Gene expression profiling demonstrates WNT/β-catenin pathway genes alteration in Mexican patients with colorectal cancer and diabetes mellitus

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Summary

Purpose: Several studies have shown a strong association between diabetes mellitus (DM) and increased risk of colorectal cancer (CRC). The fundamental mechanisms that support this association are not entirely understood; however, it is believed that hyperinsulinemia and hyperglycemia may be involved. Some proposed mechanisms include upregulation of mitogenic signaling pathways like MAPK, PI3K, mTOR, and WNT, which are involved in cell proliferation, growth, and cancer cell survival. The purpose of this study was to evaluate the gene expression profile and identify differently expressed genes involved in mitogenic pathways in CRC patients with and without DM.

Methods: In this study, microarray analysis of gene expression followed by quantitative PCR (qPCR) was performed in cancer tissue from CRC patients with and without DM to identify the gene expression profiles and validate the differently expressed genes.

Results: Among the study groups, some differently expressed genes were identified. However, when bioinformatics clustering tools were used, a significant modulation of genes involved in the WNT pathway was evident. Therefore, we focused on genes participating in this pathway, such as WNT3A, LRP6, TCF7L2, and FRA-1. Validation of the expression levels of those genes by qPCR showed that CRC patients without type 2 diabetes mellitus (T2DM) expressed significantly more WNT3Ay LRP6, but less TCF7L2 and FRA-1 compared to controls, while in CRC patients with DM the expression levels of WNT3A, LRP6, TCF7L2, and FRA-1 were significantly higher compared to controls.

Conclusions: Our results suggest that WNT/β-catenin pathway is upregulated in patients with CRC and DM, demonstrating its importance and involvement in both pathologies.

Key words: colorectal cancer, diabetes mellitus, gene expression, microarrays, Wnt/β-catenin

Introduction

DM is a metabolic disorder of carbohydrates, fats, and proteins, caused by defects in secretion or function of insulin and characterized by chronic hyperglycemia [1,2]. DM is a major health problem worldwide; its prevalence in 2014 was 8.5% and caused 1.5 million deaths [3] due to renal failure, cardiovascular events, cerebrovascular disease and cancer [4,5]. Several epidemiological studies

have shown a strong association between DM and increased risk for certain cancer types, including CRC [5-7].

CRC is among the most common digestive system neoplasms worldwide (third in men and second in women). Moreover, it is the fourth leading cause of death in men and the third in women [8]. CRC shares some etiologic factors with DM such as obesity, sedentary lifestyle and western diet. These circumstances led to the hypothesis that there may be a connection between both pathologies, and diabetes has been proposed as a causal agent of CRC [5,9]. Epidemiological studies report an increased risk of 2.1 (95% confidence interval/ CI, 1.82-2.42) for CRC in patients with DM [10].

In patients with DM, chronic hyperinsulinemia probably promotes the initiation and progression of cancer through its mitogenic effect [11-14]. Multiple oncogenic signaling pathways (PI3K, mTOR, MAPK, WNT and IGF-1), activated by insulin, stimulate cancer phenotypes such as inhibition of apoptosis, increased cell proliferation, growth, and survival [9,15,16]. Liu et al. [17] demonstrated that expression of IGF-1 and IGF-1R were significantly higher in patients with CRC and DM in comparison to CRC patients without DM. Also, it has been shown that insulin and IGF-1 promote the nuclear translocation of the β-catenin, an effector of the WNT signaling pathway [18].

The increased glucose demand is a known feature of cancer cells [19]. The augmented glucose flux favors the anabolic metabolism to produce fuel for the tumor growth. Thus, hyperglycemia in DM can ensure a high supply of glucose for inducing an additional stimulation of the neoplastic cell's growth, promoting cell progression, increased proliferation and anti-apoptotic signaling [20,21]. Hyperglycemia has been positively correlated with an increased risk for development gastrointestinal cancers, including CRC [22,23]. One of the mechanisms is enhancing signaling through the WNT/β-catenin pathway, by allowing its nuclear accumulation and transcriptional activation of some target genes [24].

