Prognostic significance of immunohistochemical expression of chemokine receptor (CXCR4) and RKIP in gastric carcinoma and premalignant lesions of the stomach

Abstract:

Background: CXCR4 and RKIP have been implicated in tumorigenesis and progression in many cancers, but their significance in gastric carcinoma remains unclear.

Aim: The aim of this study is to assess their possible significance in gastric carcinoma and premalignant lesions of the stomach.

Methods: This retrospective study was carried upon 50 cases of gastric adenocarcinoma and twenty cases from premalignant lesions. Immunohistochemistry was performed to examine the expression of CXCR4 and RKIP in both gastric adenocarcinoma and premalignant lesions.

Results: CXCR4 was found highly expressed in gastric carcinoma. It was significantly correlated with tumor grade, depth of tumor invasion, lymph node metastasis, distant metastasis and TNM stage. RKIP was negatively correlated with advanced tumor grade, depth of tumor invasion, distant metastasis and TNM stage. No significant correlation between CXCR4 and RKIP expression in studied cases was detected.

Conclusions: The results suggested that both CXCR4 and RKIP might be involved in gastric carcinogenesis. Therefore, Both CXCR4 and RKIP could be considered as prognostic markers in gastric carcinoma.

Keyword: Gastric adenocarcinoma, premalignant, CXCR4, RKIP.

Introduction

Gastric cancer is one of the most common causes of cancer related deaths in the world **(1)**. It is the second leading cause of cancer mortality in men and the fourth in women. Due to its aggressive behavior, it represents a challenging oncology session **(2)**.

In Egypt, Gastric cancer is in the eleventh rank constituting 2.1% of all cancers with slight male predilection. Patients' age ranged from 2 to 96 years old, with an average of 58 years compared to a median age of 70 years in the USA **(3)**.

Chemokines are small secreted proteins which are best known for their vital roles in mediating immune cell trafficking and lymphoid tissue development. Chemokines are the largest subfamily of cytokines and can be further subdivided into four main classes depending on the location of the first two cysteine (C) residues in their protein sequence **(4).**

CXCR4 belongs to the family of seven transmembrane G-protein-coupled receptors that transduce signals via heterotrimeric G-proteins and selectively binds the CXC chemokine Stromal Cell-Derived Factor 1 (SDF-1) **(5**).

CXCR4 is over-expressed in about many cancer cells lines and many human tumor types including lung, breast, liver, colorectal, bladder, and ovarian cancer **(6).**

Raf kinase inhibitor protein (RKIP) is a kinase inhibitor protein that regulates many signaling pathways within the cell**.** It was proved that RKIP has a significant molecule in suppressing cancer metastasis **(7).**

A variety of evidence suggests that reduced RKIP function may influence metastasis, angiogenesis, resistance to apoptosis, and genome integrity. Recent studies have

shown that the expression levels of RKIP are frequently downregulated in various cancer types, and correlate with an invasive or metastatic phenotype **(8).**

Material and Methods: This retrospective study was carried upon 50 cases of gastric adenocarcinoma and twenty cases from premalignant lesions including chronic gastritis, chronic gastritis with intestinal metaplasia and chronic gastritis with dysplasia. The studied cases included archival formalin-fixed, paraffin embedded blocks processed during the years 2015-2020 from the Pathology Department of Benha faculty of medicine. Gastric adenocarcinoma and premalignant lesions cases were endoscopic biopsies, partial gastrectomy specimens and total gastrectomy specimens.

Histopathological study: Four-micron thick sections were stained by conventional hematoxylin and eosin (H& E) stain.

Immunohistochemical study: For immunohistochemical study, anti-CXCR4 and anti RKIP immunostaining was performed for each case using Avidin-Biotin complex technique.

- One to two drops of each of the primary polyclonal antibody, either Anti-CXCR4 with concentration 1:200 or Anti-human RKIP with concentration 1:200 and slides were incubated at 4 C overnight; were applied to each section.

-Freshly prepared chromogen diaminobenzine (DAB) was used; it was incubated with slides for 3-5 minutes then washed with distilled water.

- *Immunohistochemical assessment:*

-Assessment of CXCR4 expression:

Specific CXCR4 staining was membranous and cytoplasmic in the cancer cells. According to **(9)** criteria for scoring CXCR4 expression, the intensity of

membranous and cytoplasmic staining varied from weak to strong: 0 (no staining), 1 (weak), 2 (moderate) and 3 (intense). The percentage of positive tumor cells was scored as 0 (negative), 1 (<10%), 2 (10% to 50%), 3 (>50%). Staining index was calculated by multiplying the staining intensity score and the percentage of positive cells. Staining indices 0 and 1 are considered to be negative and staining indices from2 to 9 are considered to be positive.

