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Sex-determining Region Y-related (SRY) High-mobility Group Box 4 (SOX4) Immunoexpression in Colorectal Cancer as an Unappreciated Parameter for More Individual Approach in Cancer Disease – A Preliminary Study

Katarzyna Joanna Gąsior[1]
https://orcid.org/0000-0001-6626-6141
Aleksandra Hincz[1]
https://orcid.org/0000-0003-2423-3389
Marta Jadwiga Cichoń[1]
https://orcid.org/0000-0002-7864-9112
Paweł Kapszewicz[2]
https://orcid.org/0000-0002-8438-195X
Elżbieta Sałacińska-Łoś[3]
https://orcid.org/0000-0002-3912-7143
Magdalena Orzechowska[4]
https://orcid.org/0000-0002-8301-8059
Katarzyna Taran[1]
https://orcid.org/0000-0002-0598-9079

[1] Department of Pathomorphology, Medical University of Lodz, Poland
[2] Department of Ophthalmology, Mother's Health Memorial Hospital, Lodz, Poland
[3] Department of Oncology and Pediatric Surgery, Medical University of Lodz, Poland
[4] Department of Molecular Carcinogenesis Lodz, Medical University of Lodz, Poland

Address for correspondence
Katarzyna Taran
Laboratory of Isotopic Fractionation in Pathological Processes
Department of Pathomorphology, Chair of Oncology
Medical University of Lodz
251 Pomorska St., 92-213 Lodz, Poland
katarzyna.taran@umed.lodz.pl

Abstract

Background: Colorectal cancer is one of the most important problems that medicine has to face nowadays. Despite the development of new diagnostic tools and therapeutic strategies in oncology, it still takes the leading position regarding cancer related deaths, which proves the presence of undiscovered part of its biology. Therefore, is justified to search for new biomarkers to determine better colorectal cancer biology. Sex-determining region Y-related (SRY) high-mobility group box 4 (SOX4) is currently highly investigated protein which expression was identified in many pathological processes, including malignancies with the highest mortality rates and which seems to be a promising marker related to cancer progression.
Objectives: The aim of the study was to evaluate SOX4 expression level in colorectal cancer cells and search for its potential relation to the estab-

lished prognostic parameters for more individual approach in cancer disease.
Material and methods: Immunohistochemical evaluation of the SOX4 protein expression in colorectal cancer cells supported by statistical analysis of the relation to tumour stage, grade, and presence of lymph node metastases, as well as chosen histoclinical features.
Results: Obtain results showed for the first time complex expression of SOX4 in colorectal cancer. There were revealed differences of SOX4 immunoexpression regarding tumour grade, lymph node status, and tumour ulceration, as well as potential relation between SOX4 expression and patients' age and sex and grade 1 histological malignancy of the tumour.
Conclusions: Obtained results support the role of the SOX4 in colorectal cancer biology and for the first time indicate the relation with the established prognostic factors: tumour grade and lymph node status. The findings revealed in presented studies alight SOX4 expression as a promising parameter for colorectal cancer more individual approach with prospective clinical impact.
Key words: colorectal neoplasms; biomarkers; prognosis; transcription factors

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancy worldwide and also one of the leading causes of cancer related deaths [1]. There are the established histoclinical prognostic parameters for CRC patients as clinical advancement of cancer disease (stage), level of histological malignancy (grade) and lymph node status as well as the other biomarkers with proved utility, first of all carcinoembryonic antigen (CEA) which is related to a like hood of the recurrences of colorectal carcinoma. Another widely used parameter is CA 19-9, which elevated postoperative levels indicate poorer prognosis [2]. Based on the developing knowledge of CRC biology a lot of new treatment methods were discovered and successfully applied in the past few years 5-year survival rate varies from 90% in stage I to around 10% in stage IV and recently reached the plateau [3–5]. Progressive, however still unsatisfactory, res-

ults make a growing need for further studies to reveal new biomarkers for more individual approach in CDC disease.

There are evaluated numerous potential biomarkers, nowadays, mostly nucleic acids (RNA/DNA/messenger RNA/microRNAs), cytokines, antibodies, and various proteins [6].

In the recent years the role of proteins expression in colorectal carcinoma gains in value but the results are not unambiguous. Among these, especially interested seems to be Sex-determining region Y-related (SRY) high-mobility group box 4 (SOX4) protein due to its part in cancer proliferation and growth, already proved in the other cancer including entities with the highest mortality [7–10].

