

## Prognostic Value of Immunohistochemical Expressions of the Stem Cell Biomarker "LGR5" and the Proliferation Biomarker "TPX2" in Colorectal Carcinoma

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Received: April 9, 2016

Revised: June 3, 2016

Accepted: June 7, 2016

Published online: June 29, 2016

### ABSTRACT

**AIM:** Colorectal carcinoma (CRC) is the commonest and most deadly malignant gastrointestinal tumors. Carcinogenic process starts in a group of cancer cells, called cancer stem cells (CSCs) having the ability to start and maintain tumor growth and invasion. In order to detect and remove colon CSCs, a selective biomarker "LGR5 (leucine-rich-repeat-containing G-protein-coupled receptor 5)" might be required. At the same time, an abnormal expression of TPX2 (targeting protein for xenopus kinesin-like protein 2) has been found to be related to the development and progression of tumors. We aimed to evaluate the immunohistochemical expressions of LGR5 and TPX2 in CRC in a trial to clarify their relations to proliferation and metastasis of CRC, and hence its prognosis.

**MATERIALS AND METHODS:** Immunohistochemical staining of both LGR5 and TPX2 was assessed in sections from sixty paraffin blocks of CRC. The relationships between their levels of expressions with the clinicopathological parameters and patient outcome were statistically analyzed.

**RESULTS:** The immunoeexpression of LGR5 in CRC was signifi-

cantly positively correlated with location of the tumor, grade, stage, local recurrence, lymph node and distant metastasis ( $P < 0.001$ ). The expression of TPX2 in CRC was also found to be significantly correlated with the previous parameters ( $P < 0.001$ ). Both biomarkers were up-regulated in CRC than in the adjacent non tumorous tissues. High LGR5 and TPX2 immunohistochemical expressions were strongly correlated with worse 3-year overall survival (OS), local recurrence free survival (LRFS) and distant metastases free survival (DMFS) ( $P < 0.001$ ).

**CONCLUSIONS:** High immunohistochemical expressions of both LGR5 and TPX2 and the positive correlation between both of them represent signs of poor prognosis of CRC.

**Key words:** Stem cell; Colorectal cancer; LGR5; TPX2; Metastases; Prognosis

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Harb OA, Hegazy AA, Gertallah LM, Haggag R, El Shorbagy S. Prognostic Value of Immunohistochemical Expressions of the Stem Cell Biomarker "LGR5" and the Proliferation Biomarker "TPX2" in Colorectal Carcinoma. *Journal of Tumor* 2016; 4(3): 426-434 Available from: URL: <http://www.ghrnet.org/index.php/jt/article/view/1665>

### INTRODUCTION

Colorectal cancer (CRC) is the commonest and most fatal malignant gastrointestinal tumors<sup>[1]</sup>. The major causes of CRC related death are local recurrence and lymphatic and hematogenous metastases<sup>[2]</sup>.

Mechanisms responsible for the initiation, progression, prognosis and adequate management of CRC have been studied<sup>[3]</sup>. Two theories of CRC carcinogenesis have been stated; a stochastic model, where any cell has the ability of cancer initiation, promotion and progression and a cancer stem cell (CSC) model, that only produced by transformed CSCs having the ability to self-renewal, abnormally differentiate and form a cancer<sup>[4]</sup>. Although, CSCs have been incriminated in colon carcinogenesis for several years, the complexity of their detection and isolation is still not precisely

clarified<sup>[5]</sup>. CSCs are resistant to chemotherapeutic agents and responsible for recurrence and spread of CRC. Continuous research about CRC is essential to identify novel therapeutics specifically targeting CSCs<sup>[6]</sup>. G-protein-coupled receptors (GPCRs) are transmembrane proteins which after combination to their ligand activate cytoplasmic G-proteins stimulating many intracellular enzymes. They have important roles in many cellular activities, such as tissue repair, inflammation and carcinogenesis<sup>[7]</sup>. LGR5 is a member of the largest family of cell-surface molecules of the GPCRs. It is expressed on intestinal stem cells, where it functions as a transducer of Wnt signaling<sup>[8]</sup>. LGR5+ cells have been detected in the crypt stem cells (precursor cells) and gastric mucosal lesions that progressed to cancer<sup>[9]</sup>. It is predominantly restricted to the base of mature pyloric glands in the adults<sup>[10]</sup>. Many treatment protocols have been applied to CRC, but they have not resulted in a complete cure. This may be due to colorectal CSCs that are resistant to radiation therapy and chemotherapy, and may enable the recurrence of cancers. Therefore, it is important to use therapies that target not only proliferating cells but also stem cells in order to treat the tumor<sup>[11]</sup>. In order to detect and remove such CSCs, a selective marker is required. Becker *et al* suggested that LGR5 may be a novel biomarker for CSCs in cases of CRC<sup>[12]</sup>.

At the same time, TPX2, a microtubule-associated protein was initially stated by Heidebrecht *et al*<sup>[13]</sup>. It has been found to be a nuclear proliferation-related protein which is implicated in the regulation of the cell mitosis. TPX2 is released in the early stage of mitosis; and plays a critical role in mitotic spindle formation and subsequent segregation of chromosomes at cell division<sup>[14]</sup>. In recent years, a connection between TPX2 and initiation, progression and prognosis of malignant tumor is becoming a focus of researches<sup>[15]</sup>. Previous studies showed that abnormally high expression of TPX2 has been confirmed in many tumor tissues<sup>[16,17]</sup>. TPX2 can upregulate expression of matrix metalloproteinases (MMP) family through activating PI3K/Akt pathway. Therefore, inhibiting its expression through downregulating expressions of MMP2 and MMP9 can inhibit the invasion of liver cancer cells<sup>[18]</sup>. Moreover, TPX2 expression is obviously upregulated in breast cancer in comparison to the adjacent tissues. Nevertheless, the connection between TPX2 and prognosis, and specific molecular mechanism still need further research<sup>[19]</sup>. Despite its overexpression in many tumors, its role in CRC remains to be vague.

