The prognostic roles of PYCR2 and ZBTB18 expression in tissues of colorectal carcinoma and Non-neoplastic tissues; an immunohstochemical study

Ola Harb (olaharb2015@gmail.com)
Zagazig University Faculty of Human Medicine https://orcid.org/0000-0002-4396-3101
Mariem A. Elfeky
Zagazig University
Fady M. Wadea
Zagazig university
Ahmed M. Elsayed
Zagazig University
Shereen El Shorbagy
Zagazig University
Ahmed Z. Alattar
zagazig Univserity
Ahmed F. Amin
zagazig university
Mohamed Farouk Amin
zagazig university
Abdelwahab S. Almoregy
Zagazig university

Research Article

Keywords: CRC, PYCR2, ZBTB18, immunohistochemistry, prognosis

Posted Date: October 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-981235/v1

Abstract

Background: it will be important to detect novel biomarkers which are responsible for progression and spread of CRC for better evaluation of patients' prognosis, better management and development of recent therapeutic targets. Pyrroline-5-carboxylate reductase 2 (PYCR2), in humans is encoded on chromosome 1q42.12. PYCR2 metabolic activity was linked to oncogenesis in many cancers. ZBTB18, a zinc finger transcriptional repressor was found to have tumor suppressor role and was found to be methylated in CRC.

Detailed assessment of prognostic roles of PYCR2 and ZBTB18 CRC patients are not sufficiently studied.

Aim of the study was to evaluate tissue protein expression of PYCR2 and ZBTB18 in CRC and adjacent non-neoplastic intestinal tissues to detect their roles in CRC carcinogenesis, progression and metastases.

Patients and methods: After application of inclusion criteria, 60 CRC patients were included in the study. Tissue samples from tumor and adjacent non-neoplastic tissues of included patients were stained with PYCR2 and ZBTB18.

Patients were followed up for about 30 months (range from 10-36 month). We correlate between markers expression, clinicopathological and prognostic parameters.

Results: upregulation of PYCR2 and down regulation of ZBTB18 was found in CRC than adjacent non-neoplastic colonic mucosa (p= 0.026 and p<0.001 respectively).

High expression of PYCR2 and low expression of ZBTB18 were positively correlated with large tumor size, higher grade, advanced tumor stage, presence of spread to lymph node and presence of distant metastases (p<0.001).

High PYCR2 and low ZBTB18 expression were significantly associated with poor response to therapy (p = 0.008, 0.0.17 respectively), high incidence of progression and relapse recurrence (p=0.005), unfavorable OS rate (p=0.001).

Conclusions: High expression of PYCR2 and low expression of ZBTB18 were independent predictors of CRC, progression, poor prognosis and unfavorable patient OS and PFS rates.

Introduction

Colorectal cancer (CRC) is considered the 3rd commonest cancer and 2nd commonest cause of cancer related death worldwide. CRC prognosis markedly depends on its stage at initial diagnosis [**1, 2**].

Distant metastases and cancer progression are the most important causes of death `in CRC patients **[3].** It will be critical to detect novel biomarkers which are responsible for progression and spread of CRC for better evaluation of patients' prognosis, better management and development of recent therapeutic targets.

Pyrroline-5-carboxylate reductase 2 (PYCR2), in humans is encoded on chromosome 1q42.12, considered a key housekeeping protein which consumes NAD(P) H as a coenzyme for production of proline and NAD(P)þ [**4**]. PYCR2 metabolic activity was linked to oncogenesis in many cancers [**5**], a regulator of amino acid metabolism in hepatocellular carcinoma which carry out a prognostic role [**6**]. However, its role in CRC prognosis and metastases needed to be evaluated [**7**].

Moreover, CRC progression and development of metastases is a multistep process which was observed to result from accumulation of genetic and epigenetic defects which lead to transformation of normal colonic epithelial cells to metastatic CRC [**8**]. Histone modifications in CRC as acetylation and methylation could alter gene expression which drives the colon cancer oncogenesis. There are many epigenetic events responsible for CRC progression and spread that are sufficiently unstudied [**9**].

ZBTB18, a zinc finger transcriptional repressor was found to have tumor suppressor role and was found to be methylated in CRC [**10]**.

Comprehensive assessment of prognostic roles of PYCR2 and ZBTB18 in CRC patients was sufficiently unstudied.

In the current study we aimed to evaluate expression of PYCR2 and ZBTB18 proteins in CRC and adjacent non-neoplastic intestinal tissues to detect their roles in conventional colorectal adenocarcinoma carcinogenesis, progression and metastases.

Patients And Methods

The present study was performed in Faculty of Medicine, Zagazig University hospitals where we collected samples from 60 CRC patients in the period from January 2017 to May 2020. Colonoscopic diagnosis of CRC and biopsies were performed and collected from GIT endoscopy unit of the Internal Medicine Department. Samples were delivered to Pathology Department where processing and initial diagnosis were done, operable Patients were admitted to General surgery Department where surgical excision was performed according to site of cancer. Surgical samples were delivered to Pathology Department where processing, diagnosis, grading and staging were performed. Patients were sent to Medical Oncology and Clinical Oncology and Nuclear Medicine departments for chemotherapy and/or Radiotherapy treatment protocols according to stage.

Treatment protocols: Postoperative systemic adjuvant chemotherapy is traditionally performed for pStage III and high risk pStage II colon cancer at our institute. In the present study, 5-fluorouracil-based regimens were commonly used in p Stage II, while Oxaliplatin-based and 5-fluorouracil-based regimens were

most commonly used in pStage III.

Early Rectal cancer patients received neoadjuvant concurrent chemoradiotherapy (long course radiation therapy at the dose of 45 to 50 Gray (Gy) in 25 to 28 fractions to the pelvis plus capecitabine) then post operative chemotherapy

Metastatic CRC patients received chemotherapy protocols in the form of FOLFOX or FOLFIRI.

Inclusion Criteria

Patients with complete clinical data, patients diagnosed with conventional adenocarcinoma, patients who accepted to be included in the study.

