Original article

PTGS2 Over-Expression: A Colorectal Carcinoma Initiator not an Invasive Factor

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Abstract

Background: Cyclooxygenase-2 (COX-2) main product is Prostaglandin E2 (PGE2) which cause mitogenesis and inflammation. COX-2 is the product of prostaglandin-endoperoxide synthase 2 (*PTGS2*) gene expression. COX-2 dysregulation can cause angiogenesis, differentiation, and promotion of cancer and its suppression related to control of the tumor's size, number, and cell shape. This study focused on the association of COX-2 expression with colorectal carcinoma (CRC) among Iranian patients on mRNA level and in the Cancer Genome Atlas Program (TCGA) colon and rectum RNAseq dataset, and its relation with pathological features.

Methods: *PTGS2* expression was assayed by quantitative-PCR method from 90 tissue samples collected from 45 participants. The control samples come from the non-tumor area of the same patients. The data analyzed based on ΔΔCq. The *PTGS2*-RNAseq data extracted and analyzed by UCSC Xena browser, and its association assessed the occurrence of CRC and invasive-features.

Results: *PTGS2* showed very significant over-expression in tumor tissues (p< 0.0001) with an N-fold expression of 2.25. But, there was not any significant association between *PTGS2* and CRC invasive-pathological features such as Lymphatic, vascular and perineural invasion, the Grades of cancer, and Pathologic-M in both parts of this study.

Conclusions: The increase in *PTGS2* is related to the occurrence of CRC among patient samples. But in both part of this study, *PTGS2* is not an invasive factor, and it does not affect the cell differentiation of tumors and metastasis. Based on the high N-fold for patient samples, it can be a strong candidate as a CRC initiator biomarker.

Keywords: Cyclooxygenase-2, Gene expression profiling, Neoplasm invasion, Prostaglandins, TCGA-data.

Introduction

Currently, colorectal carcinoma (CRC) is the third universal cause of death by cancer and in general, is one of the most rampant cancers in most of the countries in which Iran is included (1,2). The occurrence of CRC in Iranian men and women is 8.1 to 8.3 and 6.5 to 7.5 per 100,000, respectively (3). Also, the five years' survival rate of it is 41% among the Iranian (4).

Since CRC is heterogeneous based on the treatment response and molecular features, the molecular-based studies to characterize the molecular features in malignancy, finding biomarkers, and molecular classification of CRC have improved the individual therapy and reduced the metastatic phase (4-6). Lack of biomarkers, especially in the early stages of CRC is one of the main limitations of preventing it (7). On the other hand, the effect of long term intake of non-steroidal anti-inflammatory drugs (NSAID) on the reduction of CRC promotion risk, especially in familial groups, brought the idea of possible potential of COX-2 as a biomarker for colorectal cancer (8). Also, deeper literature in this matter showed the dysregulation

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of COX-2 in half of the adenomatous polyps which would be counted as the CRC precursors (9).

Cyclooxygenase-2 is the key enzyme of producing prostaglandins (PGs) from arachidonic acid for that the gene is called *PTGS2*. Among the PGs, the PGE2 is the dominant product of COX-2 which can cause the varieties of malignancy including colon cancer by its role in activating mitogenesis and inflammation (10-13). Even though the COX-2 expression is almost undetectable in most of the normal tissues, but its expression is more frequent in tumorigenic areas (14,15). When the $APC^{min/+}$ mouse model was treated by PGE2 the tumor loads had been increased dramatically in both large and small intestines (16). Currently, the amount of urine PGs considered as a biomarker of cancer (11). COX-2 can induce cancer duo to several ways including induces of cancer stem cells like activity, apoptotic resistance, angiogenesis, and metastasis which is approved as the mechanistic aspect of how COX-2 is working (15).