This study aimed to evaluate immunohistochemical expression of the stem cell biomarker “LGR5” and the proliferation biomarker “TPX2” in CRC in a trial to clarify the relations between their expressions with the proliferation and metastasis of the tumor, and to detect any correlation to its prognosis.

## MATERIALS AND METHODS

### Patients and tissue specimens

For this retrospective cohort study, 60 archival formalin-fixed paraffin-embedded blocks of CRC cases collected from Pathology Department, Faculty of Medicine, Zagazig University. Samples were obtained by partial and radical colectomy done in general surgery department, Faculty of Medicine, Zagazig University, between December 2012 and December 2015. The seventh edition of the American Joint Committee on Cancer staging system (AJCC-7) classification was used for pathologic staging<sup>[20]</sup>. At the same time, the World Health Organization (WHO) classification was performed for pathologic grading<sup>[21]</sup>.

We identified sex, age, tumor size, histological subtype,

grade, stage, lymph node and distant metastasis of the cases by retrospective examination of the medical records and the slide files of the pathology department. Most patients had follow-up records for three years in Medical Oncology Department, Faculty of Medicine, Zagazig University. Others died within such period of follow-up. The study was performed after patients' consents with full local ethics approval.

### Immunohistochemical staining

Immunohistochemical staining was carried out using the streptavidin-biotin immunoperoxidase technique<sup>[22]</sup>. Five  $\mu\text{m}$  thick sections were cut from paraffin-embedded blocks of all cases, put on positively charged slides, and then de-paraffinized in xylene and rehydrated in ascending grades of ethanol. Antigen retrieval was done by boiling sections in citrate buffer (pH 6.0) for 20 min. Then after washing with phosphate buffered saline (PBS), the slides were incubated with primary rabbit polyclonal Anti-GPCR GPR49/LGR5 antibody ab71225 (Abcam, Cambridge, MA, USA) diluted 1:50 in blocking solution; and primary mouse monoclonal anti-TPX2 antibody 18D5-1 ab32795 was used at a dilution of 1:200. at 4 °C overnight. The sections were then washed in PBS and incubated with Polyperoxidase-anti-mouse/rabbit IgG (Zymed Laboratories, San Francisco, CA, USA) for 20 min. After washing with PBS, 3, 30-Diaminobenzidine was used as the chromogen. Finally, the sections were counterstained with hematoxylin.

Specimens of esophageal adenocarcinoma were used as positive controls for LGR5<sup>[23]</sup>. Tissue sections that previously stained positive for TPX-2 were used as the positive controls<sup>[24]</sup>. The negative controls used the non-immune isotype serum instead of the primary antibodies.

### Evaluation of immunostaining intensity of LGR5 proteins

Evaluation was analyzed according to both the percentage of positive cells and the intensity of staining. The intensity was graded from 0 to 3; with 0 records as no staining, 1 as mild, 2 as moderate and 3 as strong. The highest intensity score was assigned when a threshold of > 10% of cells stained with that intensity. Percentage of stained cells was also scored from 0 to 3, where immunoreactivity was scored 0 if no carcinoma cells stained positive, 1 if 1% to 25% were positive, 2 if 25% to 50% were positive, and 3 if > 50% were positive. A composite expression score [0-6] was obtained by adding the intensity and percentage scores, with 1-2 recorded as weak positive [+], 3-4 as moderate positive [++], and 5-6 as strong positive [+++]. In the statistical analysis, 0 was ranked as negative and 1-6 was recorded as positive. Score of 1-2 was ranked as low expression, and 3-6 recorded as high expression<sup>[23]</sup>.

### Evaluation of immunostaining intensity of TPX2

The TPX2 labeling index (LI) was ranked according to the percentage of positive cells. The staining intensity was graded as follows; 0: no staining, 1+: mild staining, 2+: moderate staining, and 3+: intense staining. The staining was scored as the following; 0: no staining of cells, 1+ : < 10% of tissue stained positive, 2+: 10%-50% stained positive, and 3+ : > 50% stained positive. The sum of staining score (intensity + extension) index was designated as follows; 0-2: negative expression, 3-4: low expression and 5-6: high expression<sup>[25]</sup>.

### Statistical analysis

All statistics were performed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium). Continuous variables were expressed

Table 1 Correlation between clinicopathological features and LGR5 and TPX2 immunohistochemical expressions.