Exclusion criteria

Patients with insufficient data, inoperable patients, and patients diagnosed with other histopathological subtypes of CRC.

After application of inclusion criteria, 60 patients were included in the study.

We collected tissue samples from tumor and adjacent non-neoplastic tissues

Written informed consents were acquired from all included patients after taking an approval from the local ethical committee of institutional review board of Faculty of Medicine, Zagazig University.

Pathological staging of CRC patients was performed according to guidelines of The American Joint Committee on Cancer in its 7th edition **[11].**

Patients were followed up for about 30 months (range from 10-36 month) for detection of recurrence, distant metastasis and OS.

Most patients were regularly followed up by clinical examination, laboratory and radiological investigations in the outpatients' clinic. The follow-up period ended in May 2020.

Immunohistochemical staining of PYCR2 and ZBTB18

Sections contain tumor tissue and adjacent non-neoplastic tissues were taken from collected paraffin blocks of included patients were incubated with primary monoclonal anti- PYCR2 antibody (1:1000, Proteintech, USA) and primary rabbit polyclonal anti-ZBTB18 antibody (1:1000, Biorbyt, Cambridge, UK; cat# orb357631).

Evaluation of **PYCR2 and ZBTB18 expression in stained tissues**

Evaluation of staining extent and intensity scores were assessed by two pathologists who were blinded to patients' clinicopathological data.

We evaluated five representative fields of each section then assessed the extent of stain as follows: 0 if stain extent was <5%, 1 if stain extent was <30%, 2 if stain extent was 30-70%, and 3 if stain extent was >70%. We assessed the intensity of stain as follows: no stain, 0; weak stain, 1; intermediate stain, 2; and strong stain, 3. Scores of extent and intensity scores were multiplied to yield final stain scores. Final scores were from 0-9. To facilitate statistical analysis, we divided the scores into low stain which are from 0-3 and high stain from 4-9 **[12].**

Statistical analysis

We used the program of SPSS v23.0 (SPSS Inc., IL, USA) for statistical analyses. Chi-squared tests were performed for evaluation of the association between PYCR2 and ZBTB18 expression, clinicopathological characteristics and follow-up data, while association with OS and PFS rates were assessed through Cox regression analyses. $P < 0.05$ was considered a significant value.

Results

Patient data

We included 120 samples from 60 patients with CRC; 60 samples were retrieved from CRC tissues and 60 samples from adjacent non-neoplastic colonic mucosa Table 1.

Immunohistochemical results:

PYCR2 expression and its association with clinicopathological data of CRC patients

PYCR2 was expressed in 31 (52.0%) cases of CRC and in 5 (8.0%) of non-neoplastic colon mucosa. PYCR2 upregulated in CRC than adjacent non-neoplastic colonic mucosa (p= 0.026).

Significant expression of PYCR2 was observed in cancer of ascending region more than other sites (p=0.034), PYCR2 expression CRC was positively correlated with large tumor size, higher grade, advanced tumor stage and presence of spread to lymph node (p<0.001).

ZBTB18 expression and its association with clinicopathological features of CRC patients

ZBTB18 was expressed in 31 (52.0%) cases of CRC and in 60 (100.0%) of non-neoplastic colon mucosa. ZBTB18 was down regulated in CRC than adjacent non-neoplastic colonic mucosa (p<0.001).

Significant expression of ZBTB18 was observed in cancer of the recto-sigmoid region more than other sites (p=0.017),

ZBTB18 expression in CRC was associated with more limited size of the tumor, lower grade, initial stage of the tumor, absence of a lymph node and absence of distant metastases (p<0.001).

No significant correlation was observed between PYCR2 expression and ZBTB18 expression with age or sex of patients Table 2; Figs 1

Follow-up and survival data.

High PYCR2 and low ZBTB18 expression were significantly associated with poor response to therapy (p = 0.008, 0.0.17 respectively), increased incidence of progression and relapse recurrence (p=0.005), unfavorable OS rate (p=0.001).

There was an inverse association between PYCR2 and ZBTB18 expression r= -0.521(p=0.002).

In univariate analyses, High PYCR2 expression, reduced ZBTB18 expression, depth of tumor invasion, lymphatic metastasis, distant metastasis, and TNM stage were significantly related to patient OS and RFS. Multivariate analysis revealed PYCR2 expression (P < 0.001), ZBTB18 expression, distant metastasis (P < 0.001), and TNM stage (P < 0.001) to be independent predictors of patient OS and PYCR2 expression, ZBTB18 expression and distant metastasis (P < 0.001) were predictors of patient RFS.

Discussion

Detection of predictive and prognostic biomarkers and novel targeting therapies for CRC is important to improve its prognosis [**13**].

Proline metabolism and resulting products are important for many biological processes which occur in normal and oncogenic process, that lead to development and progression of broad range of cancers [**14-16**].

PYCR2 is a prolin family member that was studied and has many roles in progression of cancers of various organs, but its role in CRC oncogenesis and progression was not sufficiently studied.

In the current study we assessed the expression of PYCR2 in malignant tissues and adjacent non-neoplastic colonic mucosa and found that PYCR2 expression was upregulated in malignant more than normal tissues which indicate its role in CRC oncogenesis. Additionally, tissue protein expression of PYCR2 was upregulated in high grade and advanced stage CRC which indicated its role in cancer progression and development of metastases. Furthermore, we showed that increased PYCR2 expression was associated with shortened survival and poor outcome of CRC patients.

Our results were in line with **Yin et al., [13],** who represent the first to investigate genetic roles of PYCR2 in CRC oncogenesis, progression and shortened overall survival time.

Additionally, our results are similar to results of **Wang et al., [7]** who assessed genetic and tissue protein expression of PYCR2 in CRC and other tumor types. They have found that levels of PYCR2 are up-regulated in CRC tumor tissues at the mRNA and protein levels.

PYCR2 was found to have many roles in CRC progression mainly through controlling apoptosis in cancer cells and mutations in Wnt/b-catenin and Notch signaling pathway pathways which are incriminated in CRC oncogenesis **[T. Nakayama et al., [17], Wang et al., [7]** results.