-Assessment of RKIP expression:

Expression of RKIP is cytoplasmic. The intensity of cytoplasmic staining varied from weak to strong: 0 (no staining), 1 (weak), 2 (moderate) and 3 (intense). The percentage of positive tumor cells was scored as 0 (negative), 1 (<10%), 2 (10% to 50%), 3 (>50%). Staining index was calculated by multiplying the staining intensity score and the extent of positive cells score. For the Scores were further grouped into two categories as follows: negative (final scores below 4) and positive (final scores of 4 or more) **(10).**

Statistical analysis: Results were analyzed using SPSS (version 16). The Pearson correlation coefficient was used for statistical analysis. P value <0.05 was considered statistically significant and highly statistically significant when it was <0.01. ROC curve to predict the most suitable cutoff point of CXCR4 and RKIP.

Results:

Histologically cases were classified into 26 cases of tubular adenocarcinoma (52%), 13 cases of mucinous adenocarcinoma (26%) and 11 cases were of signet ring type in a percentage of 22%. They were graded into: 27 (54%) cases of low grade (I&II) and 23 (46%) cases of high grade gastric adenocarcinoma (III). According to tumor depth of invasion, cases were classified into: 6 (12%) cases were T1, 13(26%) cases were T2, 18 (36%) cases were T3 and 13 (26%) cases were T4. Out of the 50

adenocarcinoma cases, 14 (28%) cases were N0, 14 (28%) cases were N 1, 13 (26%) were N2 and 9 (18%) cases were N3. Sixty two percent (31cases) out of the 50 cases were M0 and the other 19 (38%) cases were M1. Also, they were staged according to TNM staging into; 7 cases were stage I (14%),13 cases were stage II (26%), 11 were stage III (22%) while stage IV cases were 19 (38%).

Immunohistochemical Results:

CXCR4 expression was significantly increased gradually with gastric disease progression from different premalignant gastric lesions to gastric adenocarcinoma **(p value < 0.01).** There was no significant correlation **(P value =0.8)** between CXCR4 expression & different histopathologic types of gastric adenocarcinoma. There was a statistically significant positive correlation between CXCR4 expression score and advanced tumor grade **(P value = 0.027)**, depth of invasion **(P value = 0.035)**, lymph node status **(P value =0.025)**, distant metastasis **(P value =0.044)** and TNM stage of gastric carcinoma **(P value=0.002)**. RKIP expression has no significant correlation with progression from different premalignant gastric lesions to gastric adenocarcinoma **(P value 0.078)**. Also, there was no significant correlation **(P value =0.09)** between RKIP expression score & different histopathologic types of gastric adenocarcinoma and lymph node metastasis **(P value =0.053)**. There was a statistically significant negative correlation between RKIP expression and advanced tumor grade **(P value =0.027)**, depth of tumor invasion **(P value =0.043),** the distant metastasis of gastric carcinoma **(P value = 0.01)** and TNM stage of gastric carcinoma **(P value =0.002**).

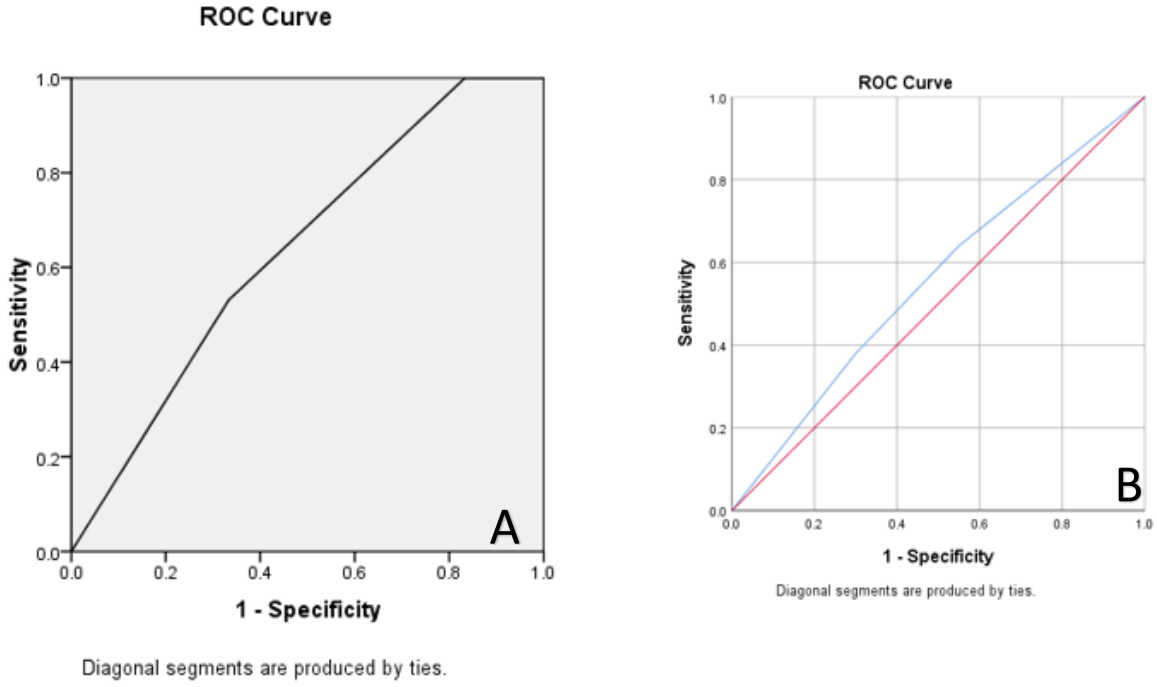
The current study did not show a significant statistical correlation between CXCR4 and RKIP expression in studied gastric non neoplastic and adenocarcinoma cases **(P value =0.178).**