SOX4 is a transcription factor protein encoded by the SOX4 gene, which belongs to SOXC subgroup of the SOX family [11]. It plays an important role in the embryonal development, influence the apoptosis, resulting in the cell death but also can be present in the tumorigenesis [12]. The expression of SOX4 was observed in over 20 types of malignancies, including breast cancer, bladder cancer and prostate cancer [7–10]. Although a lot of researches were performed the exact role of SOX4 in complicated biology of cancer cells is still unknown. The scientific attempts to determine SOX4 influence are highly limited, however they suggest the role of this protein in the proliferation of the cancer cells [13].

The study presents the first complex evaluation of SOX4 expression in relation to the established prognostic parameters (stage, grade, lymph node status) and chosen histoclinical features (patients' age and gender, tumour ulceration) to determine needed future study directions and potential value of SOX4 evaluation for more individual CDC approach.

Material and methods

There were chosen 20 representative colorectal adenocarcinoma cases for the study from the archives of the Department of Pathomorphology, Chair of Oncology Medical University of Lodz, Poland for the presented preliminary studies, according to the agreement of Bioethics Committee of the Medical University of Lodz RNN/351/19/KE. Paraffin embedded

CRC tissues were used for further evaluation – paraffin sections with a thickness of 3–4 micrometres were used to prepare routine slides stained with hematoxylin and eosin (H + E) for routine histological examination as well as and for immunohistochemical tests in the PowerVision detection system by ImmunoLogic according to the immunoperoxidase method, using the primary rabbit polyclonal antibodies directed against SOX4 by Thermo Fisher Scientific.

For immunohistochemical study, paraffin sections, after being placed on adhesive slides and dried in an incubator at 56°C for 1 hour, were subjected to dewaxing in a series consisting of xylenes and alcohols of decreasing concentrations (96%, 80%, 70%, 60%). Endogenous peroxidase activity was then inhibited with a 3% solution of perhydrol in methanol for 5 minutes. In order to restore tissue antigenicity and open the path for antibodies, the following procedure was used: Sections were heated in a Target Retrieval Solution at pH 9.0 DAKO, in a water bath at 95°C for 45 min. After cooling, the sections were washed twice in 0.05 M TRIS buffer (TBS) at pH 7.6 for 5 min. Then they were subjected to an overnight incubation in a refrigerator at 4°C in a humid chamber with appropriately diluted antibodies anti-SOX4 1:20. After incubation, the sections were washed twice in TBS buffer and the ImmunoLogic PowerVision two-step visualization system was used to visualize the antigen-antibody reaction. The first step of the reaction consisted of a 30-minute incubation in the above-mentioned conditions with a polymer labelled with peroxidase and associated with secondary goat antibodies directed against the polyclonal rabbit antibodies used. The last stage of detection was an enzymatic reaction in which a coloured product arised when the substrate for peroxidase – 3,3`-diaminobenzidine tetrachloride (DAB) was used (incubation time with DAB solution – 2 min.). Then, the nuclei were stained with Meyer's hematoxylin (2 min.) and the sections were dehydrated in a series of alcohols of increasing concentrations (70%, 80%, 96%) and passed through a series of acetones and xylenes. Thus prepared, they were embedded in the anhydrous Histokit medium. The negative control of the method were sections in which the primary antibodies were replaced with TBS buffer using the immunohistochemical procedure described hereinafter. Sections showing a previously known

strong positive response to the test antigen were used as positive controls. The microscopic slides were assessed with the use of a light microscope. Histological examination according to the currently used protocols was performed and SOX4 expression was estimated (defined as brown coloration of cancer cell nuclei) and presented as an index for each CRC case. Histoclinical analysis was done regarding patients' related features (age, gender), tumour related features (grade, ulceration), and cancer disease related features (stage, lymph node status, distant metastases, recurrences). The collected immunohistoclinical data were analysed in the R environment (R 4.0.2) with rstatix and ggpubr packages. The normality of distribution was analysed with the Shapiro-Wilk test followed by Levene's test to analyse the homogeneity of variance. The hypotheses were tested by applying a non-parametric signed rank Wilcox test or in the case of normally distributed data the t-test. Comparisons between three groups were performed with a non-parametric Kruskal-Wallis test. Correlations were evaluated with the Spearman coefficient. All of the results were statistically significant if $p<0.05$.

Summary of the study group and details of histoclinical features chosen for the study are presented in Table 1.