Mutations in Wnt pathway result in CRC stem cell expansion in addition to stimulation of epithelial mesenchymal transition process which are responsible for CRC metastases and progression [**18**].

Abnormal PYCR2 expression was associated with abnormalities in Jagged1/Notch signaling pathway in CRC that was correlated with CRC development and progression [**19, 20**].

PYCR2 was found to be correlated with MYC levels in CRC and MYC knockdown lead to reduction of CRC growth, progression and metastasis [**21, 22**].

Yin et al., [13] study demonstrated the roles of PYCR2 in apoptosis in CRC as its silencing could induce cell apoptosis by increasing levels of proapoptotic factors and decreasing levels of antiapoptotic factors.

PYCR2 has other studied roles in CRC metastases by activation of matrix metalloproteinases (MMPs) which mediate CRC cell invasion and migration through degradation of extracellular matrix (ECM) [**13, 23]**

All these previous results and results of current study showed that PYCR2 up-regulation could lead to CRC progression and metastases through several mechanisms that need to be clarified.

To clarify role of PYCR2 in CRC, we assessed levels of another biomarker which is ZBTB18 in sections from CRC and adjacent non-neoplastic tissues of colonic mucosa.

To our knowledge, There are few studies assessed its roles in CRC carcinogenesis and the current study represent the first one which assessed both PYCR2 and ZBTB18 tissue protein expression in CRC.

ZBTB18 expression was high in non-neoplastic colon mucosa and its expression is down-regulated in malignant tissues; additionally decreased ZBTB18 expression was associated with increased tumor invasion and metastases. We assessed the association of ZBTB18 with patient survival and we found that intense expression was associated with favorable outcome and longer survival time. Our results highlighted the possible tumor suppressor role of ZBTB18 in CRC patients.

Our results were similar to results of Sarah Bazzocco et al., 2021 who was first to investigate values of tissue protein expression of ZBTB18 in CRC tumorigenesis and declared that some zinc finger proteins participated in CRC tumorigenesis

ZBTB18 was formerly found to play an essential role in supporting normal myogenesis [**24**] and normal brain development [**25**], but its role in normal intestinal epithelium development and CRC carcinogenesis have not sufficiently clarified.

ZBTB18 expression reduce proliferation of brain tumor cells and promote apoptosis glioblastoma multiforme (GBM) and medulloblastoma (MB) [**26**]. Additionally, ZBTB18 down regulation was associated with aggressive phenotype, unfavorable survival and poor prognosis of GBM patients [**26**]. These results are in line with our results in CRC.

Previous reports about roles of ZBTB18 expression in CRC were in line with our results that ZBTB18 down regulation in CRC cells associated with increased growth, proliferation and invasion that consequently associated with poor prognosis.

Our results and results of previous studies collectively showed that ZBTB18 is considered a tumor suppressor gene that could be used in novel targeted therapy against CRC**.** Therefore, strategies aimed at rescuing ZBTB18 expression or down regulating some of the downstream ZBTB18 targets genes may offer therapeutic potential in colorectal cancer patients **[10, 27]**.

In addition, the present study showed that reduced ZBTB18 expression in the tumors was associated with shorter CRC patient survival which was similar to results of **Bazzocco1 et al., [10],** who showed that ZBTB18 expression was reduced in presence of lymph node metastases in comparison to the primary CRC tumors. These findings confirmed the tumor suppressor role of ZBTB18 in CRC tissues that could detect a subset of CRC patients with poor prognosis that will get benefits from aggressive targeted therapy.

Summary

Our study represents the first to study both PYCR2 and ZBTB18 expression in CRC tissues and non-neoplastic tissues. We observed an inverse association between expression of PYCR2 and ZBTB18 in tissues of CRC and non-neoplastic colon mucosa. PYCR2 carry out an oncogenic role and ZBTB18 has oncosupressor role.

High expression of PYCR2 and low expression of ZBTB18 were independent predictors of CRC, progression, poor prognosis and unfavorable patient OS and PFS rates.

However, we have certain limitations in our study as little number of studied patients due to inclusion of patients from a single institute and in addition to using only immunohistochemistry for both PYCR2 and ZBTB18 evaluation

Recommendation

Further future genetic studies including larger patients number from multi- center institutions and longer follow-up time periods with genetic evaluation of both PYCR2 and ZBTB18 levels in CRC are needed to assess roles of PYCR2 and ZBTB18 in controlling CRC proliferation, migration, invasion and apoptosis which will help in discovering recent targeted therapy to improve CRC patients' prognosis.

Declarations

Authors had no conflict of interest

References

1. **Martinez-Barriocanal A, Arango D, Dopeso H. PVT1 long non-coding RNA in gastrointestinal cancer. Front Oncol. 2020; 10:38.**
2. **Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68(6):394–424.**
3. **D.V.F. Tauriello, S. Palomo-Ponce, D. Stork, A. Berenguer-Llergo, J. Badia- Ramentol, M. Iglesias, M. Sevillano, S. Ibiza, A. Canellas, X. Hernando-Momblona, D. Byrom, J.A. Matarin, A. Calon, E.I. Rivas, A.R. Nebreda, A. Riera, C.S. Attolini, E. Batlle, TGFbeta drives immune evasion in genetically reconstituted colon cancer metastasis, Nature 554 (2018) 538e543.**
4. **J.M. Phang, J. Pandhare, Y. Liu, The metabolism of proline as microenvironmental stress substrate, J. Nutr. 138 (2008) 2008Se2015S.**
5. **F. Loayza-Puch, K. Rooijers, L.C. Buil, J. Zijlstra, J.F. Oude Vrielink, R. Lopes, A.P. Ugalde, P. van Breugel, I. Hofland, J. Wesseling, O. van Tellingen, A. Bex, R. Agami, Tumour-specific proline vulnerability uncovered by differential ribosome codon reading, Nature 530 (2016) 490e494.**