Characteristics	All (n = 60)		LGR-5		High (n = 28)		P-value	TPX-2		High (n = 26)		P-value
	No.	(%)	No.	(%)	No.	(%)		No.	(%)	No.	(%)	
Age (years)												
Mean ± SD	57.46	± 12.42	50.25	± 10.86	65.71	± 8.35	< 0.001 <sup>†</sup>	51.02	± 11.23	65.88	± 8.22	< 0.001 <sup>‡</sup>
Median (Range)	61	(35-80)	50	(35-77)	67.5	(45-80)		50	(35-77)	67.5	(50-80)	
≤ 60 years	30	(50%)	26	(81.30%)	4	(14.30%)	< 0.001 <sup>§</sup>	26	(76.50%)	4	(15.40%)	< 0.001 <sup>§</sup>
> 60 years	30	(50%)	6	(18.80%)	24	(85.70%)		8	(23.50%)	22	(84.60%)	
Sex												
Male	37	(61.70%)	18	(56.30%)	19	(67.90%)	0.356 <sup>§</sup>	20	(58.80%)	17	(65.40%)	0.604 <sup>§</sup>
Female	23	(38.30%)	14	(43.80%)	9	(32.10%)		14	(41.20%)	9	(34.60%)	
Location												
Right colon	29	(48.30%)	8	(25%)	21	(75%)	< 0.001 <sup>§</sup>	11	(32.40%)	18	(69.20%)	0.020 <sup>§</sup>
Transverse colon	6	(10%)	3	(9.40%)	3	(10.70%)		3	(8.80%)	3	(11.50%)	
Left colon	6	(10%)	6	(18.80%)	0	(0%)		5	(14.70%)	1	(3.80%)	
Sigmoid colon	19	(31.70%)	15	(46.90%)	4	(14.30%)		15	(44.10%)	4	(15.40%)	
Size (cm)												
Mean ± SD	5.68	± 2.77	3.96	± 0.89	7.64	± 2.89	< 0.001 <sup>†</sup>	4.41	± 1.90	7.34	± 2.86	< 0.001 <sup>‡</sup>
Median (Range)	5	(2-12)	4	(2-6)	8	(3-12)		4	(2-12)	8	(3-12)	
≤ 5 cm	40	(66.70%)	31	(96.90%)	9	(32.10%)	< 0.001 <sup>§</sup>	31	(91.20%)	9	(34.60%)	< 0.001 <sup>§</sup>
> 5 cm	20	(33.30%)	1	(3.10%)	19	(67.90%)		3	(8.80%)	17	(65.40%)	
Pathological Type												
Adenocarcinoma	55	(91.70%)	32	(100%)	23	(82.10%)	0.013 <sup>§</sup>	33	(97.10%)	22	(84.60%)	0.084 <sup>§</sup>
Mucinous	5	(8.30%)	0	(0%)	5	(17.90%)		1	(2.90%)	4	(15.40%)	
Grade												
Grade 1	10	(16.70%)	10	(31.30%)	0	0%	< 0.001 <sup>*</sup>	10	(29.40%)	0	(0%)	< 0.001 <sup>*</sup>
Grade 2	38	(63.30%)	22	(68.80%)	16	(57.10%)		23	(67.60%)	15	(57.70%)	
Grade 3	12	(20%)	0	(0%)	12	(42.90%)		1	(2.90%)	11	(42.30%)	
Lymph node												
Negative	30	(50%)	24	(75%)	6	(21.40%)	< 0.001 <sup>§</sup>	24	(70.60%)	6	(23.10%)	< 0.001 <sup>§</sup>
Positive	30	(50%)	8	(25%)	22	(78.60%)		10	(29.40%)	20	(76.90%)	
Stage												
Stage I	10	(16.70%)	10	(31.30%)	0	0%	< 0.001 <sup>*</sup>	10	(29.40%)	0	(0%)	< 0.001 <sup>*</sup>
Stage II	20	(33.30%)	14	(43.80%)	6	(21.40%)		14	(41.20%)	6	(23.10%)	
Stage III	18	(30%)	8	(25%)	10	(35.70%)		8	(23.50%)	10	(38.50%)	
Stage IV	12	(20%)	0	(0%)	12	(42.90%)		2	(5.90%)	10	(38.50%)	
LGR5												
Mean ± SD	34.83	± 34.04	7.81	± 10.37	65.71	± 23.55	< 0.001 <sup>†</sup>	12.64	± 21.17	63.84	± 24.46	< 0.001 <sup>‡</sup>
Median (Range)	23.5	(0-95)	0	(0-24)	75	(30-95)		0	(0-85)	70	(20-95)	
Low	32	(53.30%)						31	(91.20%)	1	(3.80%)	< 0.001 <sup>§</sup>
High	28	(46.70%)						3	(8.80%)	25	(96.20%)	
TPX2												
Mean ± SD	41.33	± 34.17	16.09	± 17.76	70.17	± 23.90	< 0.001 <sup>†</sup>	14.55	± 16.57	76.34	± 11.96	< 0.001 <sup>‡</sup>
Median (Range)	37.5	(0-90)	10	(0-55)	75	(0-90)		0	(0-45)	77.5	(55-90)	
Low	34	(56.70%)	31	(96.90%)	3	(10.70%)	< 0.001 <sup>§</sup>					
High	26	(43.30%)	1	(3.10%)	25	(89.30%)						

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD and median (range). <sup>†</sup>Mann Whitney U test; <sup>‡</sup> Chi-square test; <sup>§</sup> Chi-square test for trend; <sup>\*</sup> P < 0.05 is significant.

as the mean ± SD and median (range), and the categorical variables were expressed as a number (percentage). Percentages of categorical variables were compared using the Pearson's Chi-square test or Fisher's exact test when was appropriate. Strength of relationship between LGR5 and TPX2 and clinicopathological features were determined by computing Spearman's correlation coefficient. Stratification of OS, LRFS and DMFS was done according to all clinicopathological features and immunohistochemical markers and were estimated using the method of Kaplan-Meier plot. A P-value < 0.05 was considered statistically significant.

## RESULTS

### Patients

The investigated 60 cases were 37 males and 23 females, with age ranged from 35-80 years (mean: 57.46 ± 12.42) (Table 1). There were 55 (91.7%) cases of conventional adenocarcinoma and 5 (8.3%)

cases of mucinous carcinoma. Ten (16.7%) cases were grade I, 38 (63.3%) cases were grade II, 12 (20%) cases were grade III. At the same time, 10 (16.7%) cases were stage I, 20 (33.3%) cases were stage II, 18 (30%) cases were stage III and 12 (20%) cases were stage IV (Table 1).