Table 1. Characteristic of histoclinical features chosen for the study

Chosen parameter		Characteristic
Patients' related features	Age	Min. 54; Max. 95; Average: 71.95 (years)
	Gender	Male: 14; Female: 5
Tumour related features	Grade	Grade 1: 7 Grade 2: 7 Grade 3: 6
	Ulceration	Present: 7 Absent: 11
Cancer disease related features (stage, lymph node status, distant metastases = 1, recurrences = 0)	Stage	Stage 1: 5 Stage 2: 2 Stage 3: 6 Stage 4: 1
	Metastases in the lymph nodes	Present: 7 Absent: 7

Source: own study.

Results

SOX4 immunoexpression was found in all the examined CRC cases and appeared various with differences in gender, age, stage, grade study subgroups as well as between the tumours with and without ulceration, and with and without metastases in the lymph nodes.

The SOX4 indices appeared as follow:

Among males, the median SOX4 expression was 57.5% (mean=54.5±14.2), whereas among females it was higher and reached 64% (mean=56.7±12.7).

Median index for grade 1 (g1), grade 2 (g2), and grade 3 (g3) tumours was 58% (mean=52.4±17.3), 66% (mean=61.9±6.96), and 58% (mean=52.8±15.5), accordingly (Figure 1).

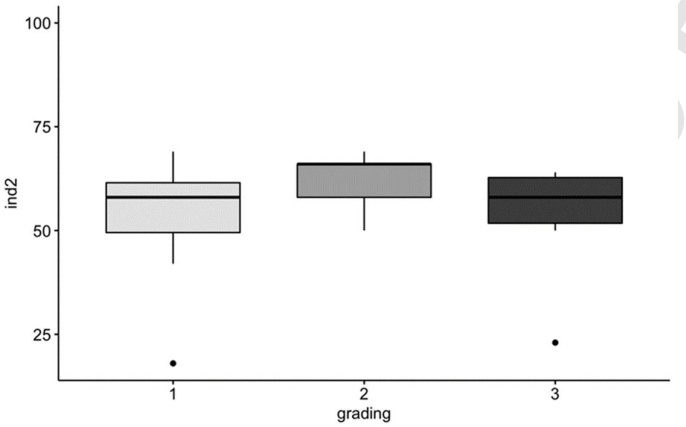

Figure 1. The box-plot presented the values of SOX4 immunoexpression indices (ind2) in tumours with different histological level of malignancy, grade 1, 2, and 3, appropriately (grading)

The median index in the stage 1 group was 57% (mean 53.6±7.64), although in the stage 3 group median index was higher (median=61.5, mean=55.2±17.1) (limited data for the other stages) (Figure 2).

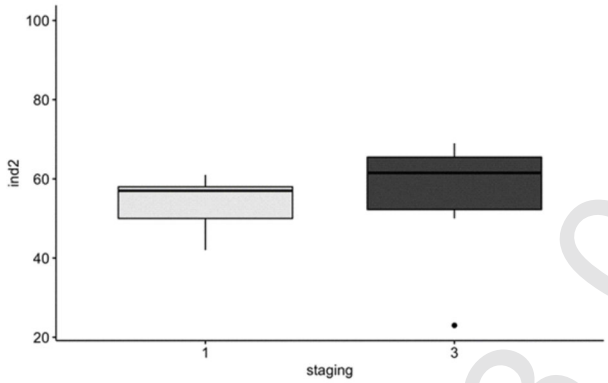

Figure 2. The value difference of SOX4 expression between stages 1 and 3

SOX4 immunoexpression index in CRC cases without lymph node metastases reached 57% (mean=50±15.9), while among patients with present lymph node metastasis achieved 61.5% (mean=60.8±6.91) (Figure 3, Figure 4 and Figure 5).

