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Original article

Immunohistochemistry expression of TCF4 protein on carcinoma, adenoma and non neoplastic colorectal mucosa☆

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ABSTRACT

Purpose: To detect and quantify the immunoreactivity of TCF4 protein in colorectal carcinoma, colorectal adenoma and non-neoplasic colorectal epithelium.
Methods: We studied 129 individuals: 40 with colorectal cancer, 52 with colorectal adenoma and 37 with non-neoplastic colorectal epithelium. The colorectal adenoma and carcinoma samples were obtained from patients who underwent surgical procedures, and colonoscopies and samples of non-neoplastic colorectal epithelium were taken from patients who died from cardiovascular diseases, without diseases of the large intestine. Samples of different tissues were included in paraffin blocks, and the immunohistochemical expression of protein TCF4 was analyzed using the technique of tissue microarray (TMA) with polyclonal antibody TCF4. The immunoreactivity was analyzed and classified as positive and negative.
Results: The immunohistochemical expression of TCF4 protein was significantly higher ($p < 0.01$) in colorectal carcinoma than in the non-neoplastic colorectal epithelium and adenoma. There was no difference ($p = 0.76$) between TCF4 protein immunohistochemical expression in colorectal adenoma and non-neoplastic colorectal tissue.
Conclusions: TCF4 protein showed a more intense expression in colorectal carcinoma than in non-neoplastic colorectal epithelium and adenoma, indicating that this protein is involved in colorectal carcinogenesis.

☆ *This study was conducted at Hospital Federal dos Servidores do Estado do Rio de Janeiro.*

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Expressão imuno-histoquímica da proteína TCF4 no carcinoma, no adenoma e na mucosa não neoplásica colorretal

Palavras-chave:
Neoplasias colorretais
Fatores de transcrição TCF
Proteínas Wnt
Imuno-histoquímica

RESUMO

Objetivos: Detectar e quantificar a imunoexpressão da proteína TCF4 no carcinoma e no adenoma colorretal e no epitélio colorretal não neoplásico.
Método: Foram estudados 129 indivíduos: 40 com carcinoma colorretal, 52 com adenoma colorretal e 37 com epitélio colorretal não neoplásico. Os tecidos de adenoma e carcinoma colorretais foram representados por amostras da lesão retirada de doentes submetidos a procedimentos cirúrgicos e colonoscópicos, e as amostras de epitélio colorretal não neoplásico foram retiradas de doentes falecidos por afecções cardiovasculares e sem comprometimento do intestino grosso. As amostras dos diferentes tecidos foram incluídas em blocos de parafina e submetidas ao estudo da imunoexpressão da proteína TCF4 pela técnica do tissue microarray (TMA) com o anticorpo policlonal anti-TCF4. A imunorreatividade foi analisada e classificada como positiva e negativa.
Resultados: A imunoexpressão da proteína TCF4 foi significantemente maior ($p < 0,01$) no carcinoma colorretal do que nos adenomas e no epitélio colorretal não doente. Não houve diferença significante ($p = 0,76$) entre a imunoexpressão da proteína TCF4 no adenoma colorretal e no epitélio colorretal não doente.
Conclusão: A maior expressão da proteína TCF4 no carcinoma colorretal em relação ao adenoma e ao epitélio não doente sugere que esta proteína possui participação na carcinogênese colorretal.

Introduction

With the increase in the average age of the world population in recent years, an increase of causes of death by neoplastic diseases was observed. Among these diseases, colorectal cancer (CRC) become prominent, especially in the western hemisphere population. About 50% of individuals will develop colorectal adenoma until the age of 70, and one in ten adenomas will progress to carcinoma.[1] In Brazil, 30,140 new cases of CRC will emerge, according to estimates: 14,180 in men and 15,960 in women, and half of these people will die from the disease.[2]

The sequence non-neoplastic colorectal tissue-adenoma-carcinoma was first described by Fearon and Volgeistein,[3] in 1978, and prompted studies to determine the genetic and epigenetic alterations of this transformation. However, the molecular changes involved in this process are still not fully understood.

The Wnt signalling pathway plays an important role in the development of the human embryo, participating in the formation and homeostasis of organs and tissues. The improper functioning of this pathway participates in the carcinogenesis of human tissues, including colorectal tissue.[4] When the Wnt pathway is activated, in most cases by mutations of proteins members of the pathway itself, the usual result is an increase of the transcription of genes important for growth, proliferation, differentiation, apoptosis, genetic stability, migration and angiogenesis.[5] About 200 genes that are influenced by this pathway, including genes c-myc, cyclin D1,[6] VEGF (vascular endothelial growth factor) and endothelin, were described.

