorectal carcinoma- Immunotherapy

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Introduction

Colorectal carcinoma is the third most common cancer globally. Despite improvements in CRC prevention, diagnosis, and therapy, the disease still has a poor prognosis, and a large majority of CRC patients experience both local recurrence and distant metastasis (Sung et al., 2021). Immunotherapy, in particular immune checkpoint inhibitors, is seen as a very promising treatment approach. Its objective is to stimulate and enhance the anticancer immune response. The major effectors of antitumor immunity are cytotoxic *CD8*+ TILs. Immuno-checkpoints, especially the B7/CD28 immuno-checkpoint family, are significant molecules that either stimulate or inhibit the function of T cells. Targeting inhibitory receptors on immune effector cells, immune checkpoint inhibitors (ICIs) reactivate the immunological response (Golshani and Zhang, 2020).

HHLA2 has been considered a newly discovered ligand of the B7 family. It is expressed on some normal tissue, such as the epithelial cells of the stomach, kidney, gall bladder, and placenta, as well as on antigen-presenting cells like macrophages and activated dendritic cells. It is widely expressed in many types of malignant tumors, including pancreas, esophagus, stomach, colon, ovary, bladder, and lung cancers. Also, it is expressed in triple-negative breast cancer, glioma, cholangiocarcinoma, osteosarcoma, renal cell carcinoma (RCC), prostate cancer, and melanoma (Li et al., 2022).

HHLA2 has a dual role in the immune response in various tumors, as it can act as co-stimulatory or co-inhibitory according to the receptors to which it binds. Overall, the studies demonstrate that *HHLA2* predominantly functions as a T-cell co-inhibitory molecule with negative effects on TCR-mediated CD4+ and *CD8*+ T cell proliferation. Also, it suppresses their cytokine production. So *HHLA2* is participating in tumor immune escape (Niu et al., 2022).

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Some studies demonstrated a significant association between *HHLA2* expression and *CD8*+ T cell infiltration in tumor microenvironments (Zhu and Dong, 2018; Yan et al., 2020). The value of *HHLA2* expression in CRC remains indefinite and needs more investigation. Furthermore, the reports on *HHLA2* are often conflicting. The current study assesses the IHC expression of *HHLA2* and *CD8* in CRC and evaluates their correlation with the patient's prognosis.

Materials and Methods

This is a retrospective study carried out on formalin-fixed, paraffin-embedded (FFPE) tissue blocks for primary CRC obtained from resection specimens of 134 CRC cases. Patients were diagnosed and operated at GISC at our institute during the period from December 2014 to December 2018. Two hundred and fifty cases were diagnosed as CRC (116 cases were excluded, including 26 cases received pre-operative adjuvant therapy, 30 cases with unavailable blocks in the archive, 11 cases with repetitive tissue loss during the antigen retrieval procedure and 49 cases had lost follow up). So the final included cases were 134 cases.

The demographic and clinicopathological data of the enrolled cases were retrospectively retrieved from the electronic medical records of the patients in Clinical Oncology and Nuclear medicine department, Mansoura university hospital and the pathology database of the Surgical Pathology Laboratory at the GISC. In addition, the clinical outcomes of the patients were followed up in terms of overall survival and disease-free survival. The follow-up period started on the date of diagnosis and ended in March 2023. DFS was considered the period from the date of primary radical surgery to the date of the first treatment failure. Overall survival (OS) was measured from the diagnosis date to the end of the follow-up period or death. PFS is the time from the end of first-line treatment until disease recurrence or progression.

The tumors were classified histopathologically basing on the most recent WHO classification of colorectal carcinoma tumors (Nagtegaal et al., 2019). Each tumor was assigned a stage according to the latest American Joint Committee on Cancer (AJCC) TNM staging criteria, 8th edition. As regards TILs, the density of TILs was estimated based on the recommendations of the International TILs Working Group (ITWG). TILs are described as the mean percentage of the invasive tumor area occupied by lymphocytes and plasma cells. So, the tumors comprised in the current study were divided into 3 grades: low TILs (0–10%), intermediate TILs (20–40%), and high TILs (50–90%). Areas with necrosis, hemorrhage, or crush artifacts were ruled out during the TIL assessment (Fuchs et al., 2020).

The tissue microarray blocks (TMA) were designed by utilizing a fully manual-validated approach (Foda, 2013). Three tissue cores were extracted from three different sites from each donor block of resected colorectal carcinoma.

Immunohistochemical Staining

Sections from FFPE tissue blocks were deparaffinized and hydrated by standard approaches. We used *HHLA2* antibodies (rabbit polyclonal, 1:100 dilutions, IgG; Abclonal, Inc. catalog number A13262) and anti-*CD8* antibodies (mouse monoclonal primary antibody, clone C8/144B, ready to use; catalogue number IR623) according to the manufacturer's instructions with proper positive and negative controls.