Although several types of researches have shown the effect of PGE2 and overexpression of COX-2 in different types of cancer cells, especially CRC (17-22) but still some studies have not observed the correlation, this controversy is even stronger about its relation with invasion and migration (23-25). So, the aspects of COX-2 in cancer and its relation with CRC that were observed by different studies make it a good point and a potent biomarker but it needs more studies on the relation of the COX-2 expression in different populations.

In the present study, because of the lack of enough studies on COX-2 expression in Iranian cancer patients, especially in CRC, despite of its potent as a prognostic biomarker candidate, the purpose was assessing of correlation of *PTGS2* expression with colorectal cancer in patients' tissues, and the CRC pathological features. Furthermore, we evaluated the *PTGS2* expression of colon and rectum samples from TCGA and its correlation with some of the invasive pathological features by use of TCGA-COAD and READ data-sets.

Materials and methods

The participants were the colorectal cancerous patients (2012 to 2016) whose been attended for the tumor removal surgery in Imam Khomeini hospital (Tehran, Iran). Forty-five patients have participated in this study and all of them have been adults (ID Number of Ethical Committee: #IR.UMZ.REC.1397.091). The tumor area and the non-tumor adjacent tissues have been used as case and control groups. The tissues have been frozen immediately after surgery in a liquid nitrogen tank. The prevalence of colorectal cancer in Iranian is 8.3 in 100,000. So based on the standard formula the number of samples in our study is proper for this study.

The patients have been classified based on the Bloom-Richardson system which is calculating the grade by adding up the scores of nuclear pleomorphism and mitotic count, and TNM staging system. The cell nuclei uniformity and the number of divided cells in the 10x high power microscope filed would be assessed in this method (26). Also, the situation of each patient was checked based on the invasion of tumor tissues to other tissues like Lymphatic, vascular, perineural, and perineal.

RNA isolation and RT q-PCR

Total RNA was extracted by the standard TRIzol™ Plus kit protocol. In order to check the quantity and quality of isolated RNA, the OD reading by NanoDrop and gel electrophoresis have been used. For cDNA the standard protocol of Thermo Scientific™ RevertAid™ first-strand cDNA kit was applied.

The COX-2 (*PTGS2*), and *ACTβ* were quantified in 3 repeats by CYBR Premix Ex Taq II (the primers of *PTGS2* (COX-2), and *ACTβ* (Gene Control) has been designed by Primer 3 [http://primer3.ut.ee/]), and Applied Biosystems 7500/7500 Fast Real-Time PCR. To be sure of no contamination, negative controls were applied. The qPCR applied with the blinding procedure. The forward and reverse primers sequences of COX-2 were 5'-AGGAGGTCTTTGGTCTGGTG-3' and 5'-ACTGCTCATCACCCCATTCA-3' respectively. To analyze the *PTGS2* expression

data, after normalising it to *ACTβ* expression, fold expression ($2^{-\Delta\Delta Cq}$) and $\log_2$ (Fold expression) were used (27).

PTGS2 expression data extraction and RNA-seq analysis

To generate the *PTGS2* expression data of colon and rectum cancer and normal tissues, we used the UCSC Xena browser (Xena.ucsc.edu). To analyze the association of *PTGS2* expression with CRC, the data of TCGA-COAD (331-samples) and READ (103-samples) were compared with TCGA-solid normal tissues, and the dataset of RNA-Seq by Expectation Maximization (RSEM) expected-count normalized by DESeq2 normalization. The correlation of *PTGS2* expression with pathological features such as Pathological-M and lymphatic invasion among 2236 TCGA-COAD-READ samples analyzed. The *PTGS2* level expressed based on $\log_2$ (norm-count+1) unit (28).

Statistical analysis

After normal distribution of $\log_2$ (Fold Expression) in general has been checked, the Mann-Whitney test was used for statistical analysis of COX-2 expression between case and control groups. For the association of COX-2 expression with the other factors like stages and invasions, depend on the character the proper non-parametrical analysis (Mann-Whitney and Kruskal-Wallis, and Dunn's multiple comparison test) has been used and *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ were considered as significant differences. Results are presented as median $\pm$ SEM by Prism 8.