The TCF/LEF family controls a great number of genes activated by Wnt. In this family, TCF4 gene is the component most commonly expressed in human colorectal tissue and is responsible for the activation of several genes linked to colorectal carcinogenesis, acting either in the more early stages (transformation of normal colorectal tissue to adenoma) as in later phases (distant metastases).[7,8] Recently, studies consistently showed evidence of the influence of this gene in colorectal carcinogenesis. This gene exhibits a mutation 2.8 times more frequent in the CRC than in non-neoplastic colorectal tissue.[9] Another study showed that when overexpression of anti-TCF-4 occurred, colorectal tumours did not present a satisfactory response to neoadjuvant therapy,[10] and when TCF4 is inhibited using interference RNA, an increased sensitivity of CRC to treatment occurs.[11]

Because of this evidence, it becomes important to define patterns of expression of this protein in various types of colorectal tissue through a simpler, cheaper and more affordable method.

The aim of this study is the detection, by immunohistochemical method, of TCF4 protein, a member of the Wnt signalling pathway in colorectal adenoma and carcinoma and in non-neoplastic colorectal epithelium.

Methods

All patients agreed to participate in the project by signing the Statement of Informed Consent. The study was approved by the Ethics Committee in Research of UNIFESP and HFSE-RJ, as protocol: CEP 000 450.

This is a retrospective study conducted at the Serviço de Coloproctologia, Hospital Federal dos Servidores do Estado do Rio de Janeiro (HFSE), in which 163 paraffin blocks were analyzed in three groups: group 1 (carcinoma) with 46 blocks of patients with CRC operated, group 2 (adenoma) with 67 blocks of patients who underwent endoscopic or surgical polypectomy, and group 3 (control) with 50 blocks of patients who died from heart disease and without indicative picture of digestive disease, and necropsied at the Serviço de Verificação de Óbitos do Município de São Paulo (SVO - Universidade de São Paulo - USP).

For the formation of groups 1 and 2, patients treated in HFSE-RJ and who underwent colonoscopy or surgical procedures and were in outpatient follow-up during the study period were included.

Subjects under 18 years of age, patients with inflammatory bowel disease or with non-adenomatous polyps and those who refused to participate in the study were excluded; 13 of 50 samples from non-neoplastic colorectal tissue were excluded due to non-recognition of the colorectal mucosa or due to autolysis found in these samples; of 67 cases of colorectal adenomas, 15 were excluded due to the observation of only hyperplasic epithelium; and, finally, of 46 carcinoma blocks 6 were excluded, due to samples that, on analysis, did not include areas with carcinomatous transformation.

From the paraffin blocks obtained by the procedures above, blocks of *tissue micro array* (TMA) were prepared. The preparation of TMA blocks followed the technique described by Pires et al.[12] Three blocks were prepared: the first with the cases of colorectal carcinoma, the second with colorectal adenomas and the third block with non-neoplastic colorectal tissue.

For the immunohistochemistry method, histological sections of 3 mm thick were obtained; they were deposited on silanized slides and subsequently treated by streptavidin-biotin method.

Incubation with primary antibody was performed in a moist chamber at 4°C for a minimum of 16-18 hours (overnight), at a 1:10 dilution. The primary antibody used was polyclonal TCF-4 (NBP1-88633) obtained from rabbit (Novus Biologicals, Littleton, CO, USA).

The immunoreactivity of TCF4 protein was analyzed and classified as positive or negative.

The following data were recorded: clinical features of sample (age and gender) for all groups of individuals. In the group of patients with colorectal adenoma, the morphological characteristics of the lesion (location, size of the longer axis, histological type, degree of cellular atypia) were recorded. In the group of patients with colorectal carcinoma, macroscopic lesion characteristics (location, appearance, size of the longer axis), microscopic features (lymph node involvement, grade of cellular differentiation, lymphatic, venous and neural infiltration), TNM classification (UICC, 2010),[13] presence of synchronous metastases and tissue immunoexpression of the antibodies used (negative or positive) were registered.

Statistical analysis of the quantitative results was reported as mean and standard deviation. The qualitative data were described as frequency. To analyze categorical variables, the Fisher exact test or the chi-square test was used; and for continuous variables, the Student's *t* test was used, after testing for its normality.

The level of significance was set at 5% ($p < 0.05$) in all tests.

Results

This is a retrospective study in which three groups of subjects were studied, of which data of two groups were collected at HFSE-RJ, and data of the third group at SVO-USP. In both institutions the data were collected sequentially, with no attempt to matching among the groups with disease and the control group. No significant differences between groups in relation to clinical characteristics were found (Fig. 1).