Immunohistochemical evaluation

slides were scored in an independent manner by two pathologists who were blinded to the patients' data. *HHLA2* is expressed in cytoplasmic and membranous reactions. Based on the ratio of positively stained cells and staining intensity, *HHLA2* staining was quantified. According to the following metrics, the percentage of positive cells is graded: score 0 (0%), score 1 (1%–5%), score 2 (6%–30%), score 3 (31%–60%), and score 4 (61%–100%). The intensity of *HHLA2* staining was recorded as negative (0), mild (1), moderate (2), and strong (3). The final H-score was calculated by the equation: H-score = percentage × intensity. The median score was utilized to detect the cut off value of high or low *HHLA2* expression (Klua et al., 2023). The interpretation of *CD8* staining reaction was based on the study of Zhu et al 2018 by counting the number of *CD8* positive cells in each core of TMA. The cutoff point of high or low expression could be detected on the median of total scores (Zhu and Dong, 2018),

Statistical analysis

SPSS software version 25 (SPSS Inc., PASW statistics for Windows version 25) carried out the data analysis. Chicago: SPSS Inc. Qualitative data were described using numbers and percentages. For non-normally distributed data, the median (the middle number between the lowest and highest values) was used to define the data. For normally distributed data, the mean±SD was used after the Kolmogorov-Smirnov test was used to check for normality. The significance of the obtained results was judged at the ($\leq$0.05) level. Chi-Square, Fischer exact test, and Monte Carlo tests were used to compare qualitative data between groups as appropriate. Mann-Whitney U and Kruskal-Wallis tests were utilized to compare between two studied groups and more than two groups, respectively, for non-normally distributed data. • Kaplan-Meier test: utilized to calculate OS and disease-free survival by utilizing log-rank tests to detect the effect of predisposing factors affecting survival. Cox regression was used to assess predictors of survival with the calculation of the hazard ratio.

Results

Patients' clinicopathological characteristics are described in Table 1. According to the aforementioned criteria for *HHLA2* and *CD8* IHC evaluation, The H-score of *HHLA2* ranged from 0 to 12, with a median of 6. H score $\geq$ 6 was defined as a high *HHLA2* expression, and an H-score <6 indicated a low *HHLA2* expression. In 134 CRC tissues, 45.5% (61/134) cases of low *HHLA2* expression (Figure 1 A, B) and 54.5% (73/134) cases of

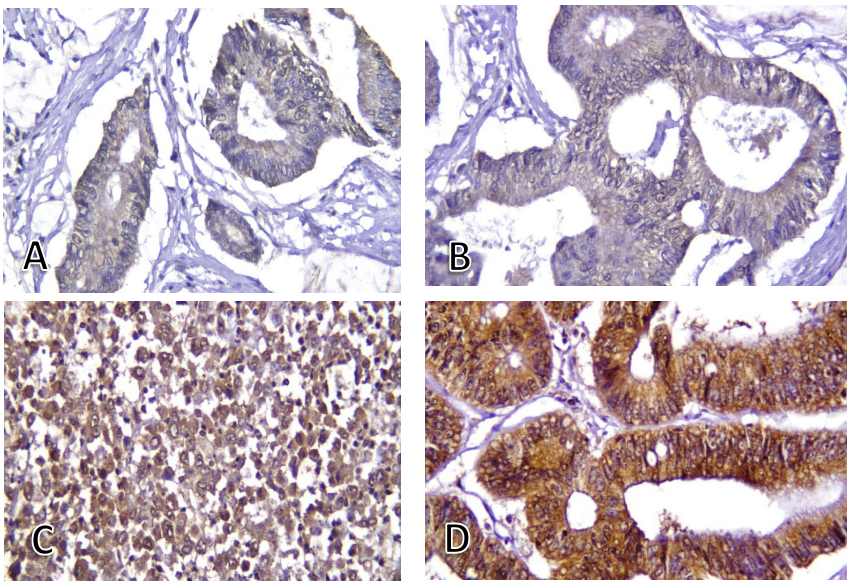

Figure 1. Immunohistochemical Staining of *HHLA2* in Various Cases of CRC: Low cytoplasmic expression (A, B). High cytoplasmic expression (C, D). Original magnification: 400X).

high *HHLA2* expression (Figure 1 C, D) were found.

As regards *CD8*, the median value of *CD8* was 60. Sixty-seven cases (50%) had a high *CD8* T-cell count (≥60) (Figure 2 A, B), and 67 cases (50%) were low (<60) (Figure 2 C, D).

As demonstrated in Table 2, there was a statistically significant association between higher *HHLA2* expression and depth of invasion (P = 0.005*), advanced N stage (P = 0.01*), higher tumor stage P = 0.002*), and distant recurrence P = 0.012*). There were no observed associations between *HHLA2* expression and other clinicopathological parameters. *CD8* expression showed a statistically significant positive association between *CD8* and TILs (P≤0.001*), M stage (P=0.029*), absence of tumor deposits (P=0.014*), and margin status (P=0.016*). There was no association between *CD8* and other clinicopathological parameters and *HHLA2*.