Results

General characters and quantitative analysis

All of the general characters of the samples are presented in Table 1. The COX-2 showed a highly significant overexpression (N-Fold Expression= 2.25) in tumor samples compare to normal tissues (Mann-Whitney $U=$ 354, $p < 0.0001$ two-tailed). This significant overexpression observed in all of the regions based on the primary site, except the Cecum groups with non-significant overexpression (Fig. 1). In both of non-tumor group and tumor tissues, there were not any significant COX-2 expression differences within the primary site of the samples (Tumor $p= 0.256$; Normal $p= 0.427$). In both groups, the most under-expressions belonged to the cecum.

Table 1: Groups characteristics analysis.

| Characters		No (Percent %)
TNM stages	Stage 0	3 (6.7%)
	Stage I	7 (15.6%)
	Stage II	9 (20.0%)
	Stage III	11 (24.4%)
	Stage IV	3 (6.7%)
	M/Unknown	12 (26.6%)
Bloom Richardson	Grade I	14 (31.1%)
	Grade II	17 (37.8%)
	Grade III	2 (4.5%)
	Grade IV	11 (24.5%)
Donor's statue	Alive	35 (77.8%)
	Deceased	10 (22.2%)
Histology	Adenocarcinoma	30 (66.7%)
	Adenosquamous carcinoma	10 (22.2%)
	Medullary carcinoma	1 (2.2%)
	Mucinous carcinoid	1 (2.2%)
	Mucinous (colloid) adenocarcinoma	3 (6.7%)
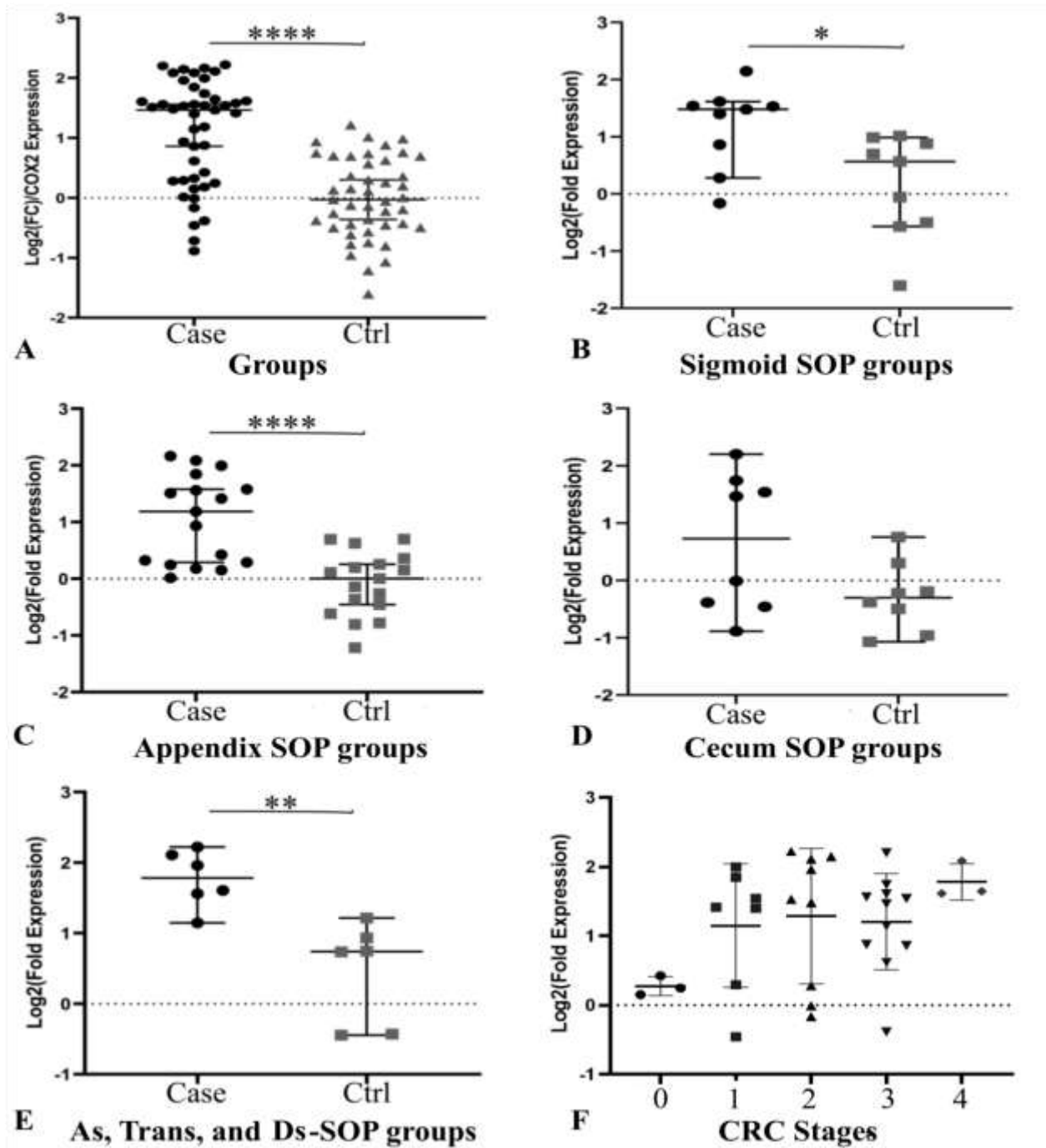
Fig. 1. Comparison of COX-2 expression in patient and control groups, and cases stages of cancer. A) The Cox-2 is significantly overexpressed in the tumor samples in general (p< 0.0001) in individual chart. B) The sigmoid primary site's COX-2 expression is significantly higher (p= 0.0188). C) There is highly significant COX-2 overexpression in Appendix tumor group compare to Appendix control (p< 0.0001). D) The cecum primary site's p-value is 0.1605. E) The p-value of the samples that were located from ascending, transverse and descending (As, Trans, and Ds) colon is 0.0043 that represent highly significant differences. F) Increase of COX-2 expression due to increase of stages is observable but it is not statistically significant.