The results of both antibodies were correlated with different clinicopathological variables of the cases examined and summarized in table 1.

Clinico-pathological parameters		CXCR4 expression		P value	RKIP expression		P value
		Negative	Positive		Negative	Positive	
Studied cases	Premalignant lesions	13(65%)	7(35%)	0.027	7(35%)	13(65%)	0.078
	Gastric adeno carcinoma	18(36%)	32(64%)		27(54%)	23(46%)	
Histological subtypes of gastric adenocarcinoma	Tubular adenocarcinoma	9(34.6%)	17(65.4%)	0.8	13(50%)	13(50%)	0.17
	Mucinous adenocarcinoma	5(38.5%)	8(61.5%)		6(46%)	7(54%)	
	Signet ring adenocarcinoma	4(36.4%)	7(63.6%)		8(72.7%)	3(27.4%)	
Grade of gastric adenocarcinoma	Low grade(I&II)	13(48%)	14(52%)	0.027	9(33.3%)	18(66.7%)	0.001`
	High grade (III)	5(21.7%)	18(78.3%)		18(78.3%)	5(21.7%)	
Depth of invasion	T1	3(50%)	3(50%)	0.019	3(50%)	3(50%)	0.018
	T2	7(53.8%)	6(46.2%)		3(23%)	10(77%)	
	T3	7(38.9%)	11(61.1%)		11(61%)	7(39%)	
	T4	1(7.7%)	12(92.3%)		10(77%)	3(23%)	
Lymph node status	N0	6(42.8%)	8(57.2%)	0.025	5(35.7%)	9(64.3%)	0.053
	N1	9(64.3%)	5(35.7%)		9(64.3%)	5(35.7%)	
	N2	2(15.4%)	11(84.6%)		7(53.8%)	6(46.2%)	
	N3	1(11.1%)	8(88.9%)		6(66.7%)	3(33.3%)	
Distant Metastasis	M0	14(45.2%)	17(54.8%)	0.044	13(42%)	18(58%)	0.029

	M1	4(21%)	15(79%)		14(73.7 %)	5(27.3%)	
TNM stage of gastric adenocarcinoma	**Stage I**	6(85.7%)	1(21%)	0.002	2(28.5%)	5(71.5%)	0.007
	Stage II	6(46%)	7(54%)		5(38.5%)	8(61.5%)	
	Stage III	2(18%)	9(82%)		6(54.5%)	5(45.5%)	
	Stage IV	4(21%)	15(79%)		14(73.7 %)	5(26.3%)	

Table (): Relationship between CXCR4 and RKIP Immunohistochemical results and clinico-pathological variables of studied cases.



A) Validity of CXCR4 to predict non neoplastic group from adenocarcinoma cases. B): Validity of RKIP in prediction of distant metastasis.