The WNT/β-catenin signaling pathway plays a significant role in the regulation of cell differentiation, proliferation and death [25-27]. Constitutive activation of WNT signaling is present in 40-90% of gastrointestinal cancers, which are the main types of cancer associated with DM [28,29]. Signaling through the WNT/β-catenin pathway also participates in other metabolic pathways, such as in homeostasis and the regulation of genes that encode to hormones as glucagon. So, any alteration in this pathway may also be associated with the development of diseases such as DM [30].

Hence, it is reasonable to assume that the patients with DM exhibit a different expression of some proteins, particularly those related to the WNT/β-catenin pathway, which would explain the increased cancer incidence. In this study, we evaluated the association of DM and CRC at the level of gene expression, focusing principally in alterations of components of the WNT/β-catenin pathway.

Methods

Study subjects and tissue samples

Patient information was obtained from the histopathology files of the Pathology Unit, Centro Médico Nacional de Occidente of IMSS, Guadalajara, México and from the Pathological Anatomy Service of the Hospital Universitario "Dr. José E. González", Universidad Autónoma de Nuevo León, México. Formalin-fixed and paraffin-embedded (FFPE) colorectal tissue samples were obtained from 10 patients with CRC and type 2 DM, 10 CRC patients without type 2 DM, and 10 individuals without CRC or type 2 DM (control group). The type 2 DM diagnosis was established according to the American Diabetes Association (ADA) criteria [4]. The CRC was diagnosed according to the tumor-node-metastasis (TNM) classification [31]. The selection criteria for all patients were: unrelated individuals with sporadic CRC and histopathological diagnosis of intestinal adenocarcinoma in stages II and III. Exclusion criteria included: patients with type 1 DM, familial adenomatous polyposis syndrome, hereditary CRC syndromes, and prior treatment with chemotherapy or radiation. The study was approved by the Research and Ethics Committee (R-2008-1305-6) of the Centro de Investigación Biomédica de Occidente, IMSS, and informed consent was obtained from each of the patients included in the study.

RNA isolation and microarrays

Total RNA was extracted from the FFPE CRC tissue samples by using the RNeasy FFPE Kit (QIAgen, México) according to manufacturer's instructions. RNA concentration and purity ratios (OD260/280) were measured with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). RNA quality was assessed using the RNA Integrity Number (RIN) measured by RNA 6000 Pico LabChip Kit on a microcapillary electrophoresis system (Agilent BioAnalyzer 2100, Waldbronn, Germany). cDNA was produced by the cDNA Synthesis System kit (Roche,Mexico) according to manufacturer's instructions. The microarrays were realized with the NimbleGen Human Gene Expression 12x135K Arrays (Roche,México), containing 45,033 genes; these arrays were utilized according to the NimbleGen arrays user´s guide.

Microarrays analysis

Microarrays were analyzed with the DEVA v1.2 (NimbleGen,Roche) and CLC Main Workbench (CLC

Bio,Qiagen) software. The image generated by Nimble-Gen MS 200 Microarray Scanner and the design files were loaded into the DEVA software. Expression values of each gene were calculated and then normalized using Robust Microarray Analysis (RMA). Later, the expression analysis was performed in CLC Main Workbench software. Statistical significance was defined as p value <0.05 and fold change value ±2. Differently expressed genes were analyzed in the KEGG database (Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/).

Quantitative real-time PCR

Genes of the Wnt/β-catenin identified as significantly and differently expressed by microarray analysis were analyzed by quantitative real-time PCR (qPCR) (Table 1). Total RNA (5 µg) was reverse-transcribed to generate first-strand cDNA using Super Script™ First-Strand Synthesis System for RT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The resulting cDNA was further used for qPCR reactions, which were carried out using the FastStart Essential DNA Green Master Kit (Roche Applied Science, Mannheim, Germany) in the LightCycler 96 System, according to the manufacturer's instructions. The expression levels were analyzed by threshold cycle (Ct), as recommended by Schmittgen and Livak [32] with the $2^{-\Delta\Delta Ct}$ method.

Expression values of *GAPDH* gene were used as a reference. The samples were loaded in triplicate and the mean value of the control group was adjusted to 1.

