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Expression of Serpin B9 as a Prognostic Factor of Colorectal Cancer

ONDREJ VYCITAL[1,2], PAVEL PITULE[2], PETR HOSEK[2], TOMAS KRIZ[2], VLADISLAV TRESKA[1] and VACLAV LISKA[1,2]

[1]*Department of Surgery, Charles University, University Hospital Pilsen, Pilsen, Czech Republic;*
[2]*Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic;*
[3]*Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic*

Abstract. *Background/Aim: Treatment of colorectal cancer (CRC) does not reflect immune interactions between tumours and macro-organisms. Serpin B9 is known as an inhibitor of Granzyme B. The aim of this study was to evaluate the impact of the expression of Serpin B9 in CRC and healthy colon tissue on prognosis. Patients and Methods: This retrospective study included 74 CRC patients in all stages. Analysis of gene expression was performed with quantitative polymerase chain reaction with reverse transcription using specific primers and master mix Xceed qPCR SG. Expression was normalized to the reference genes GAPDH, ACTB, and PSMC. Results: Increased expression of Serpin B9 in healthy tissue was significantly associated with longer overall survival (OS). This association was found both in all patients and in the group of patients with distant metastases. Conclusion: The presented results support previous evidence of positive influence of the interaction between immune system and tumour on the prognosis of CRC.*

Colorectal carcinoma (CRC) is a severe health and social issue occurring at high incidence rates (1). Recent approaches to treatment of CRC are based on the staging according to the Union Internationale Contre le Cancer (UICC), which integrates the TNM classification with other risk factors. However, these risk factors do not reflect immune interactions between tumours and macro-organisms (2).

Serpin B9 is known as an endogenous inhibitor of Granzyme B in humans, which protects against the cytotoxic granule leakage. At the molecular level, Serpin B9 inhibits the cytolytic function of Granzyme B by forming a reversible Michaelis complex (3). Serpin B9 is secreted from cytotoxic lymphocytes (4), smooth muscle cells, endothelial cells, and hepatocytes. Immunoprivileged cells, *i.e.*, trophoblast cells, Sertoli cells, granulosa cells and cells of the eye lens produce this inhibitor as well. The proposed function of Serpin B9 is the protection against cytotoxic immunity (5). In malignant cells, Serpin B9 production has been observed in breast cell carcinoma (6), melanomas (7), cervical cancer (8) and in leukemia cells (9). In addition, CRC cells also produce Serpin B9 (8). Expression of Serpin B9 is one of the ways by which malignant cells avoid cytotoxic lymphocyte-mediated killing (10). Serpin B9 expression in rectal cancer tissue has been suggested as a negative predictive factor of neoadjuvant chemoradiotherapy (11, 12).

We have previously shown that increased expression of Serpin B9 in colorectal cancer cells is associated with regional lymph node metastases (13).

The aim of the present study was to evaluate the impact of Serpin B9 expression in CRC and healthy colon tissue on the progression and prognosis of CRC. The secondary target of this study was to confirm previous results showing that increased expression of Serpin B9 in colorectal cancer cells is associated with regional lymph node metastases.

Patients and Methods

Patients. This monocentric retrospective study included 74 CRC patients, 10 patients were in stage I, 20 patients in stage II, 35 patients in stage III and 9 patients in stage IV. This study was conducted at the Department of Surgery, Charles University, Medical School and Teaching Hospital Pilsen between 2013 and 2016. A total of 36 females (median age 68.86 years, min 49.27 and max 89.81 years) and 38 males (median age 67.36 years, min 38.74 and 88.14 years), who underwent surgical therapy for CRC were included in the study. Exclusion criteria were: inflammatory bowel disease, familiar adenomatosis, insufficient number of examined lymph nodes in resected bowel or rectum (less than 13) and or previous malignancy. Patients presenting tumour rupture, acute bleeding and ileus were also not included. The follow-up of patients occurred during standard dispensarization.

Correspondence to: Ondrej Vycital, Department of Surgery, Charles University, Medical School and Teaching Hospital Pilsen, U Jam 17, Pilsen 323 00, Czech Republic. Tel: +420 776817904, e-mail: vycitalo@fnplzen.cz

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Statistics. Differences in the expression of serpin B9 between healthy tissue and tumour were analysed using Wilcoxon signed-rank test. Mann-Whitney *U*-test was used to compare Serpin B9 expression (in healthy and tumour tissue) between patients with and without regional lymph node metastasis. Differences in Serpin B9 expression between UICC stages were assessed using Kruskal-Wallis ANOVA. The association between Serpin B9 expression and overall survival (OS) or disease-free interval (DFI) was investigated using univariable Cox proportional hazards model with subsequent stratification by median expression and visualisation by Kaplan–Meier plots. The analysis was performed using STATISTICA software (version 12, Cz, StatSoft, Inc. 2013, Prague, Czech Republic).