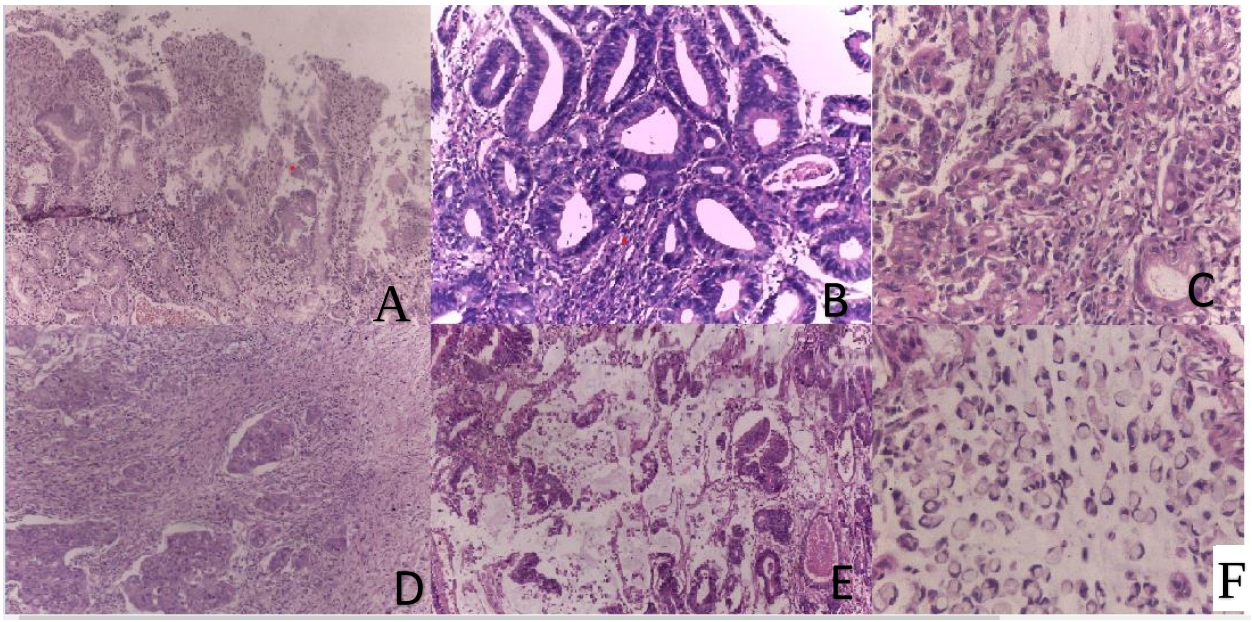


Figure (1): A) Severe chronic gastritis with surface ulceration and intestinal metaplasia with goblet cells, negative for dysplastic changes (H&EX100). B) Well differentiated tubular gastric adenocarcinoma showing well formed glands in which nuclei are pleomorphic in size and shape and hyperchromatic (H&EX200). C) Moderately differentiated tubular carcinoma with (H&EX200). D) Poorly differentiated tubular carcinoma, grade III showing solid sheets of malignant cells with absence of glandular pattern and high grade nuclear anaplasia (ABC X200). E) Moderately differentiated mucinous gastric adenocarcinoma showing irregular clusters of malignant cells floating in pools of mucin, moderately differentiated (H&EX200). F) Signet ring adenocarcinoma, grade III intracellular mucin accumulation displacing the nucleus giving the signet ring appearance (H&EX400).