Statistics

Continuous variables were expressed as mean ±standard deviations, and categorical variables were expressed as number (percentage). Statistical difference between groups was evaluated by the Mann-Whitney U test and p value <0.05 was considered statistically significant. Statistical analysis was performed using the SPSS v22 software.

Results

Study population

Table 2 shows the clinical features of the CRC patients with and without DM and the histological characteristics of the tumor. There were no significant differences between groups for age, gender, location, pathological features and TNM stage (p>0.05). Significant differences in the levels of blood glucose were observed in the CRC patients without DM in comparison with the CRC patients with DM.

Table 1. Clinical features of CRC patients with and without T2DM and histological characteristics of the tumor

Characteristics	CRC group n=10	CRC with T2DM group n=10	p value
Clinical characteristics	*n (%)*	*n (%)*	
Age, mean±SD (years)	64.90 ±12.94	65.4 ±8.92	0.2630
Gender, n (%)			1.0000
Male	3 (30)	3 (30)	
Female	7 (70)	7 (70)	
Duration of diabetes (years)			
5-9		4 (40)	
≥10		6 (60)	
Fasting blood glucose, mean±SD (mg/dl)	87.9 ±4.18	188.4 ±24.89	0.0001
Therapies			
Insulin injections		2 (20)	
Oral antidiabetic drugs		8 (80)	
Histological characteristics	*n (%)*	*n (%)*	
Location			0.9120
Ascending colon	3 (30)	3 (30)	
Transverse colon	1 (10)	2 (20)	
Sigmoid colon	3 (30)	2 (20)	
Rectum	3 (30)	3 (30)	
Pathological differentiation			0.1600
Poor	2 (20)	5 (50)	
Moderate	8 (80)	5 (50)	
TNM stage			1.0000
II A, B and C	5 (50)	5 (50)	
III A, B and C	5 (50)	5 (50)	

Microarrays analysis

In the expression analysis by microarrays, some differently expressed genes were identified among the study groups (p value <0.05 and with a fold change value ±2). In the CRC without DM group, 367 sub- and 213 overexpressed genes were identified, while in the CRC with DM group, sub-103 and 110 overexpressed genes were identified. Comparing the CRC patients with DM to the CRC patients without DM, we found 375 sub- and 487 overexpressed genes. The results between groups were also comparing the individuals without CRC nor DM (control group) (data not shown). The differently expressed genes were analyzed through genetic and molecular signaling pathways database, in the context of KEGG biological pathways, showing several relevant genes, sub- or overexpressed found in different signaling pathways like WNT, insulin and MAPK signaling, metabolic and cancer pathways, among others. Interestingly, we observed differences in genes expression that play key roles in the WNT/β-catenin pathway, like *WNT3A, LRP6, TCF7L2,* and *FRA-1* (Figure 1).

Expression levels

A qPCR procedure using SYBR-Green was done to validate the expression changes of *WNT3A, LRP6, TCF7L2,* and FRA-1. Figure 2 shows the results of expression levels of these genes in the different groups analyzed. The expression level of *WNT3A* was significantly higher in both CRC groups (CRC patients with and without DM) compared to the control group (p=0.001), but no between the CRC groups (Figure 2a). The *LRP6* gene showed also higher expression in both CRC groups (p=0.001) compared to the control group, but the expression between the CRC groups was not statistically significant (Figure 2b).

On the other hand, the expression level of *TCF7L2* was significantly higher in the CRC patients with DM compared to the control group (p=0.001); however, significant subexpression was observed in the CRC patients without DM compared to the control group (p=0.001). Additionally, we found substantial differences between the CRC patients with DM and the CRC patients without DM (p=0.001) (Figure 2c). For the *FRA-1* gene, the expression level was significantly higher in the CRC patients with DM compared to the control group (p=0.017), but considerably lower in the CRC patients without DM compared to the control group (p=0.001). We also observed significant differences between the CRC groups (Figure 2d). In Figure 3 we summarized the expression levels of the analyzed genes for the groups of CRC patients (with and without DM).