6. L. Tang, J. Zeng, P. Geng, C. Fang, Y. Wang, M. Sun, C. Wang, J. Wang, P. Yin, C. Hu, L. Guo, J. Yu, P. Gao, E. Li, Z. Zhuang, G. Xu, Y. Liu, Global metabolic profiling identifies a pivotal role of proline and hydroxyproline metabolism in supporting hypoxic response in hepatocellular carcinoma, Clin. Canc. Res. 24 (2018) 474e485.
7. Wang S, Gu L, Huang L, Fang J, Liu Z, Xu Q. The upregulation of PYCR2 is associated with aggressive colon cancer progression and a poor prognosis Biochemical and Biophysical Research Communications 572 (2021) 20-26
8. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487(7407):330–7.
9. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, et al. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet. 2009;41(2):178–86.
10. Bazzocco S, Dopeso H, Barriocanal M A, Anguita E, Nieto R, Li J, Garcia-Vidal E, Maggio V, Rodrigues P, Guimaraes de Marcondes P. Clin Epigenet (2021) 13:88,
11. Edge SB and Compton CC: The American Joint Committee on Cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol 17: 1471-1474, 2010.
12. LI J, HU B, FANG L, GAO Y, SHI SH, HE H, LIU X and YUAN C. Barrier-to-autointegration factor 1: A novel biomarker for gastric cancer ONCOLOGY LETTERS 2018.
13. Yin F, Huang X, and Xuan Y. Pyrroline-5-Carboxylate Reductase-2 Promotes Colorectal Cancer Progression via Activating PI3K/AKT/mTOR Pathway Disease Markers Volume 2021, Article
14. C. D'Aniello, E.J. Patriarca, J.M. Phang, G. Minchiotti, Proline metabolism in tumor growth and metastatic progression, Front Oncol 10 (2020) 776.
15. M.L. Craze, H. Cheung, N. Jewa, N.D.M. Coimbra, D. Soria, R. El-Ansari, M.A. Aleskandarany, K. Wai Cheng, M. Diez-Rodriguez, C.C. Nolan, I.O. Ellis, E.A. Rakha, A.R. Green, MYC regulation of glutamine-proline regulatory axis is key in luminal B breast cancer, Br. J. Canc. 118 (2018) 258e265.
16. T. Sharif, E. Martell, C. Dai, S.K. Singh, S. Gujar, Regulation of the proline regulatory axis and autophagy modulates stemness in TP73/p73 deficient cancer stem-like cells, Autophagy 15 (2019) 934e936.
17. T. Nakayama, A. Al-Maawali, M. El-Quessny, A. Rajab, S. Khalil, J.M. Stoler, W.H. Tan, R. et al., Mutations in PYCR2, encoding pyrroline-5-carboxylate reductase 2, cause microcephaly and hypomyelination, Am. J. Hum. Genet. 96 (2015) 709e719.
18. J. Zhang, X.J. Tian, J. Xing, Signal transduction pathways of EMT induced by TGF-beta, SHH, and WNT and their crosstalks, J. Clin. Med. 5 (2016).
19. R. Jackstadt, S.R. van Hooff, J.D. Leach, X. Cortes-Lavaud, J.O. Lohuis, R.A. Ridgway, V.M. Wouters, et al., Epithelial NOTCH signaling rewires the tumor microenvironment of colorectal cancer to drive poor-prognosis subtypes and metastasis, Canc. Cell 36 (2019) 319e336, e317.
20. A.A. Farooqi, M. de la Roche, M.B.A. Djamgoz, Z.H. Siddik, Overview of the oncogenic signaling pathways in colorectal cancer: mechanistic insights, Semin. Canc. Biol. 58 (2019) 65e79.
21. J.M. Phang, Proline metabolism in cell regulation and cancer biology: recent advances and hypotheses, Antioxidants Redox Signal. 30 (2019) 635e649.
22. K. Satoh, S. Yachida, M. Sugimoto, M. Oshima, T. Nakagawa, S. Akamoto, et al. Global metabolic reprogramming of colorectal cancer occurs at adenoma stage and is induced by MYC, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) E7697eE7706.
23. K. Umezawa and Y. Lin, "Inhibition of matrix metalloproteinase expression and cellular invasion by NF- κB inhibitors of microbial origin," Biochim Biophys Acta Proteins Proteom, vol. 1868, no. 6, p. 140412 2020.
24. Yokoyama S, Ito Y, Ueno-Kudoh H, Shimizu H, Uchibe K, Albini S, Mitsuoka K, Miyaki S, Kiso M, Nagai A, et al. A systems approach reveals that the myogenesis genome network is regulated by the transcriptional repressor RP58. Dev Cell. 2009;17(6):836–48.
25. Xiang C, Baubet V, Pal S, Holderbaum L, Tatard V, Jiang P, Davuluri RV, Dahmane N. RP58/ZNF238 directly modulates proneurogenic gene levels and is required for neuronal differentiation and brain expansion. Cell Death Differ. 2012; 19(4):692–702.
26. Fedele V, Dai F, Masilamani AP, Heiland DH, Kling E, Gatjens-Sanchez AM, Ferrarese R, Platania L, Soroush D, Kim H, et al. Epigenetic regulation of ZBTB18 promotes glioblastoma progression. Mol Cancer Res. 2017; 15(8):998–1011.
27. Giannakis M, Mu XJ, Shukla SA, Qian ZR, Cohen O, Nishihara R, Bahl S, Cao Y, Amin-Mansour A, Yamauchi M, et al. Genomic correlates of immunecell infiltrates in colorectal carcinoma. Cell Rep. 2016; 17(4):1206.

Tables

Table (1): Clinicopathological features, immunohistochemical markers and outcome of 60 patients with colorectal carcinoma.