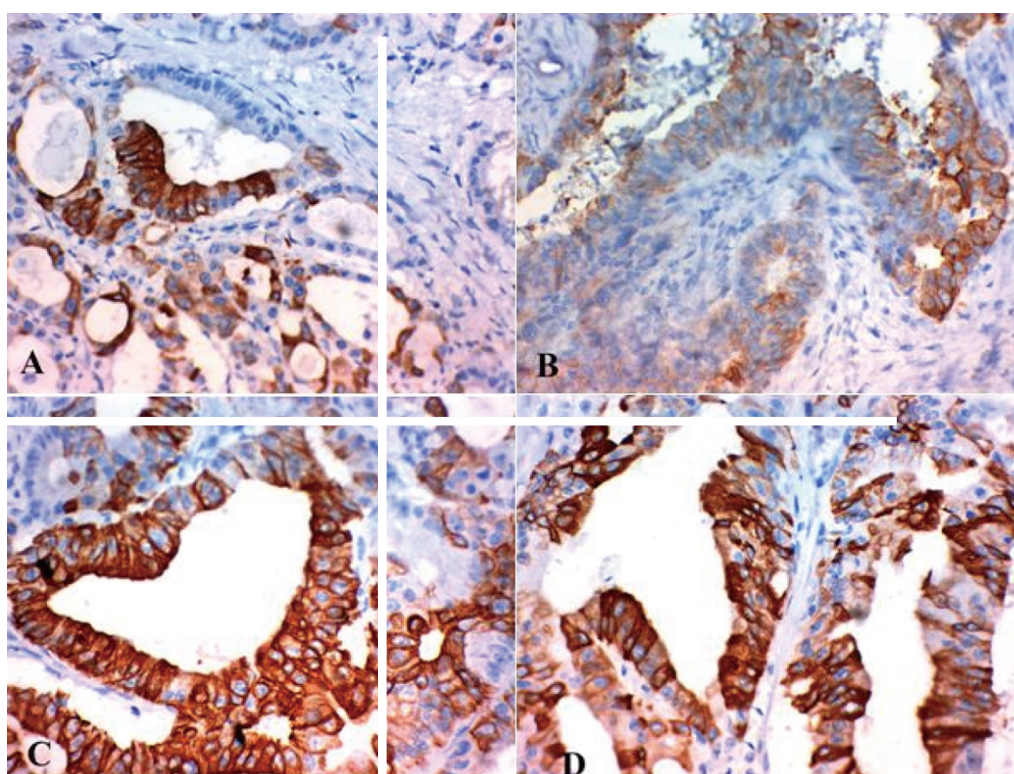
### LGR5 immunorexpression and its correlation with clinicopathological features

LGR5 was cytoplasmic and membranous. Its high immunohistochemical expression in CRC was detected in 28 (47%) cases and low expression in 32 (53%) cases. In grade I, the low expression was detected in 10/10 cases and the high expression not detected in any case. In grade II, the low expression was detected in 22/38 cases and the high expression detected in 16/38 cases. In grade III the low expression was not detected in any case and the high expression detected in 12/12 cases. In stage I, the low expression was detected in 10/10 cases and the high expression not detected in any case. In stage II, the low expression was detected in

**Table 2** Correlation between clinicopathological features, LGR5 and TPX2 immunohistochemical expressions and outcome of patients.

Characteristics	All		Local recurrence		P-value	Distant metastasis		P-value	Survival		P-value
	(n = 60)		No (n = 37)	Yes (n = 23)		No (n = 48)	Yes (n = 12)		Alive (n = 42)	Died (n = 18)	
<b>LGR5</b>											
Mean ± SD	34.83 ± 34.04	14.18 ± 20.92	68.04 ± 22.85	< 0.001 <sup>†</sup>	21.66 ± 23.79	87.50 ± 3.98	< 0.001 <sup>†</sup>	18.69 ± 24.34	72.5 ± 21.16	< 0.001 <sup>†</sup>	
Median (Range)	23.5 (0-95)	0 (0-85)	80 (30-95)		20 (0-80)	87.5 (80-95)		16.5 (0-95)	82.5 (30-90)		
Low	32 (53.30%)	32 (100%)	0 (0%)	< 0.001 <sup>§</sup>	32 (100%)	0 (0%)	< 0.001 <sup>§</sup>	32 (100%)	0 (0%)	< 0.001 <sup>§</sup>	
High	28 (46.70%)	5 (17.9%)	23 (82.1%)		16 (57.1%)	12 (42.9%)		10 (35.7%)	18 (64.3%)		
<b>TPX2</b>											
Mean ± SD	41.33 ± 34.17	20.81 ± 23.49	74.34 ± 19.49	< 0.001 <sup>†</sup>	33.22 ± 29.18	73.75 ± 34.51	< 0.001 <sup>†</sup>	26.54 ± 27.17	75.83 ± 21.70	< 0.001 <sup>†</sup>	
Median (Range)	37.5 (0-90)	20 (0-90)	80 (0-90)		30 (0-80)	90 (0-90)		25 (0-90)	82.5 (0-90)		
Low	34 (56.70%)	33 (97.1%)	1 (2.9%)	< 0.001 <sup>§</sup>	32 (94.1%)	2 (5.9%)	0.002 <sup>§</sup>	33 (97.1%)	1 (2.9%)	< 0.001 <sup>§</sup>	
High	26 (43.30%)	4 (15.4%)	22 (84.6%)		16 (61.5%)	10 (38.5%)		9 (34.6%)	17 (65.4%)		

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range). <sup>†</sup>Mann Whitney U test; <sup>§</sup>Chi-square test; <sup>‡</sup>Chi-square test for trend; P < 0.05 is significant.



**Figure 1** Immunohistochemical staining of LGR5 in the cytoplasm and membrane of CRC: (A) Low expression in grade I, stage I CRC ×400; (B) Low expression in grade II, stage II CRC ×400; (C) High Immunohistochemical expression in grade II, stage III CRC ×400; (D) High Immunohistochemical expression in grade II, stage IV CRC ×400.