Discussion

Several studies have linked DM with a variety of cancers, among them remarkably CRC. Some of the molecular mechanisms through which DM could favor CRC development include the upregu-
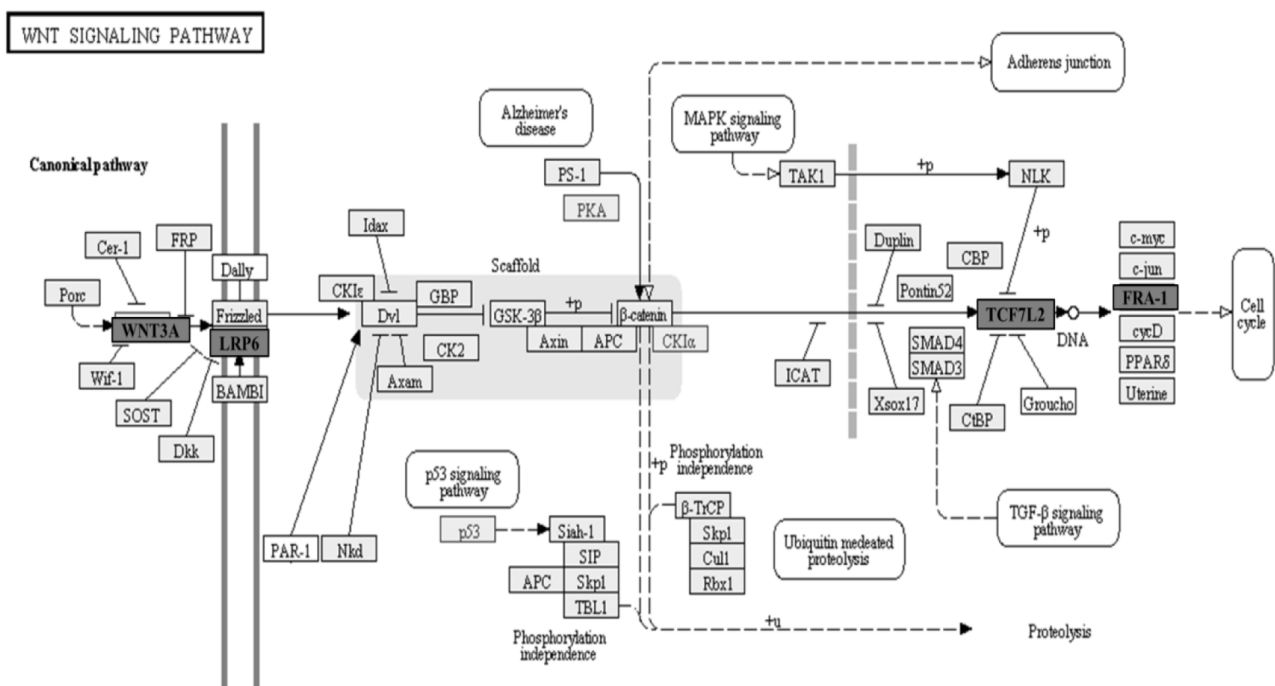