Characteristics	All patients (N=60)		Characteristics	All patients (N=60)	
	No.	%		No.	%
Sex			Distant metastasis		
Male	36	60%	Absent	43	71.7%
Female	24	40%	Present	17	28.3%
Age year			Duke's stage		
Mean±SD	58.33	±12.27	Stage A	14	23.3%
Median (Range)	60	(29-80)	Stage B	12	18.3%
Initial site			Stage C	17	30%
Ascending	19	31.7%	Stage D	17	28.3%
Transverse	6	10%	AJCC stage		
Descending	5	8.3%	Stage I	14	23.3%
Rectosigmoid	30	50%	Stage II	12	20%
Size			Stage III	17	28.3%
≤5 cm	27	45%	Stage IV	17	28.3%
>5 cm	33	55%	Follow-up months		
Pathological type			Mean±SD	23.11	±9.98
Conventional	52	86.7%	Median (Range)	25	(8-35)
Mucoid	8	13.3%	Relepase	(N=42)	
Grade			Absent	28	66.7%
-			Present	14	33.3%
Grade I	13	21.7%	Death		
Grade II	31	51.7%	Alive	40	66.7%
Grade III	16	26.7%	Died	20	33.3%
T			-		
T1	9	15%			
T2	14	23.3%			
T3	12	20%	-		
T4	25	41.7%			
LN metastasis			-		
Node negative	26	43.3%			
Node positive	34	56.7%			

Table2: correlations between PYCR2 and ZBTB18 expression and histopathological diagnosis

		Colorectal carcinoma (CRC) N= 60	Non-neoplastic Mucosa N= 60	Total N= 120	P
PYCR2	Low	29 (48.0%)	55 (92.0%)	84 (70%)	0.026
	High	31 (52.0%)	5 (8.0%)	36 (30%)	
ZBTB18	Low	29 (48.0%)	0 (0.0%)	29 (19%)	**<0.001**
	High	31 (52.0%)	60 (100.0%)	91 (81%)	

Table (3): Relation between **PYCR2 and immunohistochemistry** and clinicopathological parameters in 60 patients with colorectal carcinoma.

	All patients (N=60)		PYCR2				p-value	ZBTB18				p-v
			Low (N=29)		High (N=31)			Low (N=29)		High (N=31)		
	No.	(%)	No.	(%)	No.	(%)		No.	(%)	No.	(%)	
Sex												
Male	36	(60%)	18	(50%)	18	(50%)	0.752‡	21	(58.3%)	15	(41.7%)	0.0
Female	24	(40%)	11	(45.8%)	13	(54.2%)		8	(33.3%)	16	(66.7%)	
Age (year)												
Mean±SD	58.33	±12.27	57.10	±11.66	59.48	±12.90	0.378•	59.13	±13.90	57.58	±10.70	0.6
Median (Range)	60	(29-80)	55	(30-80)	62	(29-75)		60	(29-80)	55	(30-75)	
Initial site												
Ascending	19	(31.7%)	4	(21.1%)	15	(78.9%)	0.034‡	13	(68.4%)	6	(31.6%)	0.0
Transverse	6	(10%)	3	(50%)	3	(50%)		5	(83.3%)	1	(16.7%)	
Descending	5	(8.3%)	3	(60%)	2	(40%)		2	(40%)	3	(60%)	
Rectosigmoid	30	(50%)	19	(63.3%)	11	(36.7%)		9	(30%)	21	(70%)	
Size												
≤5 cm	27	(45%)	22	(81.5%)	5	(18.5%)	<0.001‡	5	(18.5%)	22	(81.5%)	<0.
>5 cm	33	(55%)	7	(21.2%)	26	(78.8%)		24	(72.7%)	9	(27.3%)	
Pathological type												
Conventional	52	(86.7%)	28	(53.8%)	24	(46.2%)	0.053‡	22	(42.3%)	30	(57.7%)	0.0
Mucoid	8	(13.3%)	1	(12.5%)	7	(87.5%)		7	(87.5%)	1	(12.5%)	
Grade												
Grade I	13	(21.7%)	13	(100%)	0	(0%)	<0.001§	2	(15.4%)	11	(84.6%)	<0.
Grade II	31	(51.7%)	14	(45.2%)	17	(54.8%)		14	(45.2%)	17	(54.8%)	
Grade III	16	(26.7%)	2	(12.5%)	14	(87.5%)		13	(81.3%)	3	(18.8%)	
T												
T1	9	(15%)	9	(100%)	0	(0%)	<0.001§	0	(0%)	9	(100%)	<0.
T2	14	(23.3%)	10	(71.4%)	4	(28.6%)		4	(28.6%)	10	(71.4%)	
T3	12	(20%)	4	(33.3%)	8	(66.7%)		6	(50%)	6	(50%)	
T4	25	(41.7%)	6	(24%)	19	(76%)		19	(76%)	6	(24%)	
LN metastasis												
Node negative	26	(43.3%)	22	(84.6%)	4	(15.4%)	<0.001‡	5	(19.2%)	21	(80.8%)	<0.
Node	34	(56.7%)	7	(20.6%)	27	(79.4%)		24	(70.6%)	10	(29.4%)	

positive												
Distant metastasis			-		-							
Absent	43	(71.7%)	27	(62.8%)	16	(37.2%)	<0.001‡	15	(34.9%)	28	(65.1%)	0.0
Present	17	(28.3%)	2	(11.8%)	15	(88.2%)		14	(82.4%)	3	(17.6%)	
Duke's stage			-		-							
Stage A	14	(23.3%)	14	(100%)	0	(0%)	<0.001§	2	(14.3%)	12	(85.7%)	<0.
Stage B	12	(18.3%)	7	(63.6%)	5	(36.4%)		4	(27.3%)	8	(72.7%)	
Stage C	18	(30%)	6	(33.3%)	12	(66.7%)		10	(55.6%)	8	(44.4%)	
Stage D	17	(28.3%)	2	(11.8%)	15	(88.2%)		14	(82.4%)	3	(17.6%)	
AJCC stage			-		-							
Stage I	14	(23.3%)	14	(100%)	0	(0%)	<0.001§	2	(14.3%)	12	(85.7%)	<0.
Stage II	12	(20%)	8	(66.7%)	4	(33.3%)		3	(25%)	9	(75%)	
Stage III	17	(28.3%)	5	(29.4%)	12	(70.6%)		10	(58.8%)	7	(41.2%)	
Stage IV	17	(28.3%)	2	(11.8%)	15	(88.2%)		14	(82.4%)	3	(17.6%)	
PYCR2			-		-							
Low	29	(48.3%)						8	(27.6%)	21	(72.4%)	0.0
High	31	(51.7%)						21	(67.7%)	10	(32.3%)	
ZBTB18			-		-							
Low	29	(48.3%)	8	(27.6%)	21	(72.4%)	0.002‡					
High	31	(51.7%)	21	(67.7%)	10	(32.3%)						

* Independent samples Student's t-test; • Mann Whitney U test; ‡ Chi-square test; § Chi-square test for trend; p<0.05 is significant.