The median OS was 49.32 months (44.05–54.58). It is demonstrated that high *HHLA2* expression has low OS in patients with CRC (Figure 3A, P = <0.001*). The median DFS was 45.80 months (40.96–50.65). High *HHLA2* expression had a lower DFS (Figure 4A, P = <0.0001*). The median PFS was 44.25 months (39.17–49.33). The high *HHLA2* expression had a lower PFS (Figure 5A, P = 0.003*). *CD8* shows no significant association with survival (Figures 3B, 4B, and 5B).

Univariate and multivariate analyses on OS and DFS were conducted to detect the prognostic value of *HHLA2* expression and other clinicopathological variables (Table 3). In the univariate analysis, *HHLA2* expression demonstrated a significant correlation to the OS of cases with CRC (p = 0.039*). Meanwhile, histological grade (p = 0.03*), TILS (P = 0.009*), depth of invasion (P = 0.025*), lymph node (LN) metastasis (P = 0.02*), and tumor stage (P = 0.04*) were also found to be associated with OS. *HHLA2* expression demonstrated a significant

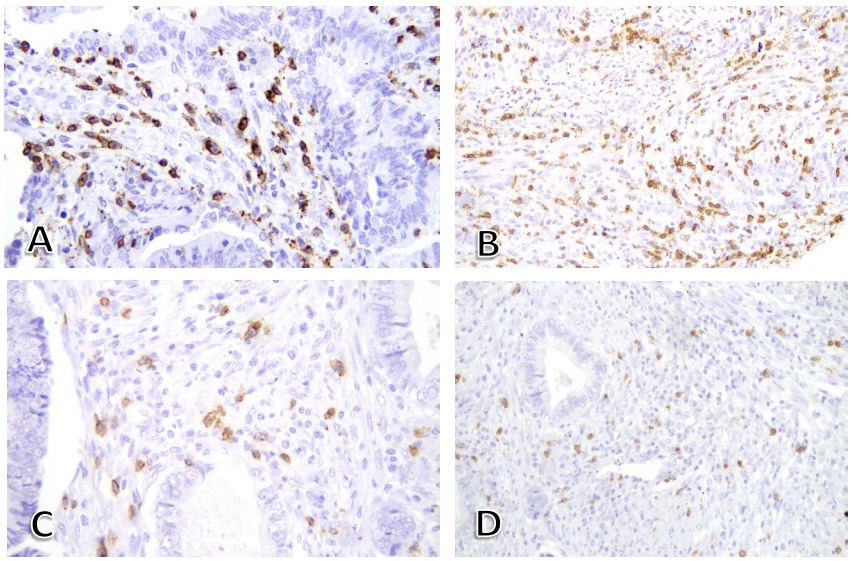

Figure 2. *CD8* IHC of *CD8* in Different Cases of CRC. High CD8 expression (A, B). Low CD8 expression (C, D). (Original magnification: A, C 400×; B, D 200×).

Table 1. Clinicopathologic Characteristics of Studied Patients

Clinicopathologic characteristics	The studied group (n=134) Number (%)
Age classes	
<60 y	88 (65.7%)
≥60 y	46 (34.3%)
Sex	
Male	75 (56.0%)
Female	59 (44.0%)
Site	
Right	49 (36.6%)
Left	85 (63.4%)
Size	
Mean ± SD	6.23±2.73
Min-Max	1.50-19.00
Histological type	
Adenocarcinoma NOS	110 (82%)
Mucinous adenocarcinoma	18 (13.4%)
Signet ring carcinoma	6 (4.5%)
Adenocarcinoma grade	
Low	107 (97.27%)
High	3 (2.7)
TILs	
Low	50 (37.3%)
Moderate	51 (38.1%)
High	33 (24.6%)
Tumor budding	
Low	90 (67.2%)
Moderate	24 (17.9%)
High	20 (14.9%)
Tumor deposits	
Present	15 (11.2%)
Absent	119 (88.8%)
Lympho-vascular emboli (LVI)	
Present	79 (59.0%)
Absent	55 (41.0%)
Perineural invasion (PNI)	
Present	37 (27.6%)
Absent	97 (72.4%)
Tumor depth of invasion	
T2	17 (12.7%)
T3	102 (76.1%)
T4	15 (11.2%)
Distant metastasis	
M 0	126 (94.0%)
M 1	8 (6.0%)
AJCC stage	
Stage I	12 (9.0%)
Stage II	63 (47.0%)
Stage III	51 (38.1%)
Stage IV	8 (6.0%)
Local recurrence	
Yes	31 (23.1%)
No	103 (76.9%)
Distant recurrence	
Yes	31 (23.1%)
No	103 (76.9%)
Patient fate	
Survived	38 (28.4%)
Died	96 (71.6%)

correlation with the DFS of patients with CRC (p=0.005*). Histological grade (P=0.026*) and tumor budding (P = 0.04*) were also associated with DFS.

The independent prognostic value was detected by multivariate analysis. *HHLA2* was independent prognostic factors with OS (P=0.03*) and DFS (P=0.008*).