Fifty-two subjects with colorectal adenoma were studied. These patients were classified according to location, size of the longest axis, histological type, degree of cellular atypia and TCF4 protein expression of colorectal adenoma. Most of the cases studied were of adenomas with < 10 mm and with low-grade cellular atypia and tubular shape (Fig. 2).

Expression of TCF4 protein in 7 (13%) cases was observed. In adenoma subtypes larger than 30 mm, with severe atypia and villous histology, higher percentage of expression of TCF4 protein (42.9%, 25% and 33.3%, respectively) was noted, but the significance of this difference could not be demonstrated (Fig. 3).

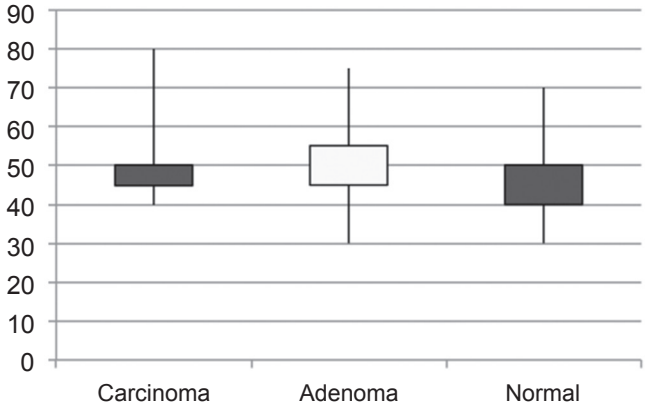

Fig. 1 – Mean age of subjects, according to the group: carcinoma, adenoma and non-neoplastic colorectal tissue.

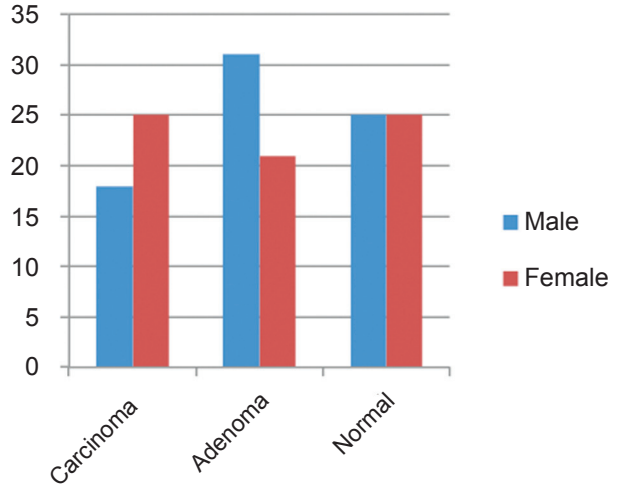

Fig. 2 – Distribution by gender of subjects according to the group: adenoma, carcinoma and non-neoplastic colorectal tissue.

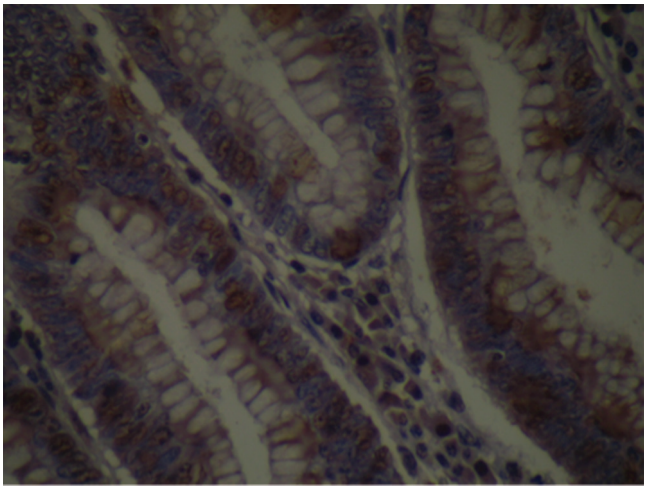

Fig. 3 – Photomicrograph of colorectal adenoma with positive immunoexpression for the TCF4 antibody, represented by a brownish colour in the cell nucleus (immunohistochemistry ×200) (photo by the author).

Of the 40 cases of patients with colorectal carcinoma, most were located in the rectum, classified as II and III stages, greater than 50 mm, moderately differentiated, without vascular compromise and without metastases.