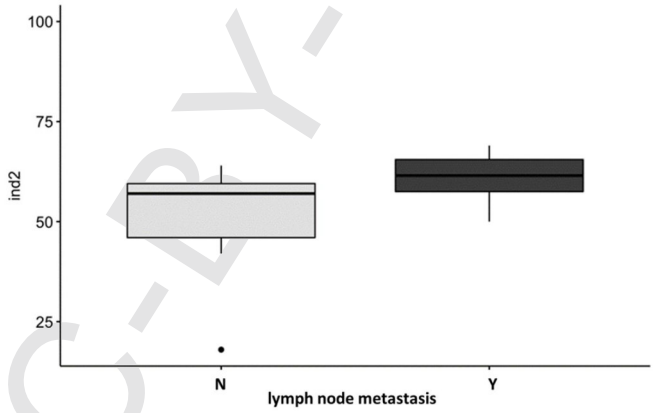

Figure 3. The box-plot presented the values of SOX4 immunoexpression indices (ind2) in tumours with different lymph node status (without metastases – N, and with lymph node metastases – T)

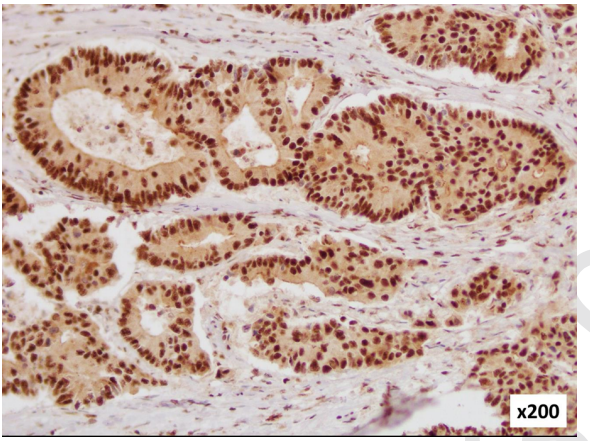

Figure 4. SOX4 immunoexpression in colorectal cancer cells (lymph nodes metastases present), orig. magn. 200x

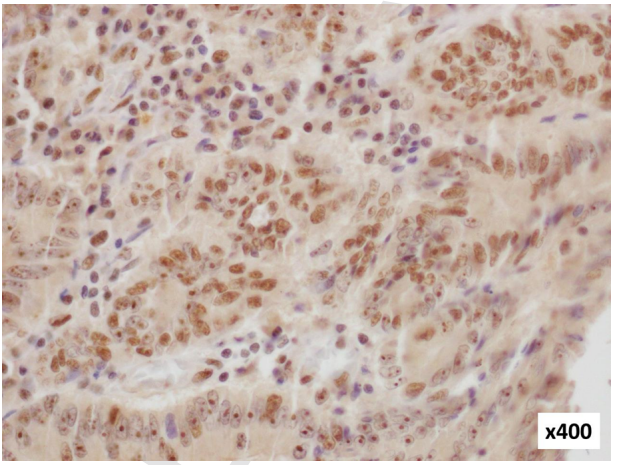

Figure 5. SOX4 immunoexpression in colorectal cancer cells (lymph nodes metastases absent), orig. magn. 400x

SOX4 immunoexpression index in CRC cases without ulceration reached 57% (mean=55.9±8.51), while in cases with ulceration the median index raised to 61% (mean=53.6±20.1) (Figure 6).

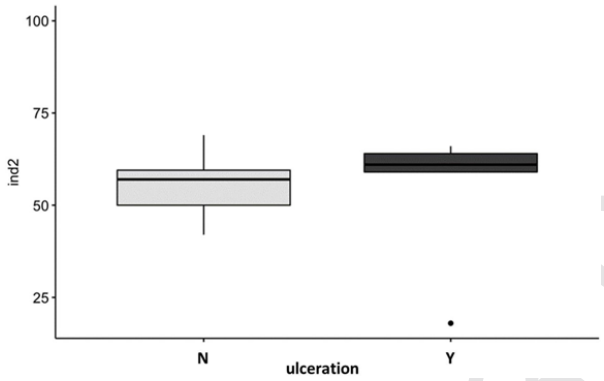

Figure 6. The box-plot presented the values of SOX4 immunoexpression indices (ind2) in tumours without ulceration – N, and with ulceration – T)

Additionally, there was observed a moderate association of the examined SOX4 index and age among females (rho=0.67), and similar correlations between the SOX4 index and patients' age among grade 1 (rho=0.57) and grade 3 tumours (rho=0.41) (Figure 7, 8, 9).