Discussion

The B7 family immune checkpoint molecule *HHLA2* is very important in the tumor microenvironment and could be a good target for human cancer therapy. The high *HHLA2* expression is associated with worse prognosis in various malignancies. These include prostate cancer, lung cancer, osteosarcoma, hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma, bladder urothelial carcinoma, stomach cancer, breast carcinoma, colorectal cancer, and esophageal cancer. On the other hand, it is associated with a better prognosis for cervical adenocarcinoma, ampullary tumors, and pancreatic cancer (Bhatt et al., 2021).

There has been debate in the few studies that have been done on *HHLA2* expression in CRC. In our study, 54% of cases had high *HHLA2* expression. This result was slightly higher than Zhu et al., (2018) (47%). In contrast, two more investigations indicated that the *HHLA2* gene was downregulated in colorectal cancer (Ying et al., 2022). This variation can result from the use of different monoclonal antibodies, a different study population, or a different scoring system.

In the current study, *HHLA2* expression level was significantly associated with depth of invasion, LN metastasis, distant metastasis, and advanced tumor stage, which was consistent with *HHLA2* expression in many cancers. According to Zhu and Dong (2018), *HHLA2* was substantially associated with the invasion depth in CRC cases. According to Janakiram et al., (2015), elevated expression of *HHLA2* was substantially correlated with local LN metastases and advanced stage at diagnosis in triple-negative breast cancer. Furthermore, advanced clinical stage, tumor invasion, lymph node metastasis, and distant metastasis were all positively linked with increased *HHLA2* expression in gastric cancer tissues (Wei et al., 2019), bladder urothelial carcinoma (Lin et al., 2019), and intrahepatic cholangiocarcinoma (Jing et al.,

Table 2. Association between *HHLA2, CD8* and Clinicopathologic Parameters

Clinicopathologic parameters	Total	*HHLA2*		χ^2	*CD8*		χ^2
		Low (n=61)	High (n= 73)	(p value)	<60 (n = 67)	≥60 (n= 67)	(p value)
Age classes							
<60 y	88	41 (46.6%)	47 (53.4%)	χ^2 =0.118	44 (50%)	44 (50.0%)	χ^2 =0.0
≥60 y	46	20 (43.5%)	26 (56.5)	p=0.731	23 (50 %)	23 (50 %)	P=1.0
Sex							
Male	75	33 (44.0%)	42 (56.0%)	χ^2 =0.159	44 (58.7%)	31 (41.3%)	χ^2 =5.12
Female	59	28 (47.5%)	31 (52.5%)	p=0.690	23 (39.0%)	36 (61.0%)	P=0.024*
Site							
Right	49	23 (46.9%)	26 (53.1%)	χ^2 =0.062	29 (59.2%)	20 (40.8%)	χ^2 =2.61
Left	85	38 (44.7%)	47 (55.3%)	p=0.803	38 (44.7%)	47 (55.3%)	P=0.106
Size							
≤6	79	37 (46.8%)	42 (53.2%)	χ^2 =0.134	38 (48.1%)	41 (51.9%)	χ^2 =0.278
>6	55	24 (43.6%)	31 (56.4%)	P=0.715	29 (52.7%)	26 (47.3%)	P=0.598
Histological type							
Adenocarcinoma NOS	110	50 (45.5%)	60 (54.5%)	MC=0.057	50 (45.5%)	60 (54.5%)	MC
Mucinous adenocarcinoma	18	8 (44.4%)	10 (55.6%)	P=0.972	11 (61.1%)	7 (38.9%)	P=0.015*
Signet ring carcinoma	6	3 (50%)	3 (50%)		6 (100%)	0 (0%)	
Adenocarcinoma NOS grade (n= 110)							
Low	107	50 (46.7%)	57 (53.3%)	FET=2.57	49 (45.79%)	58 (54.2%)	FET
High	3	0 (0%)	3 (100%)	P=0.249	1 (33.3%)	2 (66.7%)	P=1.0
TILs							
Low	50	19 (38%)	31 (62%)	χ^2 =2.27	35 (70.0%)	15 (30.0%)	χ^2 =21.38
Moderate	51	27 (52.9%)	24 (47.1%)	P=0.321	26 (51.0%)	25 (49.0%)	P≤0.001*
High	33	15 (45.5%)	18 (54.5%)		6 (18.2%)	27 (81.8%)	
Tumor budding							
Low	90	44 (48.9%)	46 (51.1%)	χ^2 =1.28	40 (44.4%)	50 (55.6%)	χ^2 =3.58
Moderate	24	9 (37.5%)	15 (62.5%)	P=0.527	14 (58.3%)	10 (41.7%)	P=0.167
High	20	8 (40%)	12 (60%)		13 (65.0%)	7 (35.0%)	
Tumor deposits							
Present	15	6 (40%)	9 (60%)	χ^2 =0.208	12 (80.0%)	3 (20.0%)	χ^2 =6.08
Absent	119	55(46.2%)	64 (53.8%)	P=0.649	55 (46.2%)	64 (53.8%)	P=0.014*
LVI							
Present	79	31(39.2%)	48 (60.8%)	χ^2 =3.06	45 (57.0%)	34 (43.0%)	χ^2 =3.73
Absent	55	30 (54.5%)	25 (45.5%)	P=0.08	22 (40.0%)	33 (60.0%)	P=0.053
PNI							
Present	37	19 (51.4%)	18 (48.6%)	χ^2 =0.700	20 (54.1%)	17 (45.9%)	χ^2 =0.336
Absent	97	42 (43.3%)	55 (56.7%)	P=0.403	47 (48.5%)	50 (51.5%)	P=0.562
Tumor depth of invasion							
T2	17	13 (76.5%)	4 (23.5%)	χ^2MC =10.58	10 (58.8%)	7 (41.2%)	χ^2 =0.635
T3	102	45 (44.1%)	57 (55.9%)	P=0.005*	50 (49.0%)	52 (51.0%)	P=0.728
T4	15	3 (20%)	12 (80%)		7 (46.7%)	8 (53.3%)	
Lymph node metastasis							
N0	77	43 (55.8%)	34 (44.2%)	χ^2 =9.17	33 (42.9%)	44 (57.1%)	χ^2 =5.45
N1	31	12 (38.7%)	19 (61.3%)	P=0.01*	16 (51.6%)	15 (48.4%)	P=0.066
N2	26	6 (23.1%)	20 (76.9%)		18 (69.2%)	8 (30.8%)	
Distant metastasis							
M 0	126	60 (47.6%)	66 (52.4%)	χ^2 =3.74	60 (52.4%)	66 (52.4%)	χ^2 =4.78
M 1	8	1 (12.5%)	7 (87.5%)	P=0.053	7 (87.5%)	1 (12.5%)	P=0.029*