Gene expression analysis

Primer design. Oligonucleotide primers for quantitative real-time polymerase chain reaction (qPCR) were designed using Primer-3 software (14). All primers span exon-exon boundaries and their specificity was tested *in silico* by BLAST (15). Reference genes *GAPDH, ACTB* and *PSMC4* were previously tested in our laboratory and were used for normalization of Serpin B9 expression. Optimal annealing temperature and specificity of reactions were evaluated by Cq values analysis, melt curve analysis and agarose gel electrophoresis.

RNA isolation. Snap-frozen samples of tumor tissue and macroscopically non-malignant mucosa were grounded in liquid nitrogen and total RNA was isolated by TRI Reagent®RT (Molecular Research Center, Inc., Cincinnati, OH, USA) according to manufacturer's protocol. RNA was resuspended in nuclease-free water and stored at –80˚C. RNA concentration (absorbance at 260 nm) and purity (230 nm/260 nm absorbance ratio) were measured with the Tecan Infinite M200 (Tecan Trading AG, Männedorf, Switzerland) using NanoQuant settings. RNA integrity was examined by agarose gel electrophoresis.

Reverse transcription. Reverse transcription of RNA was performed using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, USA) in 20 μl reaction. Five hundred ng of total RNA were first treated for 5 min with DNase I (Top-Bio, Vestec, Czech Republic) to remove potential traces of genomic DNA. Mixture of random hexamers and oligo$(dT)_{18}$ primers, each at a 2.5 μM final concentration, were used for reverse transcription. Reaction conditions were set according to the manufacturer's protocol. The quality of cDNA and the possibility of DNA contamination were examined by PCR in the absence of reverse transcriptase or cDNA samples (GAPDH primers, 40 cycles) and agarose gel electrophoresis.

Quantitative real-time PCR. Xceed qPCR SG master mix (IAB, Prague, Czech Republic) was used for qPCR. cDNA was diluted to 0.5 ng/μl and 4.75 μl of cDNA was mixed with 0.25 μl of gene specific primers and 5 μl of master mix in a final volume of 10 μl per reaction. Reference genes were analyzed in duplicate and Serpin B9 in triplicate using the CFX96 real-time PCR instrument (Biorad, Hercules, CA, USA). The following reaction conditions were used: initial denaturation at 95˚C for 5 min, 45 two-step cycles with denaturation at 95˚C for 10 sec and annealing and extension at 62˚C for 30 sec, and finally melting curve analysis with denaturation at 95˚C for 15 sec followed by continuous measurement of fluorescence between 60˚C and 90˚C with the step of 0.5˚C. Data were analyzed using Biorad CFX Manager™ software and quality checked Cq values were used for statistical analysis.

Results

No significant differences were found in the expression of Serpin B9 between healthy and tumorous tissues (Figure 1). There were also no significant differences in Serpin B9 expression between groups UICC I, II, II and IV (Figure 2). Spearman test showed a non-significant perceptible trend in the expression of Serpin B9 in tumorous tissue with increasing UICC stage (p=0.075). We did not find any significant differences between groups of patients with and without metastases in regional lymph nodes (N0 *versus* N1 and N2 lymph node status).

Increased expression of Serpin B9 in healthy tissue was significantly associated with longer OS in all patients (p=0.0023, Figure 3), as well as in the group of patients with distant metastases (p=0.0169, Figure 4). Regarding DFI, no relationship between disease progression and Serpin B9 expression in either healthy or tumour tissue was observed.

Discussion

Based on the previous results of our immunohistochemical study in CRC (13), an increased Serpin B9 expression was expected to be found in the tumour tissue of UICC III (with lymph node metastasis) patients in comparison with tumour tissues of group UICC II (without lymph node metastasis). Surprisingly, this was not the case, probably because a whole spectrum of cells from the tumour tissue were analysed together in the expression assay, and not only adenocarcinoma cells. It is known that tumour infiltrating lymphocytes produce Serpin B9 (4). The presence of tumour infiltrating lymphocytes has been revealed to be an important prognostic factor (16, 17) that could influence the results. It remains unclear if there is any causality in the association of Serpin B9 expression in non-tumorous colon tissue and OS. Information from non-tumorous tissue samples can indicate its suitability and its impact on prognosis (18).

Because we showed significantly increased expression of Serpin B9 in non-tumorous tissue in the group of patients with distant metastases and better OS, it would be interesting to investigate the predictive value of serpin B9 expression in non -tumorous tissue on the effect of oncological treatment. Currently, there are data only about the influence of Serpin B9 expression in rectal cancer cells on the response to neoadjuvant chemoradiotherapy (11, 12). We did not evaluate expression of serpin B9 as a predictive factor of oncological treatment outcomes because of the small number of patients in the UICC IV group with increased expression of Serpin B9 (9 patients in the whole group).