There was expression of TCF4 protein in 23 (57%) of subjects with colorectal carcinoma. The expression of TCF4 protein was compared, without significant difference with respect to gender ($p = 0.52$), age ($p = 0.80$), size ($p = 0.20$), grade of differentiation ($p = 1.0$), presence of metastasis ($p = 1.0$), vascular invasion ($p = 1.0$) and location ($p = 0.80$). The mean age of patients with carcinoma who expressed TCF4 protein was 65.5 ± 11.9 years; and of those who did not express the protein, the mean age was 66.4 ± 8.4 years, without significant difference between these values (Fig. 4).

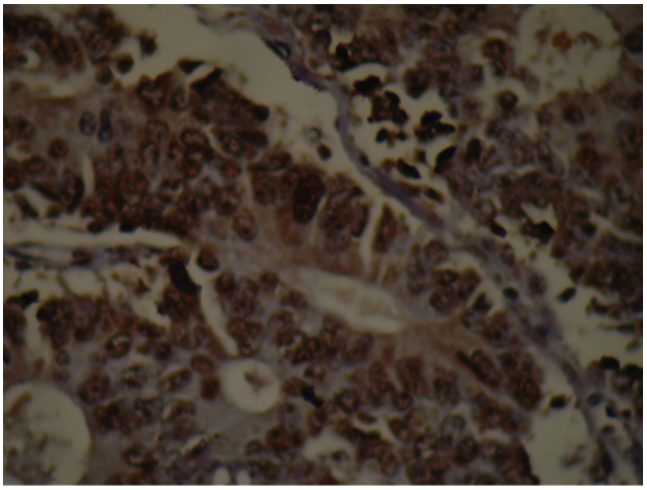

Fig. 4 – Photomicrograph of colorectal adenoma with positive immunoexpression for the TCF4 antibody, represented by a brownish colour in the cell nucleus (immunohistochemistry ×200) (photo by the author).

The analysis of non-neoplastic colorectal tissues showed expression of the protein in 6 (16%) cases (Fig. 5).

We compared the expression of TCF4 protein between the group of patients with non-neoplastic colorectal tissue and the group of patients with colorectal adenoma. This comparison showed no significant difference ($p = 0.76$) between the two groups.

The comparison of the group with colorectal carcinoma versus colorectal adenoma and versus non-neoplastic colorectal tissue showed significant difference ($p < 0.01$) (Table 1).

Discussion

Genes stimulated by β-catenin/TCF4 complex act both in carcinogenesis as in the progression and formation of metastasis of CRC.[8] In this series, a significant difference ($p < 0.01$) was observed between TCF4 protein immunoexpression in colorectal adenomas and carcinomas, suggesting a role of this protein in carcinogenesis. No significant difference was found between non-neoplastic colorectal tissue and colorectal adenoma. This finding indicates that the activity of this protein may occur in later stages of colorectal carcinogenesis.

To evaluate the role of TCF4 protein in the progression and formation of metastasis of CRC, we compared, in the cases of colorectal carcinoma studied, the expression of TCF4 protein in relation to the diameter, depth of invasion of the intestinal wall, vascular, lymph nodal and neural invasion, degree of dif-

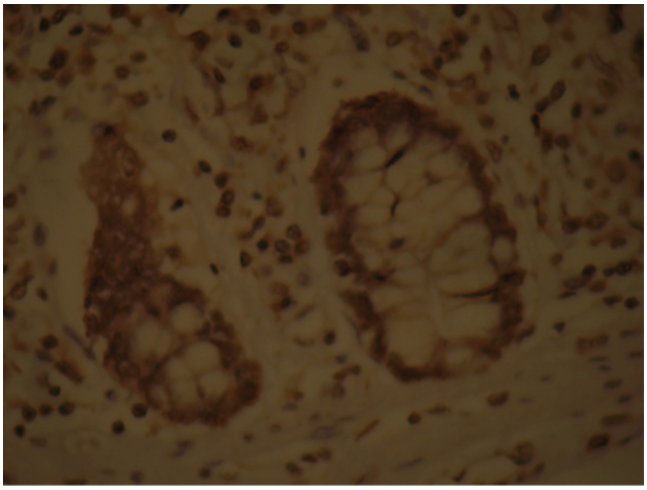

Fig. 5 – Photomicrograph of colorectal adenoma with positive immunoexpression for TCF4 antibody, represented by a brownish colour in cell nucleus (immunohistochemistry × 200) (photo by the author).

Table 1 – Comparison of the expression of TCF4 protein in colorectal carcinoma, colorectal adenoma and non-neoplastic colorectal tissue.