Table 2. Continued

Clinicopathologic parameters	Total	*HHLA2*		χ2	*CD8*		χ2
		Low (n=61)	High (n= 73)	(p value)	<60 (n = 67)	≥60 (n= 67)	(p value)
AJCC stage							
Stage I	12	10 (83.3%)	2 (16.7%)	χ^2MC =14.69	5 (41.7%)	7 (58.3%)	MC
stage II	63	33 (52.4%)	30 (47.6%)	P=0.002*	27 (42.9%)	36 (57.1%)	P=0.086
Stage III	51	17 (33.3%)	34 (66.7%)		28 (54.9%)	23 (45.1%)	
Stage IV	8	1 (12.5%)	7 (87.5%)		7 (87.5%)	1 (12.5%)	
Local recurrence							
Yes	31	15 (48.4%)	57 (55.3%)	χ^2 =0.133	14 (45.2%)	17 (54.8%)	χ^2 =0.378
No	103	46 (44.7%)	16 (51.6%)	P=0.837	53 (51.5%)	50 (48.5%)	P=0.539
Distant recurrence							
Yes	31	8 (25.8%)	23 (74.2%)	χ^2 =6.32	20 (64.5%)	11 (35.5%)	χ^2 =3.39
No	103	53 (51.5%)	50 (48.5%)	P=0.012*	47 (45.6%)	56 (54.4%)	P=0.065
Fate of patient							χ^2 =0.588
Died	96	38 (39.6%)	58 (60.4%)	χ^2 =4.82	46 (47.9%)	50 (52.1%)	P=0.443
Survived	38	23 (60.5%)	15 (39.5%)	P=0.028*	21 (55.3%)	17 (44.7%)	
CD8							
<60	67	33(49.3%)	34 (50.7 %)	χ^2 =0.752			
≥60	67	28(41.8%)	39 (58.2%)	P=0.386			

χ2, Chi-Square test; FET, Fisher's Exact Test; MC, Monte carlo test P, Probability value; *, statistically significant (P<0.05).

2019). Additionally, in cases with clear cell RCC, *HHLA2* overexpression in tumor tissues exhibited a positive link with a number of clinicopathological parameters like tumor size, clinical stage, and histologic grade (Chen et al. 2019; Zhou et al., 2020). Furthermore, Ding et al., (2022) observed that high *HHLA2* was associated with a higher grade and stage of hepatocellular carcinoma.

In contrast, other studies demonstrate that *HHLA2* has no significant correlation with clinico-pathologic parameters in CRC cases (Kula et al., 2023) and in pancreatic adenocarcinoma cases (Yan et al., 2019). In cervical adenocarcinoma (AC), Byun et al., (2021) observed that *HHLA2* expression demonstrated a significant negative link with lymph node metastases but did not demonstrate a significant correlation with stage, tumor grade, LVI, tumor size, or invasion depth.