As it is displayed in Figure 1-F, even though the level of COX-2 expression is going up from lower stages to the higher stages of CRC, yet it was not significant (Exact p= 0.19, df= 4, No= 33). Also, the COX-2 expression has no participation in cell differentiation either (Fig. 2).
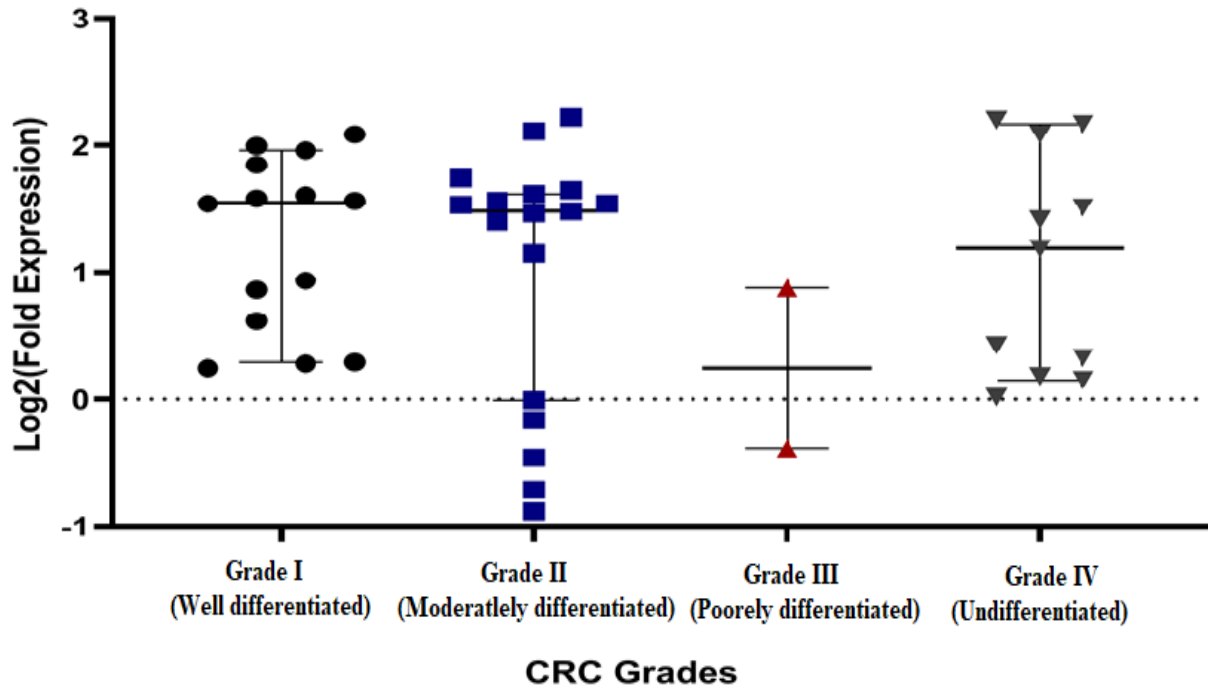
Fig. 2. The COX-2 expression in different Grades of CRC; The COX-2 expression is not significantly difference between different grades of cell differentiation.