14/20 cases and the high expression detected in 6/20 cases. In stage III, the low expression was detected in 8/18 case and the high expression detected in 10/18 cases. Finally in stage IV, the low expression was not detected in any case and the high expression detected in 12/12 cases. Low immunohistochemical expression of LGR5 was detected in all adjacent non-tumor tissues compared to CRC tissue that mean that LGR5 was down regulated in non-tumor tissues and up-regulated in CRC tissue.

The high immunohistochemical expression of LGR5 in CRC was significantly positively correlated with location of the tumor, grade, stage, lymph node and distant metastasis ( $P < 0.001$ ) (Figure 1; Tables 1 and 2).

#### **TPX2 immunopexpression and its correlation with clinicopathological features**

Only manifest nuclear staining was defined as a positive reaction of TPX2. Its high immunohistochemical expression in CRC was

detected in 26 (43%) cases and low expression in 34 (57%) cases. In grade I, the low expression was detected in 10/10 cases and the high expression was not detected in any case. In grade II, the low expression was detected in 23/38 cases and the high expression detected in 15/38 cases. In grade III, the low expression was detected in 1/12 case and the high expression detected in 11/12 cases. In stage I, the low expression was detected in 10/10 cases and the high expression was not detected in any case. In stage II, the low expression was detected in 14/20 cases and the high expression was detected in 6/20 cases. In stage III, the low expression was detected in 8/18 case and the high expression was detected in 10/18 cases. In stage IV, the low expression was detected in 2/12 cases and the high expression detected in 10/12 cases. Low immunohistochemical expression of TPX2 was detected in all non-tumor tissues compared to CRC tissue that mean that TPX2 was down regulated in non-tumor tissue and up regulated in CRC tissue.

The high expression of TPX2 in CRC was significantly positively

**Table 3** Association and correlation between LGR-5, TPX-2 and clinicopathological parameters in 60 patients with colon carcinoma.

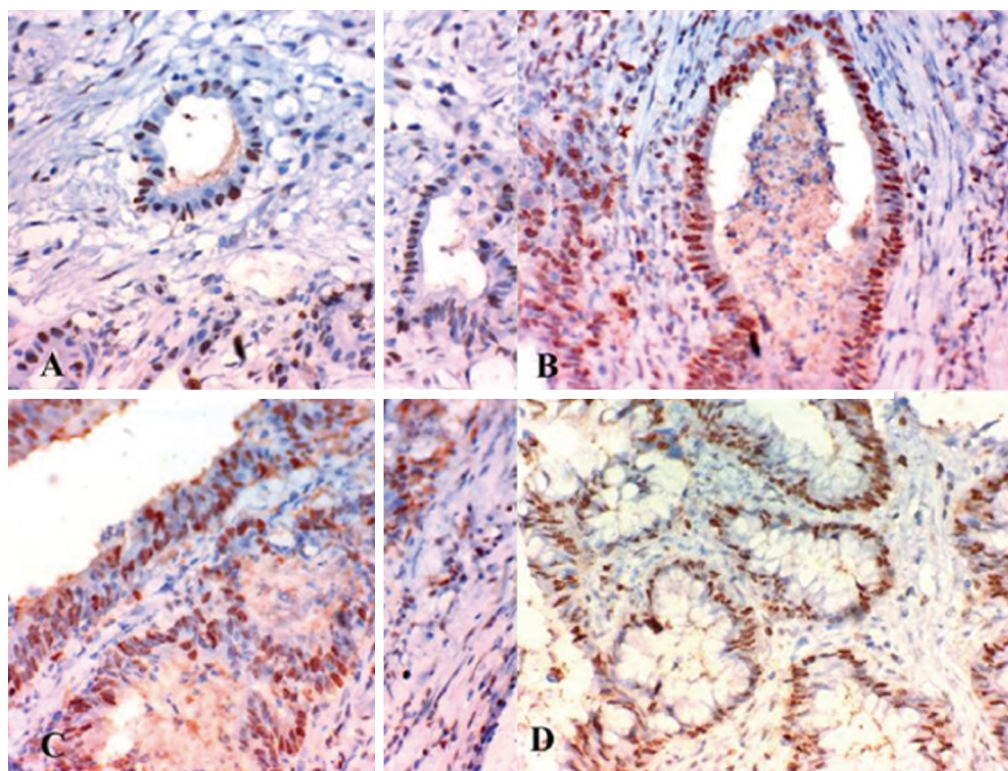
	LGR-5 (%)		LGR-5		TPX-2 (%)		TPX-2	
	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value
Age (years)	+0.665	< 0.001	+0.626	< 0.001	+0.637	< 0.001	+0.597	< 0.001
Sex	-0.156	0.235	+0.118	0.356	-0.097	0.462	+0.067	0.604
Location	-0.337	0.008	+0.48	< 0.001	-0.362	0.004	+0.375	0.02
Size (cm)	+0.703	< 0.001	+0.667	< 0.001	+0.573	< 0.001	+0.529	< 0.001
Type	+0.484	< 0.001	+0.307	0.013	+0.264	0.042	+0.218	0.084
Grade	0.511	< 0.001	+0.604	< 0.001	+0.501	< 0.001	+0.553	< 0.001
pT	0.561	< 0.001	+0.762	< 0.001	+0.484	< 0.001	+0.624	< 0.001
Lymph node	+0.686	< 0.001	+0.471	< 0.001	+0.551	< 0.001	+0.426	< 0.001
Stage	+0.739	< 0.001	+0.707	< 0.001	+0.643	< 0.001	+0.6	< 0.001
LGR-5 (%)	---	---	---	---	+0.849	< 0.001	+0.751	< 0.001
LGR-5	---	---	---	---	+0.796	< 0.001	+0.655	< 0.001
TPX-2 (%)	+0.849	< 0.001	+0.796	< 0.001	---	---	---	---
TPX-2	+0.751	< 0.001	+0.655	< 0.001	---	---	---	---

*r* correlation coefficient; *P* < 0.05 is significant.