In our study, patients with CRCs who had a high *HHLA2* expression had the worst OS and DFS. *HHLA2* was found by multivariate analysis to be an independent predictor of OS and DFS in CRC patients. This finding supports a prior study by Zhu and Dong (2018) that found *HHLA2* to be an independent predictive factor of the OS in CRC patients. Also, it was in agreement with preceding research in stomach cancer (Wei et al., 2019), lung adenocarcinoma (Chen et al., 2020; Farrag et al., 2021), osteosarcoma (Koirala et al., 2016), bladder cancer (Lin et al., 2019), intrahepatic cholangiocarcinoma (Jing et al., 2019), clear cell RCC (Chen et al., 2019; Zhou et al., 2020), hepatocellular carcinoma (Ding et al., 2022), spinal chordoma (Xia et al., 2022), and medullary thyroid carcinoma (Niu et al., 2022). On the other hand, some research suggested that *HHLA2* was associated with better

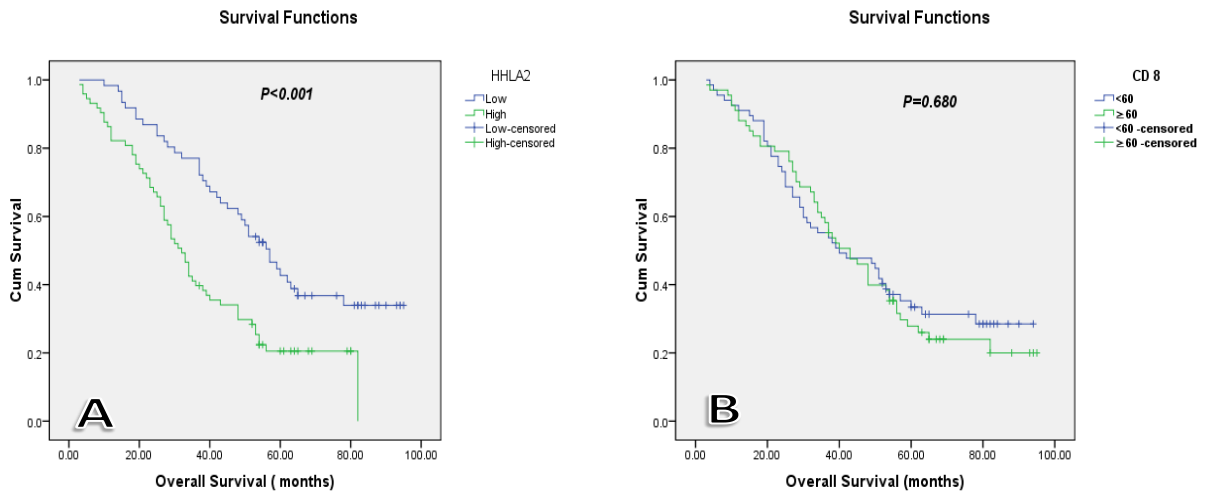

Figure 3. Kaplan-Meir Survival Curves for *HHLA2, CD8* with OS of Colorectal Carcinoma Cases. Significantly lower OS in patients with high HHLA2 expression (A). No statistically significant association between CD8 and OS(B).

Table 3. Univariate and Multivariate Survival Analysis of OS and DFS in CRCs

Variables	Overall survival					Disease free survival				
		Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis	
	Median OS	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value	Median DFS	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Age classes										
<60 y (R)	38	1				48	1			
≥60 y	45	0.943 (0.615-1.45)	0.787			51	1.24 (0.683-2.25)	0.478		
Sex										
Male (R)	43	1				52	1			
Female	38	1.095 (0.729-1.64)	0.662			48	1.27 (0.587-2.75)	0.544		
Site										
Right (R)	45	1				40	1			
Left	40	0.893 (0.582-1.37)	0.603			48	0.723 (0.369-1.41)	0.343		
Size										
<6 (R)	37	1				42	1			
>6	51	0.756 (0.497-1.152)	0.193			53	0.900 (0.497-1.63)	0.727		
Histological type										
Adenocarcinoma NOS	43	1.007 (0.548-1.85)	0.981			51	1.29 (0.681-2.47)	0.429		
Mucinous adenocarcinoma	34	1.48 (0.599-3.67)	0.394			55	2.13 (0.764-5.95)	0.148		
Signet ring carcinoma (R)	27	1				32	1			
Adenocarcinoma grade										
Low	43	0.265 (0.078-0.901)	0.03*	0.333 (0.095-1.17)	0.086	51	0.171 (0.036-0.809)	0.026*	0.451 (0.117-1.73)	0.246
High(R)	12	1		1		12	1		1	
TILs										
Low (R)	29	1		1		33	1			
Moderate	53	0.517 (0.315-0.849)	0.009*	1.24 (0.703-2.2)	0.454	54	0.834 (0.325-2.14)	0.706		
High	53	0.598 (0.340-1.05)	0.074	0.825 (0.428-1.59)	0.565	54	0.654 (0.256-1.67	0.374		
Tumor budding										
Low (R)	48	1				51	1		1	
Moderate	34	0.742 (0.418-1.318)	0.309			34	2.37 (1.04-5.43)	0.04*	1.79 (0.911-3.54)	0.091
High	30	1.35 (0.705-2.55)	0.364			38	0.708 (0.188-2.66)	0.609	0.945 (0.391-2.29	0.9

Kaplan-Meier test, used to calculate OS and DFS to detect effect of risk factors on survival. Cox regression was used to assess predictors of survival with calculation of Hazard ratio