Figure 1. WNT signaling pathway. Schematic representation of participation of WNT3A, LRP6, TCF7L2 and FRA-1 in the WNT signaling pathway (taken and modified from Kyoto Encyclopedia of Genes and Genomes).
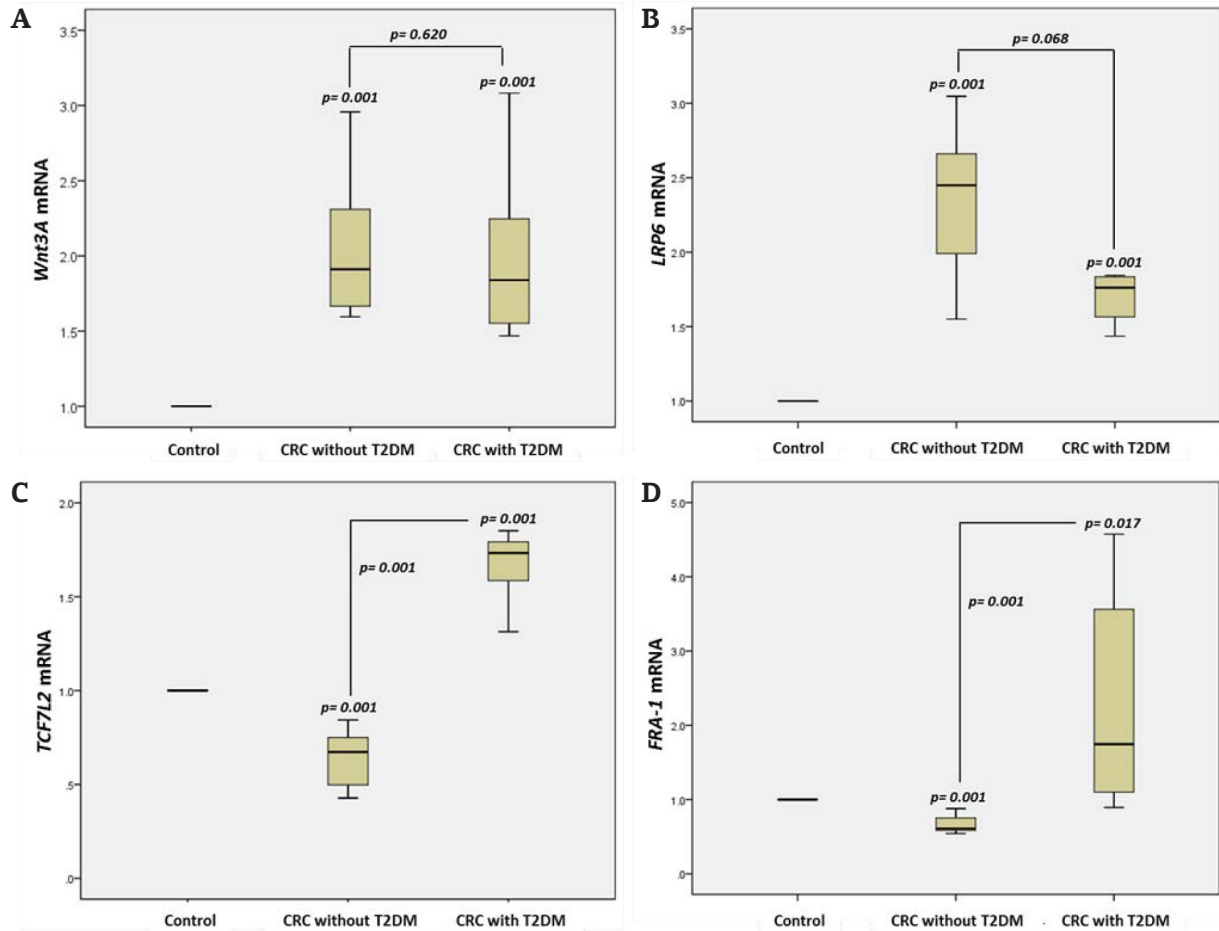
Figure 2. Expression levels of the analyzed genes. Comparative expression of WNT3A, LRP6, TCF7L2 and FRA-1 in CRC patients with or without T2DM and the control group.
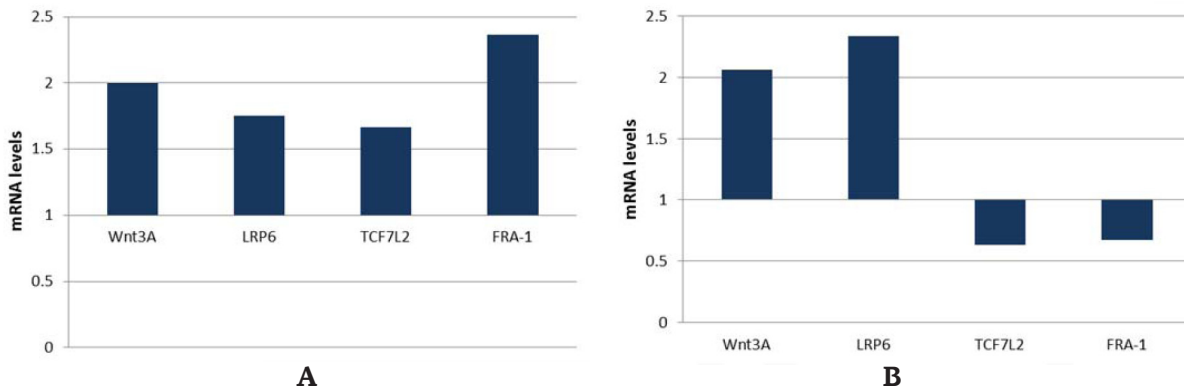