**Table 4** Diagnostic performance of immunohistochemical markers as a predictor for advanced stage colon carcinoma.

Markers	TP	FP	TN	FN	SN %	SP %	PPV %	NPV %	Accuracy
	No (%)	No (%)	No (%)	No (%)	(95% CI)	(95% CI)	(95% CI)	(95% CI)	(95% CI)
LGR5 (high)	22 (36.7%)	6 (10%)	24 (40%)	8 (13.3%)	73.30% (57.5-89.2)	80% (65.7-94.3)	78.60% (63.4-93.8)	75% (60-90)	76.70% (66-87.4)
TPX-2 (high)	20 (33.3%)	6 (10%)	24 (40%)	10 (16.7%)	66.70% (49.8-83.5)	80% (65.7-94.3)	76.90% (60.7-93.1)	70.60% (55.3-85.9)	73.30% (62.1-84.5)
LGR5 (high) and TPX-2 (high)	22 (36.7%)	7 (11.7%)	23 (38.3%)	8 (13.3%)	73.30% (57.5-89.2)	76.70% (61.5-91.8)	75.90% (60.3-91.4)	74.20% (58.8-89.6)	75% (64-86)

TP: True positive; FP: False positive; TN: True negative; FN: False negative; SN: Sensitivity; SP: Specificity; PPV: Positive Predictive Value; NPV: Negative Predictive Value, 95%CI: 95% Confidence Interval; *P* < 0.05 is significant.



**Figure 2** Immunohistochemical nuclear staining of TPX2 in CRC: (A) Low expression in grade I, stage I CRC  $\times 400$ ; (B) High expression in grade II, stage II CRC  $\times 400$ ; (C) High expression in grade II, stage III CRC  $\times 400$ ; (D) High expression in mucinous carcinoma grade II, stage III CRC  $\times 400$ .

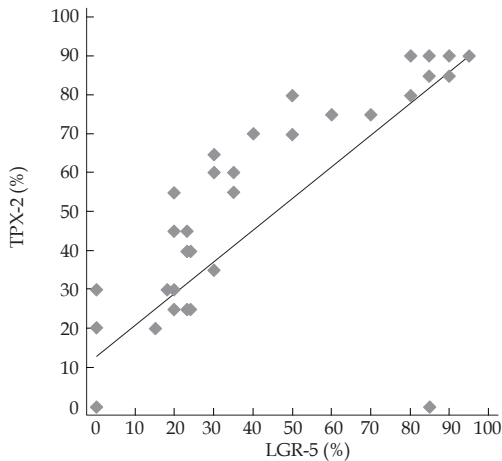
correlated with location of the tumor, grade, stage, lymph node and distant metastasis (*P* < 0.001) (Figure 2; Tables 1 and 2).

**Immunoexpression correlations of LGR5 with TPX2**

Immunohistochemical expressions of LGR5 and TPX2 were

significantly positively correlated with each other correlation coefficient *r* = +0.849 (*P* < 0.001) (Figure 3; Table 3).

The sensitivity of combination of both markers as predictors for advanced stage of CRC was 73.3%; and the specificity was 76.7% (Table 4).



**Figure 3** Scatter plot with regression line shows a significant strong direct correlation between LGR-5 and TPX-2 ( $r = +0.849$ ,  $P < 0.001$ ).

#### Follow-up results

The 3-year overall survival (OS) rate was 69.2% in all cases and 24.1% in high LGR5, 100% in low LGR5, 23.2% in high TPX2 and 100% in low TPX2 expressions.

The 3-year local recurrence free survival (LRF5) rate was 61.7% in all cases and 17.9% in high LGR5, 100% in low LGR5, 15.4% in high TPX2 and 97.1% in low TPX2 expressions.

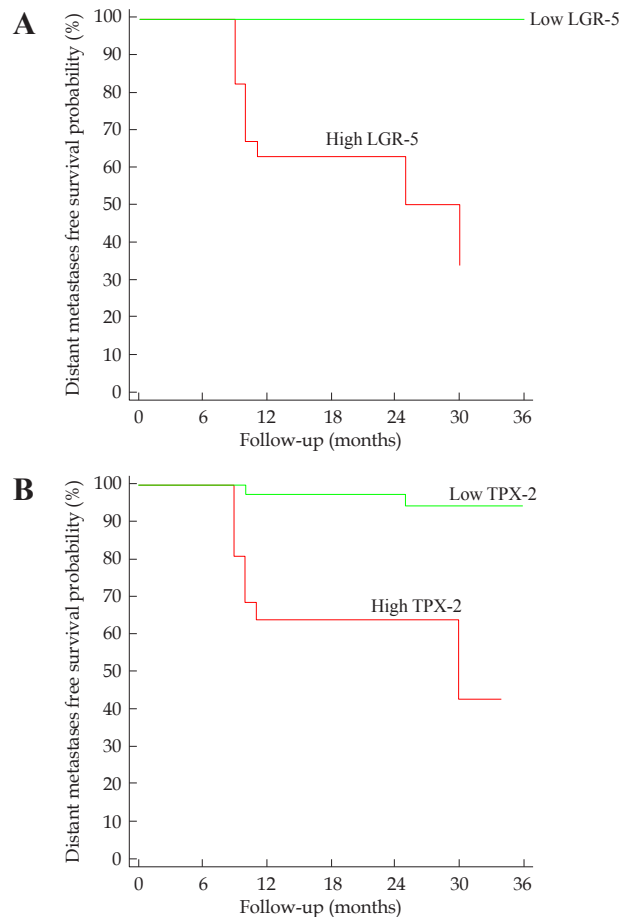
The 3-year distant metastases free survival (DMFS) rate of our cases was 78.5% in all cases and 33.6% in high LGR5, 100% in low LGR5, 42.7% in high TPX2 and 94.1% in low TPX2 expressions.