Table 3. Continued

Variables	Overall survival					Disease free survival				
		Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis	
	Median	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value	Median DFS	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
LVI										
Absent (R)	54	1				54	1			
Present	34	1.04 (0.565-1.91)	0.903			43	0.986 (0.496-1.96)	0.968		
PNI										
Absent (R)	48	1				50	1			
Present	34	0.946 (0.580-1.54)	0.823			48	1.18 (0.576-2.43)	0.646		
T (depth of invasion)										
T2	59	1.93 (0.809-4.60)	0.078	1.71 (0.418-7.02)	0.457	55	0.796 (0.134-4.72)	0.805		
T3	39	4.43 (1.20-16.35)	0.025*	0.895 (0.416-1.93)	0.778	48	0.455 (0.161-1.29)	0.139		
T4(R)	49	1		1		53	1			
N (lymph node stage)										
N0	54	1.815 (0.171-19.23)	0.799			54	0.14 (0.08-7.2)	0.99		
N1	27	1.41 (0.460-4.32)	0.621			37	4.56 (0.850-24.51)	0.08		
N2(R)	32	1					1			
M (Metastasis stage)										
M 0	45	0.427 (0.144-1.27)	0.125			50	7.90 (3.1-9.8)	0.99		
M 1(R)	24	1				50	1			
AJCC stage										
stage I	52	2.9 (0.28-13.7)	0.067	7.9 (0.25-12.69)	0.881	56	0.897 (0.059-13.52)	0.937		
stage II	53	0.069 (0.005-0.893)	0.04*	8.2 (0.15-10.44)	0.876	53	0.962 (0.087-10.60)	0.975		
Stage III	34	0.302 (0.03-2.75)	0.288	9.1 (0.14-13.19)	0.867	34	1			
Stage IV ®	24	1		1						
CD8										
<60	40	0.769 (0.470-1.26)	0.298			51	0.643 (0.316-1.31)	0.222		
>60 (R)	43	1				48	1			
HHLA2										
Low (R)	57	1		1		55	1		1	
High	32	1.74 (1.03-2.96)	0.039*	1.81 (1.06-3.07)	0.03*	29	2.06 (1.24-3.42)	0.005*	1.90 (1.18-3.06)	0.008*

Kaplan-Meier test, used to calculate OS and DFS to detect effect of risk factors on survival. Cox regression was used to assess predictors of survival with calculation of Hazard ratio

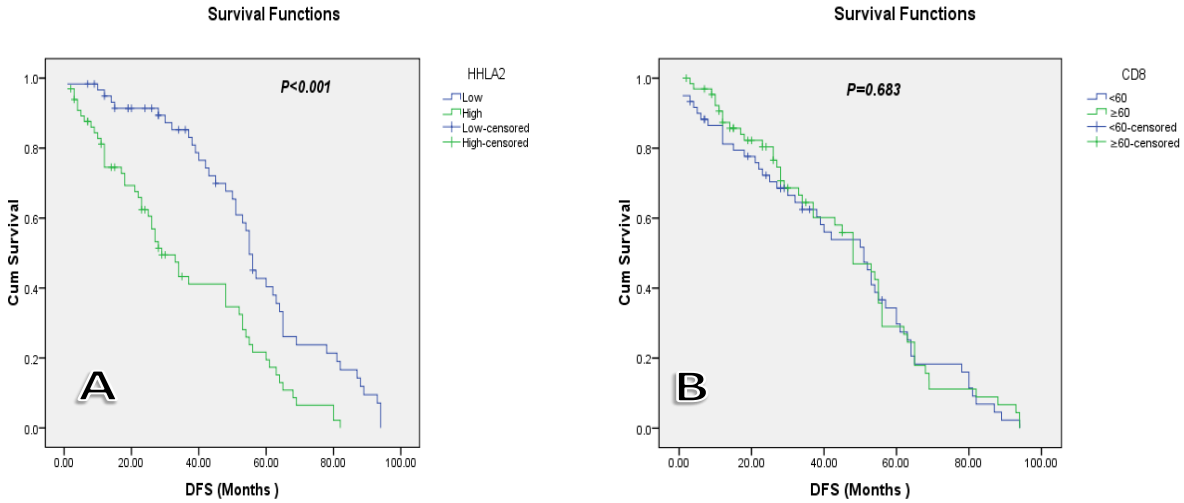

Figure 4. Kaplan-Meir Survival Curves for *HHLA2, CD8* with DFS of Colorectal Carcinoma Cases. Significantly lower DFS in patients with high *HHLA2* expression (A). No statistically significant association between *CD8* DFS (B)