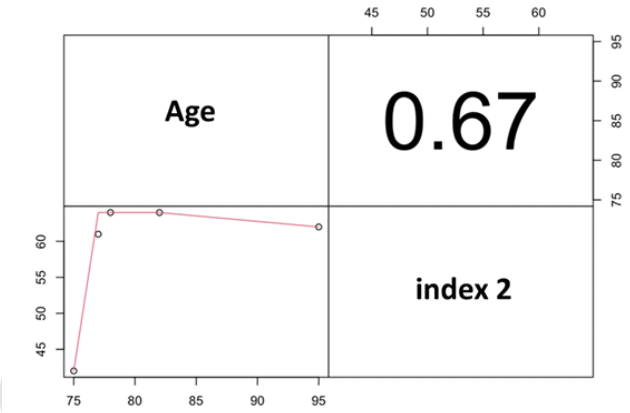

Figure 7. Correlation between SOX4 immunoexpression (index 2) and patients age among females – rho Spearman

Figure 8. Correlation between SOX4 immunoexpression (index 2) and age among grade 1 tumours – rho Spearman

Figure 9. Correlation between SOX4 immunoexpression (index 2) and age among grade 1 tumours – rho Spearman

The observed differences in SOX4 immunoexpresion in gender, age, stage, grade study subgroups and between the tumours with and without ulceration, and with and without metastases in the lymph nodes, have not

appeared to be statistically significant, the most probably they have not achieved the minimum threshold of statistical significance due to the limited studied subgroups, where only huge differences within homogeneous groups may be proven, however they illustrated the complex immunophenotypes of CRC tumours and indicate the direction of future evaluations in more numerous groups.

Discussion

Colorectal adenocarcinoma is associated with high mortality and morbidity worldwide, e.g.: according to the American Cancer Society, about 135,430 people were diagnosed with colon cancer in one year (2014) and that same year, about 50,260 people died from the disease, what perfectly illustrated why CRC is found to be one of the leading medical problems nowadays, regarding the relation between incidence and mortality rates. On the other hand, prognosis for CRC patients has improved in the last several years, and according to the Colorectal Cancer Coalition, the mortality rate for people with colon cancer has decreased by roughly 30 percent from 1991 through 2009.

Despite remarkable progress in currently used treatment modes is still far from expected results and completely personalized strategies. Many factors affect the prognosis in individual cases, these widely established according to the National Cancer Institute, include: stage of cancer disease, tumour grade, lymph node involvement, and the others less understood and able to be analysed and compared as general health status or colon blockage.

Although CRC is highly investigated malignancy, complete understanding of its biology and the same possibilities of prognostication are limited. The molecular features that could potentially become the prognostic and predictive factors are believed to be one of the most important discoveries of the modern medicine. No wonder they appear in many fields of biological sciences and are considered almost equally important as histological or clinical image of the disease. The perfect example of such a molecule being a transcription factor is Sex-determining region Y-related (SRY) high-mobility group box 4 (SOX4). The literature shows

that SOX4 is overexpressed in variety of tumours and suggested that it plays a crucial role in their development and progression [14]. It that has been proved to be associated with tumour progression and poor clinical outcome in several cancers [15]. Numerous malignancies were examined in the context of SOX4 overexpression, in prostate cancer SOX4 expression has been shown to correspond to high Gleason score and the presence of distant metastasis, which is strongly bounded with a poor prognosis in this neoplasm [16]. Its important role was also confirmed in research on the patients with hepatocellular carcinoma and proliferating effect was also evidenced in breast adenocarcinoma [10, 17]. Mostly there was observed and analysed the difference of SOX4 expression between normal and cancerous cells without histoclinical context based on comparison with the normal tissue, to show upregulation of the examined process in cancer [18, 19]. In presented studies there was performed the complex analysis which included the assessment of SOX4 expression in the cancer cells. In presented studies the first attempt was made to establish the diversity in expression of SOX4 in colorectal carcinoma, and search for CRC immunoprofile relation to complex spectrum of parameters including both, well excepted prognostic markers, as well as chosen histoclinical features to assess potential prognostic impact of SOX4 immunoexpression evaluation.

SOX4 expression was identified in numerous types of malignancies [14]. In presented studies it was observed in 100% of CRC cases, what stays in compliance with the literature, however immunoprofiles of CRC cases differed in relation to the examined features.

Stage of cancer disease is the most universal prognostic parameter in oncology age According to the National Cancer Institute (US), patients with stage 1 CRC have about 93% of survival rate while stage II is between 72% and 85%, and stage III is still as high as 83%. The prognosis of stage IV is the poorest and only 8% five years' survival rate is observed.

Regarding the stage, the examined SOX4 immunoexpression in CRC cases appeared to change in subsequent stages of cancer disease which stays in compliance with the other authors findings in literature –in sev-

eral other cancers SOX4 expression has been proved to be associated with tumour progression and poor clinical outcome [15].