There was a highly significant inverse relationship between both high LGR5 and high TPX2 immunohistochemical expressions and 3-year OS, LRF5 and DMFS rates ( $P < 0.001$ ) (Figures 4 and 5; Table 5).

## DISCUSSION

In our study regarding the stem cell biomarker “LGR5”, its increased expression in CRC in comparison to non-tumorous tissue and was significantly positively correlated with location, stage, grade of the tumor, lymph node and distant metastasis ( $P < 0.001$ ). These results suggest that high expression levels of LGR5 receptors are usually correlated with more malignant and metastatic potential of CRC. We also found that the immune-expression of LGR5 in CRC was significantly associated with shortened progression-free OS and DMFS ( $P < 0.001$ ). Our results suggest that LGR5 is involved in the growth and progression of CRC and it may be an important biological marker of its invasion and metastasis. Our results are consistent with previous studies proving that overexpression of LGR5 was associated with poor prognosis in CRC<sup>[26-29]</sup>. The current results also are consistent with studies done in other organs stating that LGR5 overexpression is associated with poor prognosis and metastasis-initiating potentials in breast cancer, glioblastoma, lung cancer and esophageal adenocarcinoma<sup>[30-33]</sup>.

Similar to the previous report proved by Becker *et al.*<sup>[12]</sup>, we found that LGR5 is a promising biomarker of SCs and cancer stem cells (CSCs). It was up-regulated in CRC in comparison to non-tumorous tissue. There was increased immunoeexpression of the biomarker with increasing grade and stage and associated with poor prognosis. LGR5-positive CRC may contain more CSCs than non-tumorous tissue. Therefore, in agreement with CSC theories, the



**Figure 4** Kaplan-Meier plot of distant metastasis free survival: (A) stratified according to LGR5; (B) stratified according to TPX2.

residual CSCs may lead to metastasis and recurrence of colorectal cancer through multilineage differentiation and self-renewal after surgical resection of the primary cancer<sup>[34]</sup>. Discovering a novel stem cell biomarker such as LGR5 which its expression was associated with poor prognosis, recurrence and metastases of CRC can help to detect therapeutic modality targeting CSCs through eradicating LGR5 positive CSCs and leading to decrease progression recurrence, metastases and improving the CRC prognosis. Since year 2002, George *et al* was trying to prove the role of therapeutic drugs that target GPCRs in clinical use<sup>[35]</sup>. Then, Hanahan *et al*, found that targeting cancer stem cells (CSCs) might be a novel approach to improve patient prognosis<sup>[36]</sup>.

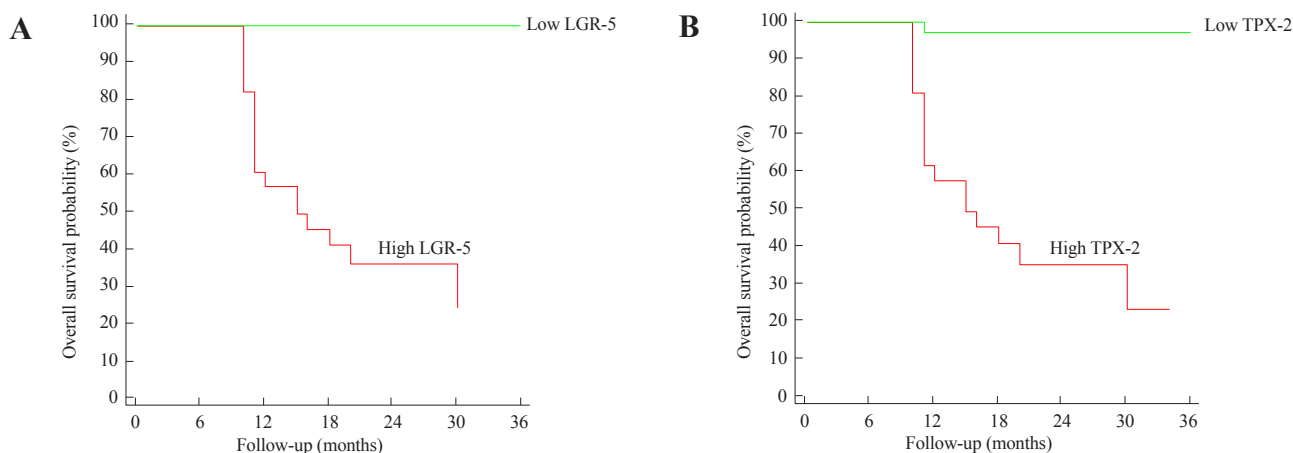
Concerning the immune-expression of TPX2 in CRC, it was up-regulated in CRC tissue in comparison to non-tumorous tissue and was significantly positively correlated with location of the tumor, stage, grade, lymph node and distant metastasis ( $P < 0.001$ ), and was significantly positively correlated with shortened OS and DMFS ( $P < 0.001$ ). Our results are consistent with that of Wei *et al.*<sup>[25]</sup>, suggesting that overexpression of TPX2 is associated with poor prognosis in colon cancer. Our results also are consistent with studies done in other organs proving that TPX2 overexpression is associated with poor prognosis in cancer of various organs including cervix, bladder and esophagus<sup>[16,37,38]</sup>.