Table (4): Relation between **PYCR2 and** ZBTB18 immunohistochemistry and outcome of treatment of 60 patients with colorectal carcinoma.

	All patients		PYCR2		p-value	ZBTB18		p-value
			Low	High		Low	High	
	No. (%)		No. (%)	No. (%)		No. (%)	No. (%)	
Response	(N=22)		(N=1)	(N=21)		(N=16)	(N=6)	
PD	8 (36.4%)		1 (100%)	7 (33.3%)	0.008‡	6 (37.5%)	2 (33.3%)	0.017‡
SD	6 (27.3%)		0 (0%)	6 (28.6%)		6 (37.5%)	0 (0%)	
PR	3 (13.6%)		0 (0%)	3 (14.3%)		2 (12.5%)	1 (16.7%)	
CR	5 (22.7%)		0 (0%)	5 (23.8%)		2 (12.5%)	3 (50%)	
Relapse	(N=42)		(N=28)	(N=14)		(N=15)	(N=27)	
Absent	28 (66.7%)		21 (75%)	7 (50%)	0.005‡	2 (13.3%)	26 (96.3%)	<0.001‡
Present	14 (33.3%)		7 (25%)	7 (50%)		13 (86.7%)	1 (3.7%)	
Relapse Free Survival	(N=42)		(N=28)	(N=14)		(N=15)	(N=27)	
Mean (months) (95%CI)	28.49 months (25.52-31.46)		30.31 months (27.03-33.59)	24.86 months (19.30-30.41)	0.002†	18.13 months (13.85-22.42)	34.19 months (32.62-35.75)	<0.001†
12 month RFS	85.7%		89.3%	78.6%		60%	100%	
24 month RFS	69.1%		78.6%	50%		20%	96.3%	
30 month RFS	66.4%		74.8%	50%		13.3%	96.3%	
Death	(N=60)		(N=29)	(N=31)		(N=29)	(N=31)	
Alive	40 (66.7%)		25 (86.2%)	15 (48.4%)	0.002‡	9 (31%)	31 (100%)	<0.001‡
Died	20 (33.3%)		4 (13.8%)	16 (51.6%)		20 (69%)	0 (0%)	
Overall Survival	(N=60)		(N=29)	(N=31)		(N=29)	(N=31)	
Mean (months) (95%CI)	26.85 months (23.92-29.79)		31.90 months (29.06-34.74)	22.12 months (17.69-26.54)	0.001†	17.93 months (13.75-22.11)	35 months	<0.001†
12 month OS	73.3%		93.1%	54.8%		44.8%	100%	
24 month OS	66.5%		86.2%	47.9%		29.5%	100%	
30 month OS	66.5%		86.2%	47.9%		29.5%	100%	

‡ Chi-square test; † Log rank test; $p<0.05$ is significant.

Table 5: Multivariant analysis of Overall and Disease -Free Survival in relation to some studied parameters

.

Variables		3-year Disease-Free survival Rate (%)	p-value	3-year Overall survival Rate (%)	p-value
Age group	<60y	68%	**0.568**	80.8%	**0.120**
	>60y	58.3%		62.2%	
Sex	Male	57.4%	**0.287**	65.6%	**0.180**
	Female	78.6%		83.3%	
Size	<5 Cm	87.8%	**< 0.001**	100%	**< 0.001**
	>5 Cm	26.7%		43.3%	
Grade	I	90.9%	**0.109**	100%	**0.021**
	II	55%		69%	
	III	50%		50%	
Duke Stage	A	91.7%	**< 0.001**	100%	**< 0.001**
	B	81.8%		100%	
	C	37.5%		58.8%	
	D	0.0%		30.0%	
LN Met	No	87.5%	**< 0.001**	100%	**< 0.001**
	Yes	31.3%		45.6%	
Distant Metastases	No	66.3%	**< 0.001**	82.5%	**< 0.001**
	Yes	0.0%		30%	
PYCR2	Low	66.3%	**< 0.001**	82.5%	**< 0.001**
	High	0.0%		30%	
ZBTB18	Low	66.3%	**< 0.001**	82.5%	**< 0.001**
	High	0.0%		30%	
Stage AJCC	Stage I	91.7%	**< 0.001**	100%	**< 0.001**
	Stage II	83.3%		100%	
	Stage III	33.3%		56.2%	
	Stage IV	0.0%		30.0%	