The association of COX-2 with some other malignancy factors including lymphatic invasion, vascular invasion, and the statue of patients examined, even though there was a real significant difference between the tumor and non-tumor samples but there weren't any between the lymph-vascular invasion and COX-2 expression; besides the lymphatic extension positive represents lower expression compared to the non-invasive. However, COX-2 almost overexpressed in the invasive perineural and deceased groups related to the non-invasive and alive (Table 2).

Table 2. The COX-2 mRNA expression and its association with different malignancy characterize.

Name of groups		No	Mean ± SD	Median	p-value
General	Case	45	1.088±0.878	1.466	<0.0001*
	Control	45	-1.1e-10±0.67	-0.029	
Stages	Stage 0	3	0.274±0.1397	0.2461	0.192
	Stage I	7	1.148±0.8957	1.416	
	Stage II	9	1.284±0.9771	1.531	
	Stage III	11	1.203±0.6964	1.466	
	Stage IV	3	1.783±0.2631	1.646	
Grades	Low Grades	31	1.118±0.876	1.531	0.464
	High Grades	13	0.9353±0.888	0.876	
Vascular invasion	Positive	23	1.185±0.798	1.561	0.844
	Negative	17	1.215±0.781	1.416	
Lymphatic invasion	Positive (NOS)	21	1.365±0.693	1.581	0.117
	Positive (Extensive)	9	0.811±0.747	0.4261	
	Negative	8	1.242±0.844	1.434	
Perineural invasion	Positive	6	1.54±0.98	0.46	0.086
	Negative	34	1.043±0.84	0.27	
Extracapsular node extension	Positive	4	1.63±0.736	1.85	0.13
	Negative	35	1.079±0.824	1.40	
Statue of patients	Alive	34	1.087±0.84	1.434	0.170
	Deceased	9	1.508±0.499	1.581	