There were several mechanisms that can explain the role of TPX2 in carcinogenesis process. It plays an important role in DNA damage stress, initiation, progression and spread of cancer<sup>[15]</sup>. It can also

**Table 5** Correlation between clinicopathological features, LGR5 and TPX2 immunohistochemical expressions and survival in cases with CRC.

Characteristics	All (n = 60)	LR free survival		P-value <sup>§</sup>	DM free survival		P-value <sup>§</sup>	Overall survival		P-value <sup>§</sup>
		Median LRFS (months)	3 years LRFS		Median DMFS (months)	3 years DMFS		Median OS (months)	3 years OS	
All patients		NR	61.70%		NR	78.50%		NR	69.20%	
Pathological type										
Adenocarcinoma	55 (91.70%)	NR	67.30%	< 0.001	NR	85.80%	< 0.001	NR	73.90%	< 0.001
Mucinous	5 (8.30%)	9	0%		10	0%		11	0%	
Grade										
Grade 1	10 (16.70%)	NR	100%	< 0.001	NR	100%	0.004	NR	100%	< 0.001
Grade 2	38 (63.30%)	NR	65.80%		NR	83.90%		NR	76%	
Grade 3	12 (20%)	12.5	16.70%		25	0%		15	0%	
T										
T1	6 (10%)	NR	100%	< 0.001	NR	100%	0.059	NR	100%	0.014
T2	4 (6.70%)	NR	100%		NR	100%		NR	100%	
T3	27 (45%)	NR	77.80%		NR	83.90%		NR	77.40%	
T4	23 (38.30%)	15	26.10%		NR	58%		20	46.10%	
Lymph node										
Negative	30 (50%)	NR	86.70%	< 0.001	NR	100%	< 0.001	NR	93.30%	< 0.001
Positive	30 (50%)	13	36.70%		NR	53%		16	43.80%	
Stage										
Stage I	10 (16.70%)	NR	100%	< 0.001	NR	100%	< 0.001	NR	100%	< 0.001
Stage II	20 (33.30%)	NR	80%		NR	100%		NR	90%	
Stage III	18 (30%)	NR	50%		NR	100%		NR	66.70%	
Stage IV	12 (20%)	9	16.70%		10	0%		11	0%	
LGR5										
Low	32 (53.30%)	NR	100%	< 0.001	NR	100%	< 0.001	NR	100%	< 0.001
High	28 (46.70%)	12	17.90%		30	33.60%		15	24.10%	
TPX2										
Low	34 (56.70%)	NR	97.10%	< 0.001	NR	94.10%	< 0.001	NR	100%	< 0.001
High	26 (43.30%)	13	15.40%		30	42.70%		15	23.20%	

Categorical variables were expressed as number (percentage); NR denote not reached yet; <sup>§</sup>Log rank test; P < 0.05 is significant.



**Figure 5** Kaplan-Meier plot of overall survival: (A) stratified according to LGR5; (B) stratified according to TPX2.

up-regulate the expression of matrix metalloproteinases (MMP) family especially MMP2 and MMP9 so increases the invasion and metastases of cancer cells<sup>[18]</sup>.

Aberrant expression of TPX2 leads to improper spindle assembly and chromosomal instability; and these processes might be partly responsible for carcinogenesis<sup>[14]</sup>. However, the precise role of TPX2 in cancer progression is still not fully understood. It has been found that elevated expression of TPX2 promotes tumor growth in pancreatic cancer<sup>[39]</sup>. Also, high levels of TPX2 expression are correlated with the aggressiveness of ovarian and salivary gland cancers<sup>[17,40]</sup>.

TPX2 expression is obviously up-regulated in breast cancer and that TPX2 inhibition can decrease the proliferation, invasion and

migration of breast cancer cells through inhibiting expressions of MMP2 and MMP9. Nevertheless, the connection between TPX2 and prognosis, and specific molecular mechanism still need further research<sup>[19]</sup>.

Our results proved that TPX2 plays an important role in promoting tumorigenesis and metastasis of human CRC. It could be used to predict the proliferation capacity of cancer cells and may represent a novel prognostic biomarker and therapeutic target for the disease. Also, the study showed that stem cell biomarker LGR5 expression was positively correlated with the proliferation biomarker TPX2 ( $r = +0.489$ ) ( $P < 0.001$ ), a recognized nuclear antigen-specific marker of cellular proliferation, suggesting that LGR5-positive cancer cells have higher proliferative activity. As the role of both the stem cell

biomarker LGR5 and the proliferation biomarker TPX2 is still a point of research and this is the first study to prove the relation between them in CRC progression and prognosis, future studies with more cases are recommended to confirm the benefit of combination of both immunoeexpressions in detecting CRC and assessing its prognosis.

## CONCLUSIONS

In conclusion, the stem cell biomarker “LGR5” is highly expressed in colorectal cancer cells and; may be considered a selective and promising biomarker of SCs and CSCs in the intestinal epithelium. Also, the proliferation biomarker “TPX2” is involved in colon cancer carcinogenesis and metastasis. Moreover, the LGR5 expression is positively correlated with the recognized nuclear antigen-specific marker of cellular proliferation “TPX2” ( $r = +0.489$ ) ( $P < 0.001$ ). This raises the suggestion that LGR5-positive cancer cells have higher proliferative activity.

Thus, LGR5 may be a potential new therapeutic target for the treatment of colorectal cancer patients, particularly those with advanced cases. TPX2 could serve as a novel prognostic biomarker to predict risk of distant metastases in patients with radical colectomy. Our results might be helpful for understanding the mechanism by which tumorigenesis is initiated, and for enabling targeted treatment of CSCs in order to prevent its progression and improve the patient outcome. Whether LGR5 up-regulation itself contributes to cancer progression or simply is a biological biomarker, this needs further studies on large number of cases to confirm its role in cancer progression.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

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