Figures

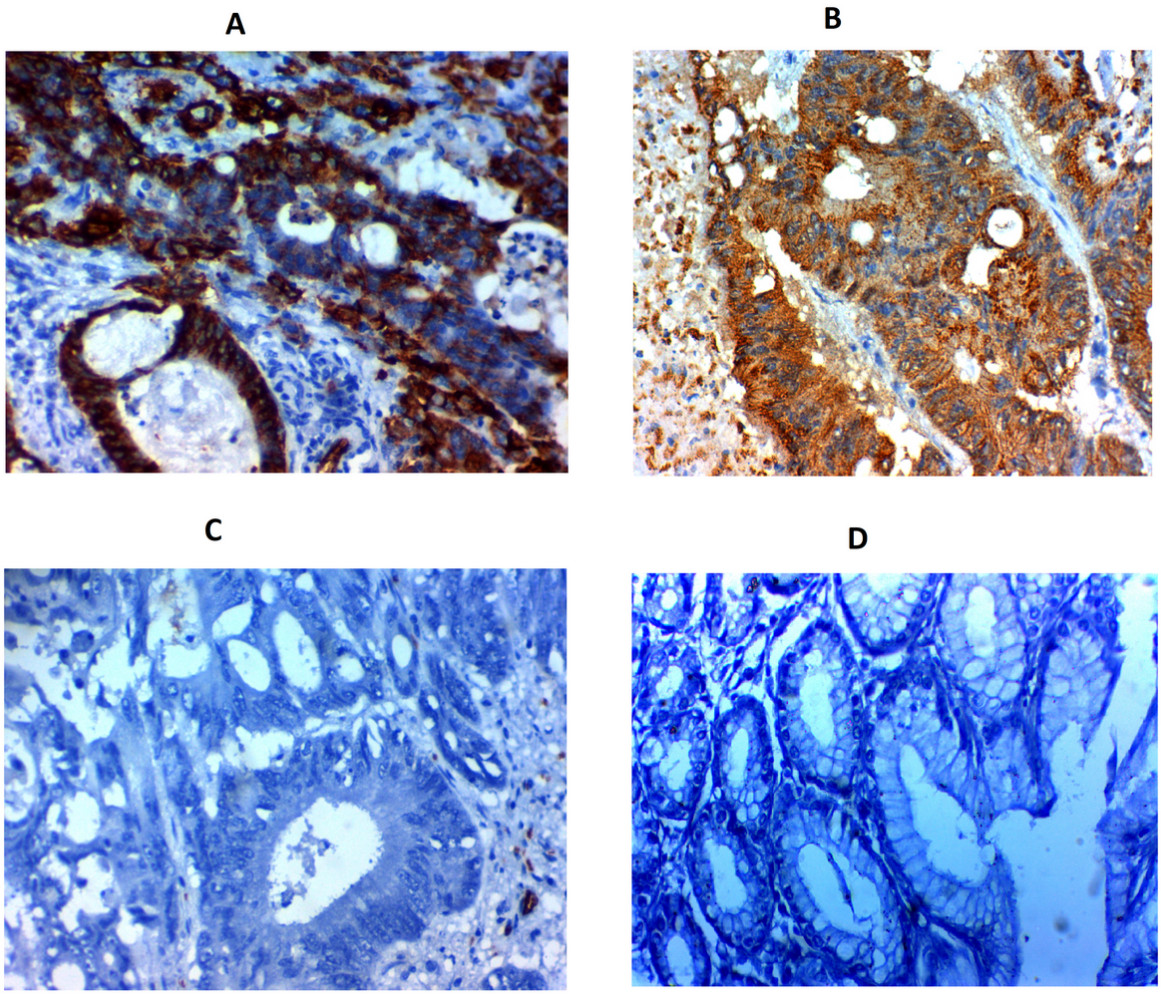

Figure 1

PYCR2 cytoplasmic expression in tissues of colorectal carcinoma (CRC) and adjacent non-neoplastic tissues A; high PYCR2 expression in CRC grade III, stage III x400, B; high PYCR2 expression in CRC grade II stage III x400, , C; Negative PYCR2 expression in CRC grade I stage I x400, C; negative PYCR2 expression in non-neoplastic mucosa of the colonx400.