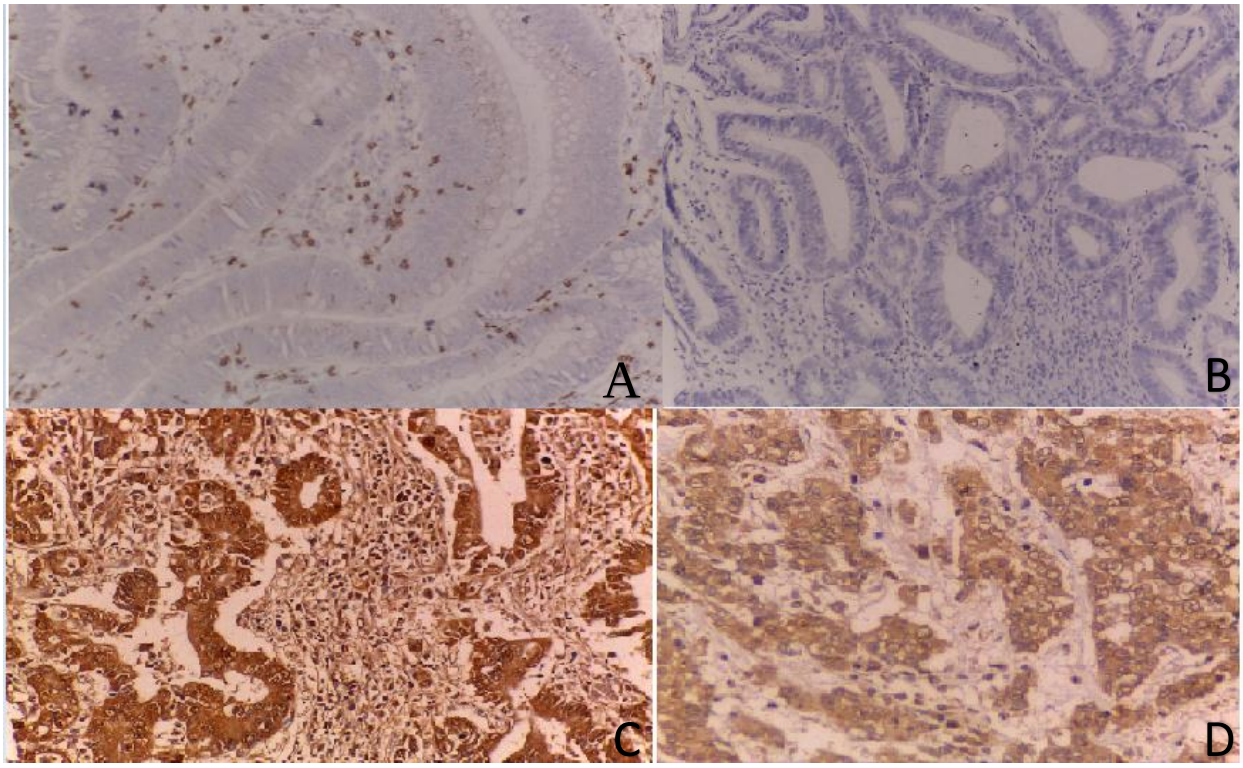


Figure (2): A) Chronic gastritis with intestinal and focal low grade dysplasia showing negative cytoplasmic staining, for CXCR4 (H&EX400). B) Well differentiated tubular gastric adenocarcinoma showing negative cytoplasmic staining for CXCR4 (ABCX400). C) Moderately differentiated tubular carcinoma, showing positive cytoplasmic staining for CXCR4 (ABCX200). D) Poorly differentiated tubular carcinoma, grade III showing positive cytoplasmic staining for CXCR4 (ABCX400).