Figure 3. Summary of the expression of the genes analyzed by study group. Overall expression levels of WNT3A, LRP6, TCF7L2 and FRA-1 in CRC patients with and without T2DM. **(A)** CRC patients with T2DM; **(B)** CRC patients without T2DM.

lation of mitogenic signaling pathways activated by insulin and glucose, such as MAPK, PI3K, mTOR, and WNT, which participate in cell proliferation, growth, and survival, and consequently favoring the development of cancer [5,9].

The microarray analysis shows statistically significant differences in the expression of some critical genes of the WNT/β-catenin signaling pathway, like *WNT3A, LRP6, TCF7L2*, and FRA-1, suggesting an involvement of DM and CRC. Currently, it is known that some oncogenes such

as FRA-1, *c-Myc*, and *CCND1* are target genes of WNT/β-catenin pathway; accordingly, the abnormal activation of this pathway can lead to the development of CRC [26]. On the other hand, the WNT/β-catenin pathway also participates in other processes related to homeostasis and regulation of gene expression of some hormones; hence, this pathway has also been associated with metabolic disorders such as DM [16]. Several authors propose that activation of the WNT/β-catenin pathway represents an important link between DM

and risk for developing CRC. This link is related to hyperglycemia and hyperinsulinemia along with insulin resistance (clinical characteristics of DM) which can potentially promote constitutive activation of canonical WNT/β-catenin pathway, inducing cell proliferation and survival [5,24,33].

Some studies have reported that *WNT3A*, a highly conserved ligand in the β-catenin pathway (Figure 1), is associated with the epithelial-mesenchymal transition (EMT) in several cancer cells [34-37]. In the present study we observed a significant overexpression of *WNT3A* in CRC patients with and without DM, when compared with healthy controls. These results suggest that the WNT signaling pathway is being activated by the *WNT3A* ligand in both CRC and DM patients. Similarly, Qi et al. [38] reported that the levels of expression of *WNT3A* were increased in colon cancer tissue, which showed a lower grade of differentiation, with more advanced clinical stages and extensive metastases. They also observed higher levels of vimentin protein, lower levels in E-cadherin protein, and wider distribution of nuclear β-catenin, suggesting that *WNT3A* can induce EMT and increase invasiveness and progression of CRC.

On the other hand, *LRP6* is a member of the low-density lipoprotein receptor (LDLR) family, acting as coreceptor for WNT ligands and inducing intracellular signal transduction through the WNT/β-catenin pathway (Figure 1) [39]. In their study on the 293T cell line Brennan et al. [40] demonstrated that mutations causing a truncated *LRP6* protein could activate the Wnt/β-catenin signaling pathway in a WNT and Frizzled-independent mode, proposing *LRP6* as a candidate oncogene. In our study, the expression level of *LRP6* was significantly increased in both CRC groups compared with healthy controls, suggesting a higher activation of the WNT/β-catenin pathway in these patients. Also, Watanabe et al. [41] comparing the gene expression profile of non-neoplastic rectal mucosa between patients with ulcerative colitis-associated CRC and patients with ulcerative colitis without cancer, they found a significant overexpression of *LRP6* in the CRC group compared to the ulcerative colitis without cancer group.