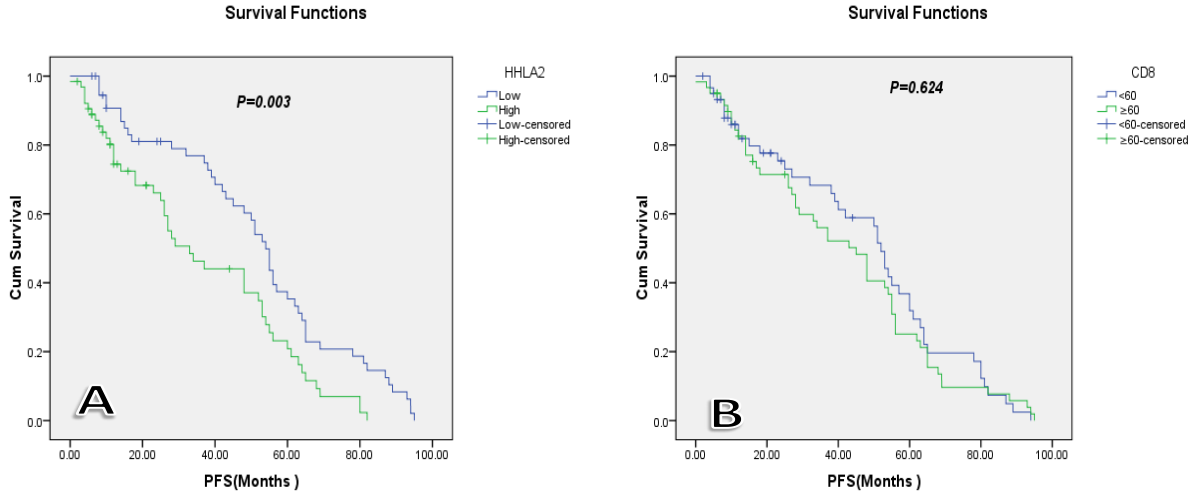

Figure 5. Kaplan-Meir Survival Curves for *HHLA2, CD8* with PFS of Colorectal Carcinoma Cases. Significantly lower PFS in patients with high *HHLA2* expression (A). No statistically significant association between CD8 and PFS (B)

outcomes in various malignancies such as CRCs (Khan et al., 2021), cervical AC (Byun et al., 2021), ovarian cancer (Xu et al., 2021), gastric cancer (Shimonosono et al., 2018), and hepatocellular carcinoma (Liao and Zhang, 2022). Additionally, elevated *HHLA2* expression was also associated with an improved post-surgical prognosis in pancreatic and ampullary cancers (Boor et al., 2020).

In our study, the relationship between *HHLA2* and TILs and *CD8*+ T cells is not statistically significant. This is in line with the findings of a study by Kula et al. (2023), which found no relation between *HHLA2* and *CD8*. According to Chen et al.'s (2019) research, there was no statistically significant correlation between *HHLA2* overexpression and the quantity of *CD8*+ T cells in RCC.

In CRCs, on the other hand, the *HHLA2* high-expression group had a lot fewer *CD8*+ cells than the *HHLA2* low-expression group (Zhu and Dong, 2018). On the other hand, positive *HHLA2* expression had higher levels of *CD8*+ T cells in pancreatic cancer (Yan et al., 2020) and in RCC (Zhou et al., 2020). The current study demonstrated that there is a statistically significant correlation between *CD8* expression and gender and certain favorable prognostic factors such as absence of tumor deposits, TILs, lack of metastasis, and histological type. However, *CD8* does not significantly correlate with other clinico-pathologic parameters or survival. These findings concur with those of Ko and Pyo (2019), who found a substantial correlation between *CD8* and the absence of metastasis. However, a significant association was reported between *CD8* and depth of invasion, lymph node stage, LVI, PNI, and histological grade.

According to Zhu and Dong (2018) and Barbosa et al. (2023), there is no correlation between *CD8* and tumor site and OS, but Barbosa et al., (22,023 recorded a significant association between *CD8* and lymph node status and depth of invasion. Furthermore, Yin et al., (2022) discovered

that there was a strong link between *CD8* and survival but not between *CD8* and tumor site, histological grade, or depth of invasion.

Our study had some limitations as factors influencing *HHLA2* expression, which include other T-cell subsets, and the expression of other co-inhibitory molecules, such as PD-L1, were not assessed. Additional research is needed to determine the biological significance, detect mechanisms of high *HHLA2* expression in CRC, and explain its contributions to tumor immune escape.

In conclusion, this study suggests that *HHLA2* is an independent prognostic predictor for the survival of CRC patients, associating with higher stage, advanced depth, and nodal stage. Therefore, *HHLA2* could be considered a promising prognostic and therapeutic target in the CRC.

Author Contribution Statement

All authors contribute equally; all authors share in the writing, data processing, and collecting of data, and they reviewed the whole work and approved the final version of the manuscript.

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Compliance with Ethical Standards

The present study was carried out after obtaining approval from the committed Institutional Research Board (IRB) at the Faculty of Medicine, Mansoura University, Egypt (Code Number: MDP/20.06.41). The study was processed under the ethical standards of the Helsinki Declaration.

Availability of data and material

All the clinical, radiological, and pathological data used in this manuscript is available on No scientific organization had approved this research, and it was not a component of any accepted student thesis. Mansoura University medical system (Ibn Sina Hospital management system) http://srv137.mans.edu.eg/mus/newSystem/.

Conflict of interest statement

The authors declare no relevant financial affiliations or conflicts of interest.

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