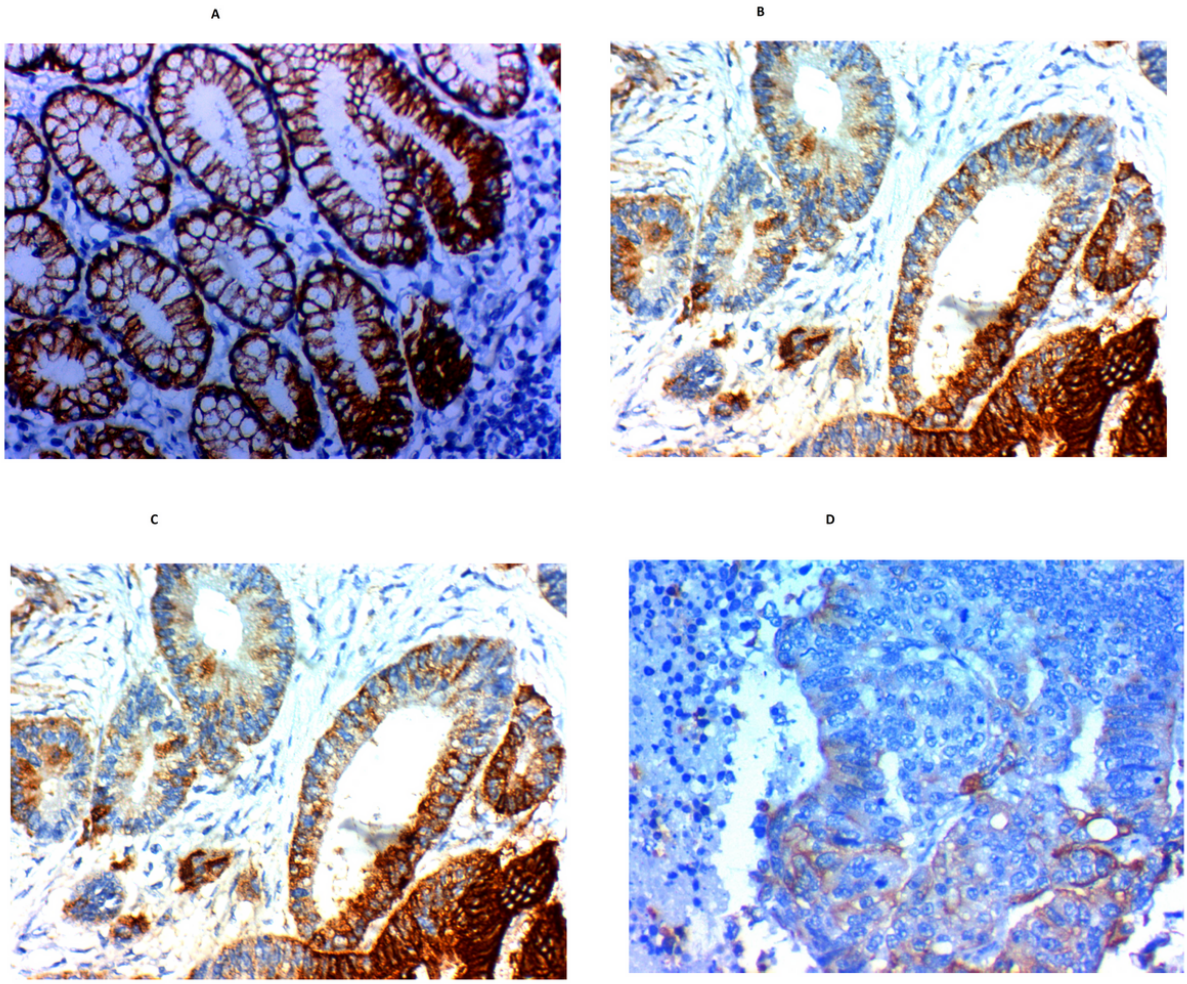

Figure 2

ZBTB18 cytoplasmic expression in tissues of colorectal carcinoma (CRC) and adjacent non-neoplastic tissues A; high ZBTB18 expression in non-neoplastic colon mucosa x400, B; high PYCR2 expression in CRC grade I stage I x400, , C; Low PYCR2 expression in CRC grade II stage II x400, C; negative PYCR2 expression in CRC grade III stage III x400

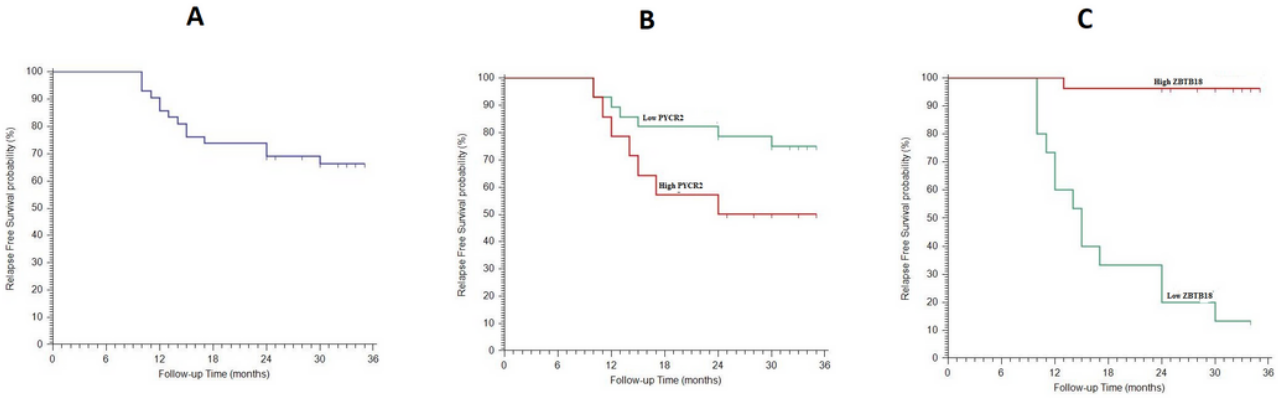

Figure 3

Kaplan Meir survival curves of Recurrence free survival (RFS) of colorectal carcinoma (CRC) patients (A) RFS rate of all CRC cases (B) RFS rate of cases stratified according to PYCR2 expression (C) RFS rate of cases stratified according to ZBTB18 expression.

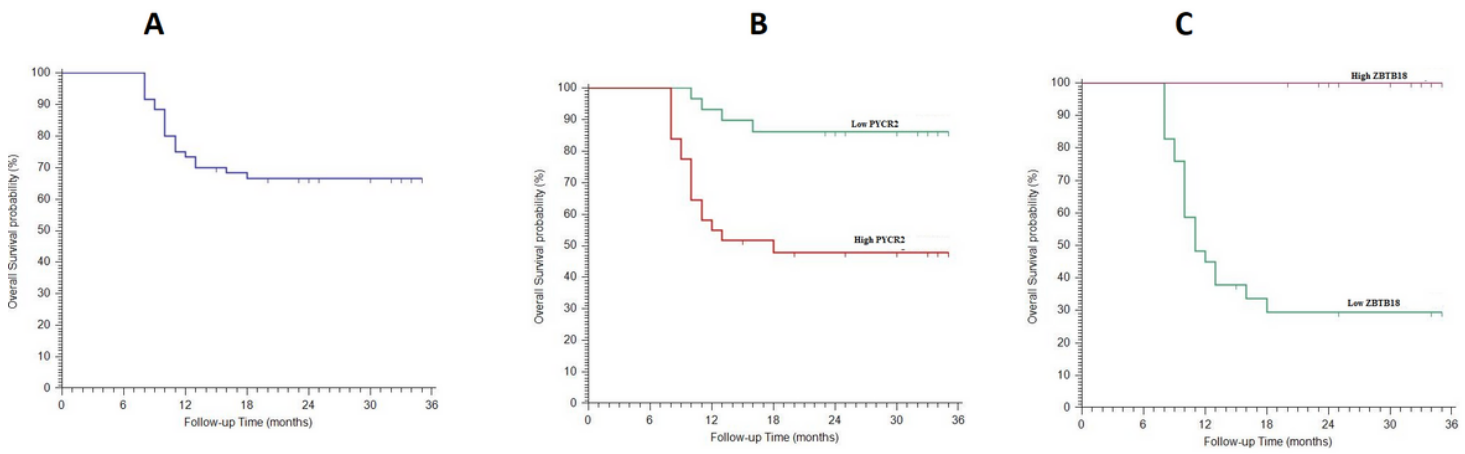

Figure 4

Kaplan Meir survival curves of Overall Survival (OS) rate of colorectal carcinoma (CRC) patients (A) OS rate of all CRC cases (B) OS rate of cases stratified according to PYCR2 expression (C) OS rate of cases stratified according to ZBTB18 expression.