Similarly, *TCF7L2* (together with β-catenin) acts as the primary effector of the WNT/β-catenin pathway, activating the expression of target genes involved in cell proliferation [41,42]. *TCF7L2* has also been involved in glucose homeostasis [43]. In our study, a significant overexpression of *TCF7L2* in CRC patients with DM, compared with healthy controls was observed, giving evidence of the hyperactivation of the WNT/β-catenin path-

way in these patients. Consistent with our findings, Lysenko et al. [45] quantified the expression levels of *TCF7L2* in pancreatic islets of patients with and without T2DM and observed significant overexpression in the T2DM group compared to the control group. Our results and those reported by Lysenko et al. demonstrate a relationship between DM and *TCF7L2*. On the other hand, the comparison between CRC patients without DM to healthy controls show a significant subexpression of *TCF7L2* in the CRC group. These findings may seem controversial owing to the accepted role of *TCF7L2* in the activation of genes that promote cell growth and proliferation in CRC. Nevertheless, Tang et al. [46] identified *TCF7L2* as a negative regulator of the WNT/β-catenin pathway. Moreover, they demonstrated that the silencing of *TCF7L2* in the DLD-1 and HCT116 CRC lines results in an increased and aberrant activity of the WNT/β-catenin pathway, along with an increased cell growth. These observations suggest that *TCF7L2* may play a tumor suppressor role in CRC [46]. Further studies on the specific functions of *TCF7L2* in CCR and its relationship with DM are required.

Finally, the *FRA-1* gene is a target of the Wnt/β-catenin pathway, activated by *TCF7L2*. *FRA-1* is considered an oncogene and a potent regulator of migration and invasiveness in a variety of tumor cells [47,48]. Increased expression of *FRA-1* in CRC patients has been previously reported congruently [49]; in this study, we observed overexpression of *FRA-1* in the CRC with DM patients, compared with the healthy controls; such results were expected due to increased expression of *TCF7L2* in this group. On the other hand, our results show a significant subexpression of *FRA-1* in CRC patients without DM compared to the healthy controls. Although apparently different, the diminished expression of *FRA-1* seems to be a direct consequence of the decreased levels of *TCF7L2*. The results of the *TCF7L2* and *FRA-1* expression in patients with CRC and DM suggest that both genes play different roles in both pathologies.

The overexpression of *WNT3A, LRP6, TCF7L2*, and *FRA-1* observed in the group of CRC patients with DM, is explained by hyperactivation of the WNT/β-catenin pathway. On the other hand, the results found in the CRC patients without DM (overexpression only for *WNT3A* and *LRP6*, and subexpression for *TCF7L2* and FRA-1), can be explained by the hypothetical model of activation of the WNT/β-catenin caused by high glucose concentrations, proposed by Chocarro-Calvo et al. These authors found that in different cell lines, including CRC (HT-29), in the absence of the

WNT ligand, β-catenin is located near to the cell membrane but not into the nucleus. Likewise, the WNT pathway target genes were found bound to its corepressor complex, silencing their expression. After the WNT stimulation, destruction of β-catenin was inhibited, with consequent accumulation in the cytoplasm but not inclusion into the nucleus. On the other hand, in these same cell lines, the presence of high glucose concentrations enhanced the WNT/β-catenin pathway, allowing nuclear accumulation of β-catenin and inducing transcriptional activation of the target genes of this pathway [24].

In our patients, a similar mechanism could be occurring. Thus, in CRC patients without DM we observed overexpression of *WNT3A* and *LRP6* but not of *TCF7L2* or FRA-1, suggesting that the activation of *WNT3A* through the *LRP6* co-receptor favors the cytoplasmic accumulation of β-catenin, but not its accumulation in nucleus; consequently, there is no transcriptional activation of target genes, demonstrated by the subexpression of FRA-1. The high concentrations of blood glucose, present in the CRC patients with DM, would be the additional stimuli required for nuclear accumulation of β-catenin and transcriptional activation of target genes in these patients.

Conclusions

To the best of our knowledge, this is the first study to evaluate the gene expression profile quantitatively in cancer tissues of CRC patients, with and without DM. The results show overexpression of the *WNT3A*, *LRP6*, *TCF7L2* and *FRA-1* genes in CRC patients with DM, demonstrating that the Wnt/β-catenin pathway is upregulated. However, additional studies with a large sample size and wider gene expression analysis in this pathway are needed to accumulate evidence regarding the importance of DM in the CRC carcinogenesis.

Acknowledgements

Conflict of interests

The authors declare no confict of interests.

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