PTGS2 expression Multi-Omics and bioinformatics analysis

Based on the analysis of TCGA-COAD and READ versus TCGA-solid normal tissues, the *PTGS2* expression of primary tumor samples have not any significant differences with solid normal tissues (Fig. 3). Though among 103 rectum samples the solid normal tissue (Log_2 norm-count+1= 9.75) represented higher (non-significant) *PTGS2* expression compares to the primary tumor (Log_2 norm-count+1= 8.07), but it is the opposite in 331 colon samples (Solid-normal Log_2 norm-count+1= 7.54; Primary-tumor Log_2 norm-count+1= 8.09).
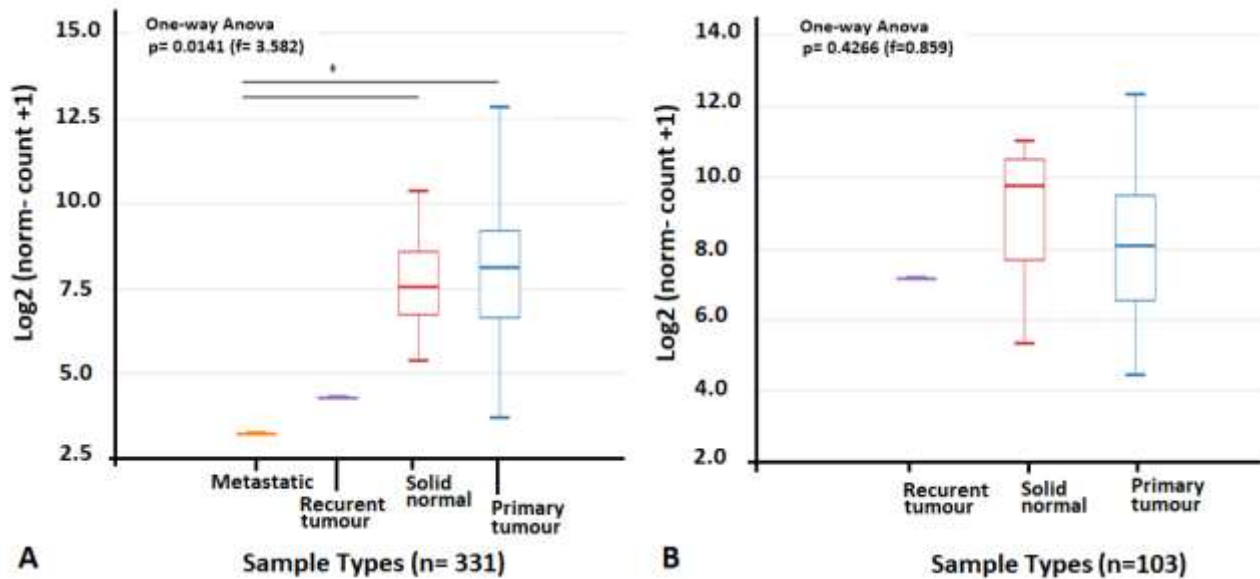

Fig. 3. The *PTGS2* expression comparison of TCGA-COAD, -READ and normal solid tissues. A) *PTGS2* expression of TCGA-COAD-primary tumor and TCGA-Solid Normal Tissues were not any significant differences; the metastatic sample (n= 1) showed significant lower expression compare to the other 2 groups. B) *PTGS2* expression of TCGA-READ and TCGA-Solid Normal Tissues does not show significant differences.

The correlation of *PTGS2* expression with some pathological features evaluated. In here, we specifically focused on metastasis and lymphatic invasion as two invasive factors. There was not any significant correlation between *PTGS2* expression and invasive-pathological features (Fig. 4A-B). But READ datasets presented significant differences of *PTGS2* expression among samples with the history of colon cancer compared to without it (Fig. 4C), while there is no association between history-of-colon-cancer with *PTGS2* level among COAD samples.

Discussion

The COX-2 is an inducible type of its family and its expression is mostly limited to the central nervous system, and women reproduction system while its overexpression in the tumor environment exhibited in different studies (14, 15, 29). On the other hand, there is the PGE2 as the main product of COX-2, which is the cause of inflammation and several aspects of malignancies such as cell proliferation, angiogenesis, migration, and invasiveness. Also, inflammation itself can ignite the beginning of the adenocarcinoma and the carcinogenesis process. As we can see in previous studies removing of COX-2 is reducing the number of polyps in $APC^{\Delta 716}$ epigenetic mice models (30). In some of the studies on patient samples, the relation of COX-2 protein overexpression with tumor recurrence, invasion, and metastatic proved, while some others have not shown these correlations in protein or mRNA (10, 24, 31, 32).