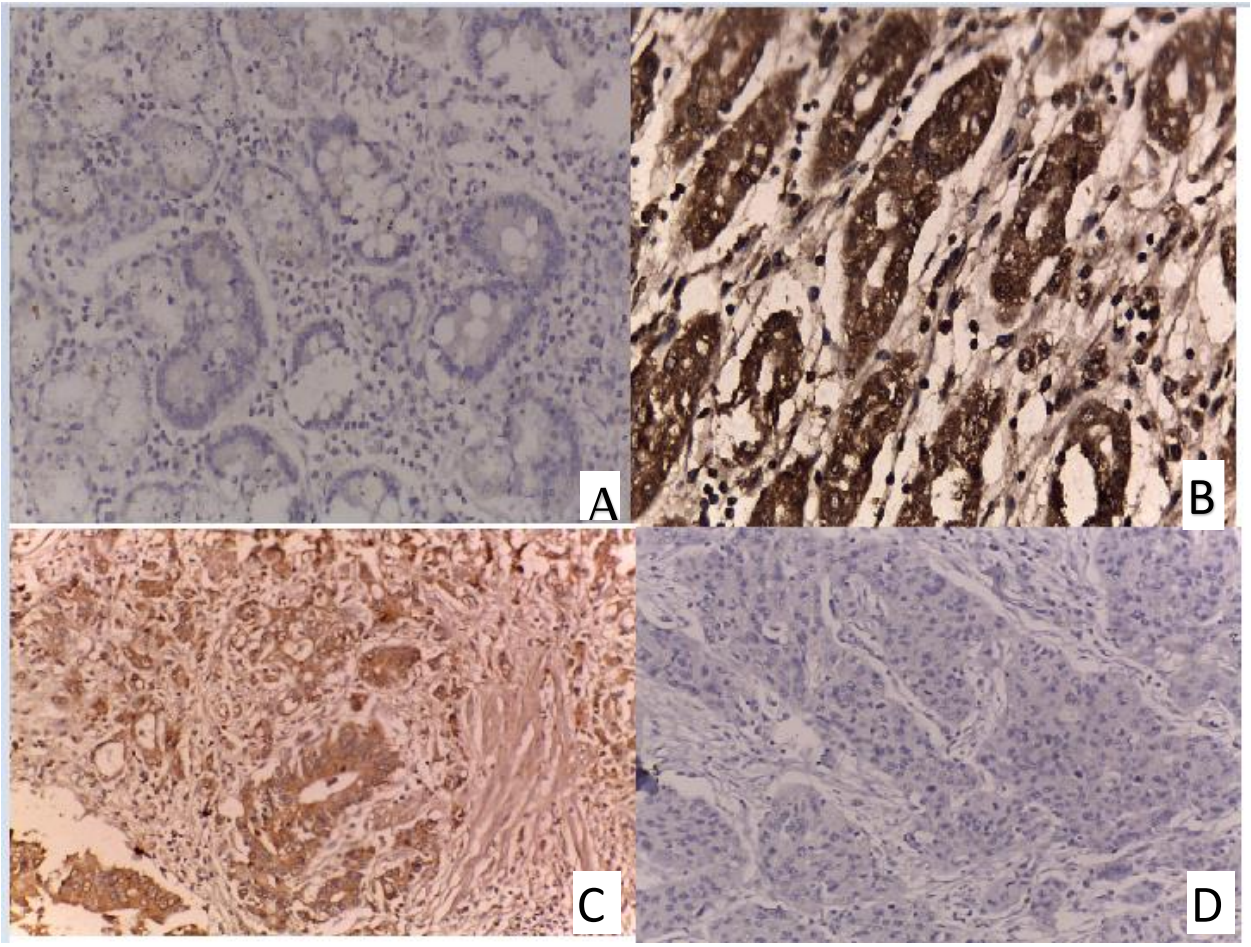


Figure (3): A) Chronic gastritis with intestinal metaplasia showing negative, score 3 cytoplasmic staining, for RKIP (H&EX400). B) well differentiated gastric adenocarcinoma showing positive cytoplasmic staining, score (9+) for RKIP (ABC X200).C) Moderately differentiated gastric adenocarcinoma showing positive cytoplasmic staining, score (9+) for RKIP (ABC X200). D)Poorly differentiated gastric adenocarcinoma showing negative cytoplasmic for RKIP (ABC X200).

Discussion

In our present study, CXCR4 expression score significantly increased gradually with progression from different non neoplastic gastric lesions to gastric adenocarcinoma (P value = 0.027). These results indicate that up regulation of CXCR4 may have an important role in the pathogenesis of gastric carcinoma.

These results were parallel to the results provided by **Zheng et al., (11)** who stated that CXCR4 in the gastric adenocarcinoma was remarkably higher than those in the premalignant gastric lesions.

Also **Nikzaban M et al., (12)** in their study stated that their qRT-PCR data showed that CXCR4 was highly expressed in tissue samples from patients with gastric cancer than premalignant lesions (2.4 times higher, *P* value < 0.05).

Other studies were also carried upon CXCR4 in other cancers and their precursor lesions. For example, **Huang Y et al., (13)** proved that CXCR4 in the oral squamous cell carcinoma was significantly higher than those in normal oral epithelium and premalignant lesions as oral leukoplakia.

Also, study by **Huang Y et al., (14)** demonstrated that the expression level of CXCR4 in endometrial cancer tissue was higher as compared to atypical hyperplasia, simple hyperplasia and normal cycling endometrium cells.

Also, **Zheng Y et al., (15)** found that CXCR4 was clearly detected in the cytoplasm of neoplastic epithelial cells, and the distribution and intensity of its expression increased as neoplastic lesions progressed through CIN1, CIN2, and CIN3 to invasive cancer.

These results can be explained by that the chemokine receptor CXCR4 has been suggested to play an important role in the initiation of many types of

cancer. Findings by **Oh et al. (16)** indicate that high levels of hypoxia in gastric carcinoma cells compared to premalignant lesions lesion is the responsible for upregulating CXCR4 expression in gastric cancer cells.

In this study, we found that there was no significant correlation **(P value =0.8)** between CXCR4 expression score & different histopathologic types of gastric adenocarcinoma. These results are similar to the results of **Kovelskaya et al., (17)**. However, study of **Shan S. et al., (18)** found that CXCR4 mRNA expression was significantly higher in signet ring adenocarcinoma than other histopathological types. This may be caused by difference in number of study cases and different laboratory technique used for evaluation as RT-PCR.

In this current study, there was a statistically significant positive correlation between CXCR4 expression score and advanced tumor grade **(P value = 0.027)**

These results are parallel to the results provided by **Yu S. et a., (19)** who demonstrated that CXCR4 expression was related to poor differentiation of cancer cells, whereas **Arigami et al., (20)** concluded that well differentiated gastric adenocarcinoma showed stronger CXCR4 expression than the poorly differentiated cases.

Data provided by **Zheng L et al., (21)** demonstrated that CXCR4 expression was associated with significantly correlated with histological differentiation of endometrial carcinoma.