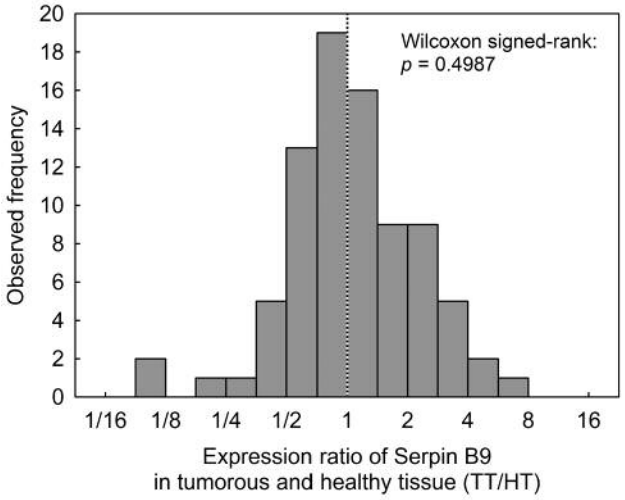

Figure 1. *No significant differences were found in Serpin B9 expression between healthy and tumorous tissues.*

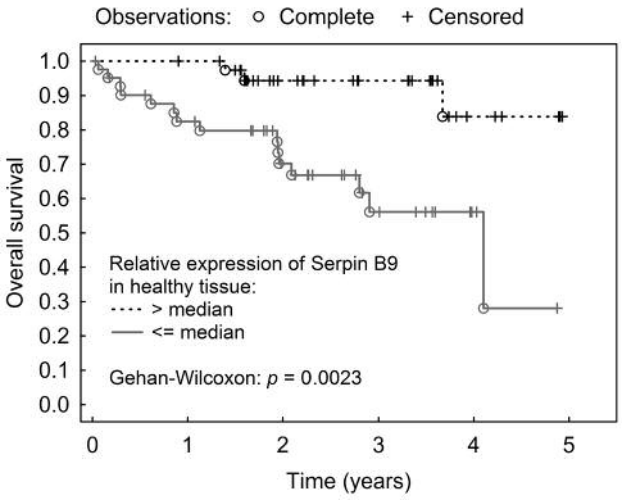

Figure 3. *Positive effect of increased Serpin B9 expression in healthy tissue on OS of all patients in the study.*

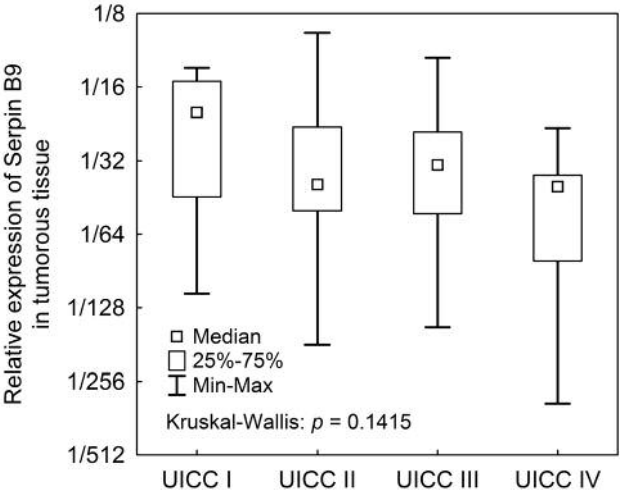

Figure 2. *No significant differences in Serpin B9 expression were observed between the cohorts UICC I, UICC II, UICC III and UICC IV in tumorous tissue.*

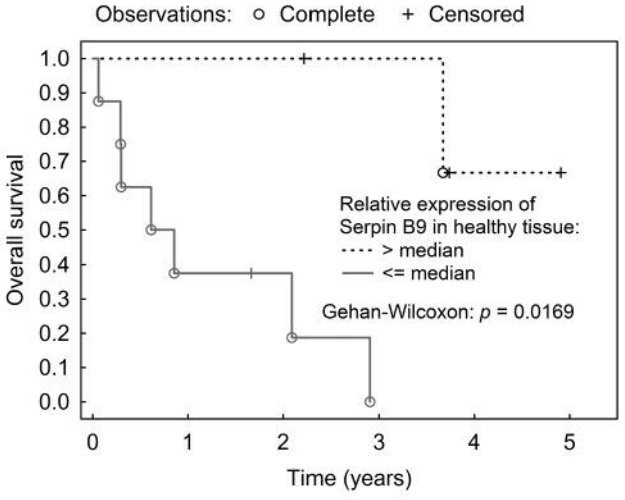

Figure 4. *Positive effect of increased Serpin B9 expression in healthy tissue on OS in the group of patients with distant metastases.*

Conclusion

The present results support previous evidence indicating a positive influence of the interaction between immune system and tumor on the prognosis of CRC as exemplified by the effect of the expression of granzyme B inhibor – Serpin B9. On the other hand, the mechanism by which Serpin B9 influences the prognosis of CRC remains unclear. However, there is a plethora of prognostic factors available for CRC and it is now important to analyze them in order to select patients who will benefit from intensive oncological treatment.

Conflicts of Interest

The Authors dcclare no conflicts of interest regarding this study.

Authors' Contributions

Ondrej Vycital – corresponding author, methodology, data and specimen collection, data analysis, data curation, writing – original draft; Pavel Pitule – gene expression analysis; Tomas Kriz – data collection; Hosek Petr – data analysis, data curation, performance of the statistical analysis; Liskav Vaclav - supervision, validation, investigation; Treska Vladislav – supervision, validation.

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