Based on the results of this study on patient samples, we can see the very significant association of COX-2 overexpression with the tumorigenesis by the fold expression of 2.25. The overexpression of *PTGS2* mRNA is obvious in malignant tissues compared to normal tissues
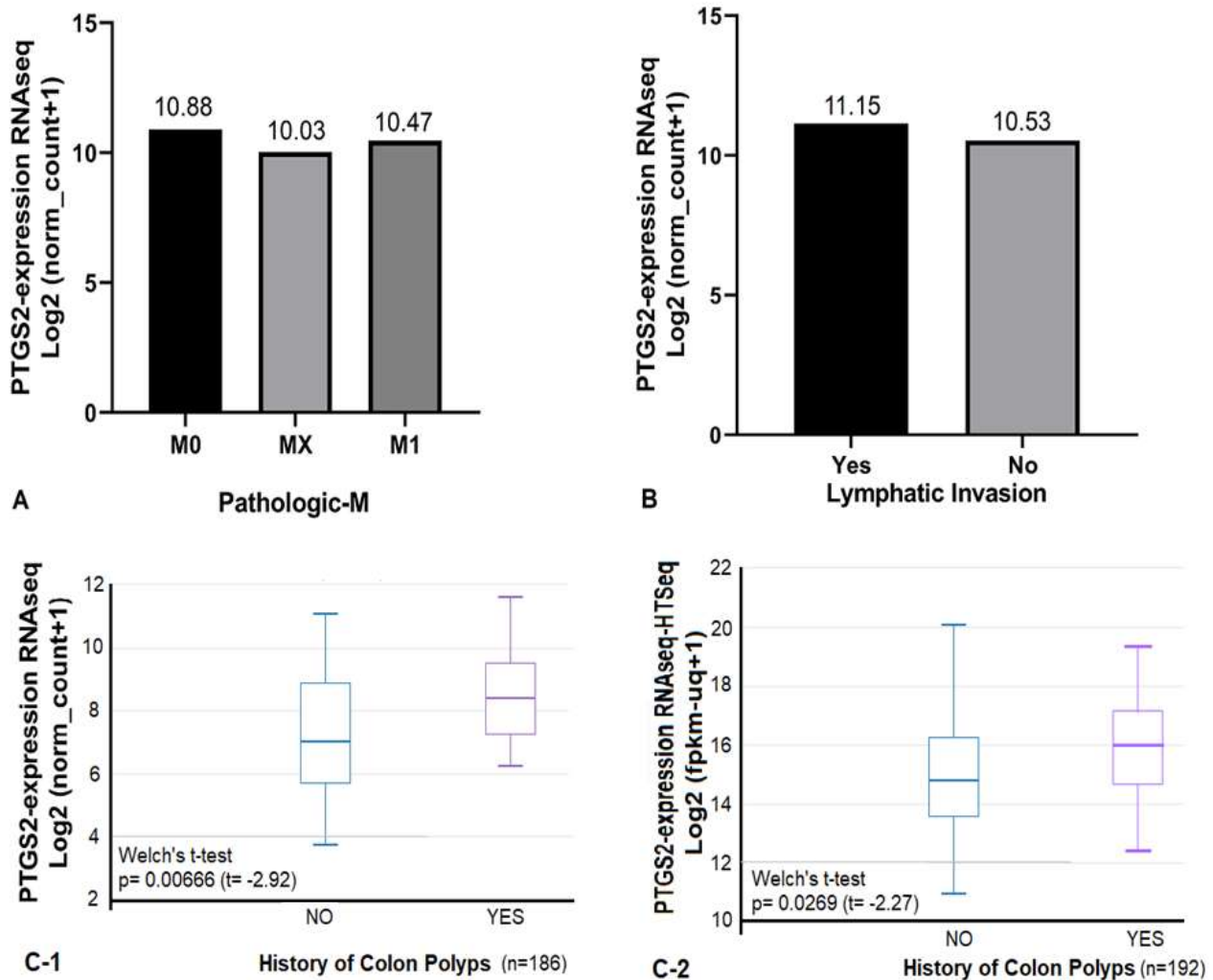
Fig. 4. Correlation of *PTGS2* expression with pathological features in TCGA-COAD and –READ. A) Among 1519 samples, the *PTGS2* expression did not represent any significant correlation with Pathologic-M feature. B) The *PTGS2* expression in lymphatic invasive group is close to the non-invasive one. C) The TCGA-READ datasets showed significant higher *PTGS2* expression in group with the history of colon polyps.