Furthermore, the expression of CXCR4 was associated significantly with the histologic grade of cervical carcinoma as proved by **Huang Y et al., (15).**

There was a statistically positive significant correlation between CXCR4 expression & depth of invasion of gastric carcinoma **(P value =0.035)**. These results are parallel to the results provided by **Zhao Y. et al., (22).**

However, study of **Liu B. et al., (23)** found that there was no significant correlation between CXCR4 expression score & depth of invasion of gastric carcinoma. CXCR4 expression levels were associated with deep myometrial invasion in endometrial carcinoma as stated by **Teng et al., (24)**.

We found that there was a statistically significant correlation between CXCR4 expression and the lymph node status in gastric carcinoma **(P value =0.025).**

These results were similar to results by **Ziang et al., (25)** who found that CXCR4 mRNA and immunohistochemical expression was significantly upregulated in cases of gastric adenocarcinoma with lymph node metastasis. On the other hand, **Sun et al., (26)** suggested that there was under expression of CXCR4 inpatients with axillary lymph node metastasis in patients with breast ductal carcinoma.

Our work proved that there was a statistically significant correlation between CXCR4 expression and the distant metastasis in gastric carcinoma **(P value =0.044)**.

These results were similar to results by **Mortezaee M. et al., (27)** who Proved that CXCR4 is a key signaling for metastasis of gastric carcinoma.

Fujita T. et al. (28) have even identified CXCR4-positive stem cells of gastric carcinoma, which can penetrate gastric wall, migrate to peritoneum, and result in the formation of peritoneal tumor nodes and malignant ascites in a mouse model.

There was a highly statistically significant positive correlation between CXCR4 expression &TNM stage of gastric carcinoma (P value=0.002). These results are parallel to the results provided by **Wang et al., (29)**, but the results provided by **IWASA S. et al., (30)** showed that there was no statistically significant correlation between CXCR4 expression score &TNM stage of gastric carcinoma (diffuse type).

The study made by **Gao et al., (31)** found that CXCR4 expression was found to be strongly associated with lymph node metastasis, TNM stage, and liver metastasis in colorectal carcinoma.

Our results could be explained by that Chemokine and chemokine receptor pairs have been identified to play vital roles in cancer initiation and progression including the migration, adhesion, proliferation and survival of tumor cells, and the formation of tumor-associated vessels and invasion. CXCR4 mediates rapid phosphorylation of ERK and Akt, which suppresses apoptotic signals of caspase-9, caspase-3, and Bcl-2 and subsequently contributes to the proliferation and survival of gastric carcinoma **(32).**

Furthermore, the binding of CXCL12 to CXCR4 was shown to stimulate the activation of several downstream signaling pathways that regulate the progression and metastasis of various tumors. The mitogen-activated protein kinase and phosphoinositide 3-kinase pathways are the two most significant downstream pathways that are regulated by the CXCL12-CXCR4 interaction. Binding of CXCL12 to CXCR4 and CXCR7 on tumor cells leads to anti-apoptotic signaling through Bcl-2 and survivin upregulation, as well as promotion of the epithelial-to-mesechymal transition through the Rho-ROCK pathway and alterations in cell adhesion molecule **(29)**.

ROC curve was used to detect validity of CXCR4 predict non neoplastic group from adenocarcinoma case and AUC of CXCR4 was 0.688 (Fair) for adenocarcinoma. These results were near to results by **He et al., (33)** who found that AUC of CXCR4 was 0.709 (Good) and **Yang et al., (34)** who found that AUC of CXCR4 was 0.646 (Fair).

In this study, RKIP expression has no significant correlation with progression from different premalignant gastric lesions to gastric adenocarcinoma **(P value 0.078).**

These results were parallel to the results provided by ***Yang et al., (35)***. However, **Martinho et al., (36)** stated that RKIP was significantly lower in gastric carcinoma than in premalignant lesions. A study by **Kim et al (37)** who used quantitative real-time polymerase chain reaction and Western blot analysis proved that RKIP mRNA and protein expression was significantly downregulated in breast cancer tissues compared with the surrounding normal tissues.

In this study, we found that there was no significant correlation **(P value =0.09)** between RKIP expression score & different histopathologic types of gastric carcinoma.

These results are similar to the results of **Wei, H et al., (38)**. However, study of **Martinho et al., (39)** found that RKIP is differently expressed between the different WHO histological types (p=0.03) being highly expressed in tubular type and lost in mucinous and signet-ring cell carcinomas.