The relationship between expression of SOX4 and the level of histological malignancy (grade) was studied on the examples of not only epithelial neoplasms but also mesenchymal origin malignancies such as osteosarcoma and revealed the connection between SOX4 expression and tumour grade, what made the survival of cancer patients poorer in comparison to population with normal levels of SOX4 protein expression [20].

In the literature it was also suggested that overexpression of nuclear SOX4 can appear prognostic marker for colon cancer and can be used as a predictive marker in this group of patients, either [19]. The results of presented studies stay in compliance with a utility of SOX4 expression as a prognostic parameter in CRC group of malignancies. According to the authors' knowledge there were no studies which covered the complex evaluation of SOX4 expression with other relevant histoclinical factors, including widely accepted prognostic markers. Although our studies did not show statistical significance in this matter (the most probably they have not achieved the minimum threshold of statistical significance due to the limited study subgroups), there were observed differences within CRC subgroups which seems to be promising and indicate the direction of future studies in more numerous groups with potential prognostic impact.

Another crucial factor which influences the survival rate of CRC patients is the presence of metastases in the lymph nodes. Although the topics of lymph node staging are still under discussion (e.g.: the definition and controversies in tumour deposits, isolated cancer cells and micrometastasis, lymph node ratio as a prognostic stratification factor) the histological evaluation of lymph nodes status remains crucial for postoperative treatment and prognosis prediction [21].

In presented studies the difference in SOX4 expression between the CRS tumours with positive lymph nodes status and without metastases to the lymph nodes were observed. It also stays in compliance with literature. After the analysis of still limited studies on the relation between cancer disease advancement and SOX4 expression we found the data which support the relation of SOX4 expression to the presence of metastases in both: regional lymph nodes and distant organs. It is believed

that the association of SOX4 expression and metastatic processes is related to migration and invasion of the cell line, what was proved on the example of the development and metastasizing in renal cell carcinoma [22]. SOX4 was evidenced to have an impact on growth and formation of metastases in lymph nodes in breast cancer, either [23]. The connection between lymph node metastasis and the advancement of the tumour and its correlation with upregulation of SOX4 was also described in gastric cancer [24]. Surprise, regarding the fact that colorectal carcinoma is one of leading causes of death due to cancer diseases worldwide no studies of correlation of SOX4 expression and presence of lymph node metastasis in this malignancy were found. Since the lymph node metastasis have their role in prognosis in colorectal cancer the relation of their appearance with the expression of SOX4, which role was already proved in other malignancies should undergo research with a broader scope. We hope that further studies in this area could give an answer to many questions about the course of the neoplastic process and potentially make the prediction if metastasis occur in specific patient possible, including CRC patients with potential benefits of more personalized treatment strategies based on SOX4 evaluation as suggested in presented studies.

The presence of ulceration – the next examined feature is believed to be typical for malignant growths and proved to be a significant prognostic factor in some of them with influence even on survival rate [25]. In colorectal cancer, besides the well-known prognostic factors, the presence of ulceration seems to also play a role in the course of the disease and influences the prognosis for CRC patients [26, 27]. Not many data are possible to find in literature which present the prognostic significance of the fact of colon ulceration and majority of them reflects inflammatory processes, mostly inflammatory bowel disease (ulcerative colitis and Crohn disease). However, in CRC it was observed that the appearance of the ulceration in majority of cases accompanied advanced stages of cancer disease. In presented studies the difference in SOX4 immunoexpression was observed between tumours with and without ulceration. Although without confirmation of statistical significance, the idea of investigation of the relationship between SOX4 expression and ulceration should not be forgotten in the future researches, similarly to the last sur-

prising findings: a moderate association of SOX4 index and age among females, and correlations between the SOX4 index and patients' age among grade 1 and grade 3 tumours which hard to be explained in this preliminary step of investigation may appear important for CRC biology and patients' fate in future.

Concluding remarks

For the first time the complex SOX4 immunoprofile of colorectal cancer was showed as well as its differences in relation to complex spectrum of histoclinical features including the established prognostic parameters. The study alights previously unknown part of CRC biology and brings promising findings which potentially may contribute to better understanding of this disease and improvement of treatment strategies due to their personalization. Regarding the fact that colorectal cancer as one of the most important challenge of today's medicine, each new direction of studies which potentially may benefit with reduction of CRC patients' mortality by influence of disease progression processes should be considered.

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