which belonged to the same participants, but in between the invasive and non-invasive groups, it is not significant. So, these results are related to starting of tumorigenesis rather than angiogenesis and invasion. On the other hand, the analysis of TCGA colon and rectum datasets did not show significant differences in *PTGS2* expression, not as an invasive factor and not as a tumorigenesis one. But among the TCGA-READ samples that have history of colon polyps, the expression of *PTGS2* was significantly higher. Therefore, in this study, the results are more met with the inflammatory impact of *PTGS2* at the beginning of CRC, and several analysis by the current study make it clear that *PTGS2* overexpression has no

participation in invasion and metastasis of CRC in patient samples. Also, there is no difference among the non-differentiated group and well-differentiated ones, and the TCGA data analysis did not show any differences between metastatic groups and M0. When we are looking at the results of *PTGS2* expression in the deceased group, the average of *PTGS2* expression is higher than the alive group though it is not significant either.

The results of this study on patient samples are following the previous studies on COX-2 mRNA expression in another population and the TCGA data analysis that was done before but our results on TCGA datasets are different from the previous

study (1,32,33). Though the other study on adenocarcinoma in Iranian patients, the COX-2 protein expression showed increasing as same as our results; but they observed a significant relationship between COX-2 expression and lymph node invasion unlike our study at both level of patient samples and TCGA analysis (31). In the other study, COX-2 expression was assessed based on semi-quantitative method while, our study was quantitative base. In the present study, the assessment was done in mRNA level, but the aforementioned study was on the protein which can be another reason for the contrary on the results. Also, in this study, there are no differences in the COX-2 expression among the 4 Grades. Hence, the loss of cell differentiation that is one of the main factors for further development of the tumor is not related to the COX-2 expression based on the current study.

In general, there are conflicting results in the matter of COX-2 association with the clinicopathological factors of the patients in CRC. Also, one of the previous studies on cell lines has shown the higher expression of COX-2 on the differentiated carcinoma group same as us. By an overview of various studies on both cell lines and tissues, and the current study, the COX-2 overexpression affects the initial cell growth (10,31-36). Furthermore, based on several studies

the COX-2 inhibitors have a significant effect on cell growth and proliferation with which the result of this study on patient samples is consistent (37,38). Furthermore, we showed that COX-2 overexpression on mRNA level has no role in the further development of CRC.

In conclusion the results of the current study, are approving the highly significant relation of COX-2 mRNA expression and occurrence of CRC, and there is up-regulation in tumor tissues for COX-2 in Iranian populations. The TCGA-COAD and –READ analysis showed no overexpression related to CRC. But the *PTGS2* overexpressed in TCGA-READ samples with a history of colon polyps that matched with COX-2 inflammatory effect. Therefore, it is a highly potent candidate as a CRC biomarker at early grades of tumorigenesis among Iranian patients, but the new finding is that it doesn't affect the further development of invasive features of tumors based on both steps of this study.

Acknowledgements

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