TCF-4 protein expression	Colorectal carcinoma	Colorectal adenoma	Non-neoplastic colorectal tissue
Negative	17 (42,5%)	45 (86,5%)	31 (83,8%)
Positive	23 (57,5%)	7 (13,5%)	6 (16,2%)

ferentiation, lymph node metastasis, and distant metastasis; in none of them a significant difference was found.

Despite evidence of the participation of TCF4 protein in the homeostasis of normal colorectal tissue and in colorectal carcinogenesis, we found only one trial in literature that compared the expression of TCF4 protein in adenomas and colorectal carcinomas by immunohistochemistry studies.[14] The authors of this trial studied 68 cases of colorectal tumours (19 adenomas, 14 adenomas with intraepithelial neoplasia and 35 carcinomas), and compared these tumours with the mucosa adjacent to the lesion. These authors found a difference between expression of TCF4 protein in the adenoma and in the adjacent normal mucosa, but no difference between the expression in the adenocarcinoma and in the adjacent mucosa. This result may have been obtained by these authors thanks to the comparison made with the mucosa from the same individual and not from individuals with non-neoplastic colon epithelium. Other reasons that may explain this difference may be linked to the smaller number of cases analyzed, as well as the different types of antibodies used, including with different dilutions (1:200).

In the present study, we observed that the non-neoplastic colorectal tissue and the colorectal adenoma expressed TCF4 protein in less than 20% of cases. Although without significant difference, the adenomas with higher probability of malignant transformation (larger ones, with a higher degree of atypia, and of histologic villous type) tended to express more frequently the protein. Possibly due to the high percentage of cases that did not express TCF4 protein among adenomas (> 80%), we were unable to establish this difference. These data suggest that a future study with a larger sample may establish significant differences with respect to the expression of TCF4 protein among tumours with low probability of neoplastic transformation and with high probability of transformation.

Kriegl et al.[15] correlated the survival of patients operated on for colorectal carcinoma with expression of TCF4 protein. They identified the expression of TCF4 protein in 46% of cases, whose survival was worse. In the current series, although the prognosis has not been studied, there was expression of the protein in 57% of cases of carcinoma, a result similar to that obtained by Kriegl et al.[15] Comparing the positivity of immunoexpression of TCF4 protein in relation to depth of invasion of the intestinal wall, lymph node invasion and presence of metastasis – data that may indicate a worse prognosis –, there was no significant difference. Such data can corroborate the fact that this protein is linked more to colorectal carcinogenesis, not participating in the processes considered as occurring in a later period in the progression of neoplasia, for instance, the invasion of adjacent tissue and occurrence of metastasis.

The difference in the expression of TCF4 protein between colorectal adenomas and carcinomas suggests that this protein participates in colorectal carcinogenesis, which is in agreement with the findings of van de Wetering et al.[16] These authors stated that the β-catenin/TCF4 complex is the main regulator of proliferation and differentiation of the non-neoplastic colorectal tissue and in colorectal carcinomas; these authors also showed that the activation of TCF4 target genes, induced by mutations in the Wnt signalling pathway, constitute the transforming event of the normal colorectal mucosa in neoplastic mucosa.

Kendiziorra et al.[33] studied colorectal carcinoma cell lines and found that tumour cells that showed overexpression of TCF4 protein did not respond satisfactorily to radiotherapy. Moreover, the authors reported that the silencing of this overexpression renders these tumour cell lines more radiosensitive. The confirmation of these data by other studies can confirm the clinical importance of evaluating the expression of this protein for planning the treatment of CRC.

Chen et al.[17] published studies on resveratrol, a substance found in plants, which could suppress the Wnt signalling pathway. In vitro analysis showed that this action occurs due to rupture of the β-catenin-TCF4 complex, suggesting that this mechanism of action may be a potential target for therapy of CRC. The reported studies show promise for the use of TCF4 protein as a therapeutic target in CRC. Considering that immunohistochemistry is a cheap and affordable method, it is important to determine patterns of TCF4 protein immunoexpression in non-neoplastic colorectal tissue and in colorectal adenomas and carcinomas.

The results reported in this study may help in the understanding of TCF4 protein activity and Wnt signalling pathway in colorectal carcinogenesis, and thereby increase the prospect to finding new therapeutic targets for drug development for the treatment of colorectal carcinoma.

Conclusions

Under the conditions of this study, the results showed that:

1) The immunoexpression of TCF4 protein in colorectal carcinoma was superior versus in adenoma and non-neoplastic colorectal tissue.
2) There was no difference of immunoexpression of TCF4 protein in adenoma and in non-neoplastic colorectal epithelium.

Conflicts of interest

The authors declare no conflicts of interest.

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