In this current study, there was a statistically significant negative correlation between RKIP expression score and advanced tumor grade **(P value = 0.027).**

These results are parallel to the results provided by **Liu. D et al., (40)** who detected that RKIP expression was detected in 58.1, 52.1 and 26.8% of cases in the well-differentiated, moderately differentiated and poorly differentiated groups, respectively ($P<0.05$). **Abdi, E. et al., (41)** also proved a negative statistical correlation between RKIP expression and the grade of gastric carcinoma, whereas **Zhang et al. (42)** found that RKIP expression was not correlated to the differentiation of cancer cells.

Furthermore, the expression of RKIP was negatively associated with the histologic grade of endometrial adenocarcinoma as proved by **Faloppa, C et al., (43) and Nie et al., (44)** who used PCR beside cell culture technique and immunohistochemical staining found that the staining intensity of RKIP protein decreased with the reducing differentiation of colorectal carcinoma.

There was a statistically negative significant correlation between RKIP expression & depth of invasion of gastric carcinoma **(P value =0.043)**. These results are parallel to the results provided **Yang Y. et al., (35)** who stated that the expression of RKIP in gastric cancer stem cells was suppressed in gastric carcinoma with deep invasion.

Also, A study by **Afonso et al., (45)** stated that Low RKIP expression was associated with deep muscle invasion of urothelial carcinoma and **Faloppa C et al., (43)** found that RKIP expression was lost with deep myometrial invasion of endometrial carcinoma.

As regard the relation between RKIP expression and the lymph node status in gastric carcinoma, there was no a statistically significant correlation between RKIP expression and the lymph node status in gastric carcinoma **(P value =0.053)**.

These results were parallel to results by **Sheng, N et al., (35)**, but conflicted by **Dong et al., (46)** who found that RKIP expression was downregulated in cases of gastric carcinoma with lymph node metastasis.

Our work proved that there was a statistically significant negative correlation between RKIP expression and the distant metastasis of gastric carcinoma (P value = 0.01) and TNM stage of gastric carcinoma (P value =0.002).

These results were similar to results **Yaqing et al., (47)** who Proved that RKIP protein expression was negatively linked distant metastasis and advanced stages of gastric carcinoma.

Our results could be explained by that RKIP is well known for its important role in EMT and in cancer metastasis suppression in various cancer types. A variety of evidence suggests that reduced RKIP function may influence metastasis, angiogenesis, resistance to apoptosis, and genome integrity. Moreover, recent data implicated RKIP depletion in chemotherapeutic resistance both in vitro and in vivo and overexpression of RKIP results in the inhibition of metastasis and invasiveness in various tumor models **(48).** RKIP regulates the activity of and mediates the cross talk between several important cellular signaling pathways, including the Raf–mitogen activated protein kinase ERK pathway, the nuclear factor kB (NF-kB) pathway, and the G protein pathway **(58)**. Some studies found that RKIP also negatively regulated the invasion of the different cancer cells through three-dimensional extracellular matrix barriers by controlling the expression of matrix metalloproteinases (MMPs), particularly, MMP-1 and MMP-2. Some previous studies showed that RKIP overexpression results in the direct activation of pro-caspase 8 which plays a central role in the execution-phase of cell apoptosis **(49**).

By using ROC curve, AUC of RKIP was 0.662 (Fair) for metastasis.
Sensitivity of RKIP (True positive cases) was 64 and Specificity of RKIP (True negative cases) was 45, positive predictive value was 74.4 and negative predictive value was 33.3. However, study by **Papale et al., (50)** revealed that AUC of RKIP was 0.93 (Excellent) for prediction of metastasis of clear cell renal cell carcinoma.

The current study did not show a significant statistical correlation between CXCR4 and RKIP expression in studied gastric non neoplastic and adenocarcinoma cases **(P value =0.178)**.

No similar published data about the relation between CXCR4 and RKIP expression in gastric carcinoma, however **Zhu et al., (51)** stated that BACH1 expression was positively correlated with C-X-C Motif Chemokine Receptor 4(CXCR4) in tumor tissues and cell lines and BACH1 depletion causes an increase in RKIP expression. Moreover, in chronic lymphocytic leukemia (CLL), inhibition of RKIP by locostatin led to a decreased expression of the chemokine receptor CXCR4 and reduced the migratory capacity of CLL cells toward stroma-derived factor 1a (SDF-1a) as proved by **Crassini et al., (52)**. **Yun et al., (53)** was able to demonstrate that RKIP inhibits expression of MMP-1, CXCR4, and OPN thus affecting the ability of metastatic cells to create an osteolytic bone environment via crosstalk with stromal cellular and noncellular components in metastatic breast cancer.

Conclusion: CXCR4 and RKIP could be considered as prognostic markers in gastric carcinoma and may have a vital role in chemoresistance of gastric adenocarcinoma

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