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Original Scientific Paper

PROGNOSTIC VALUE OF TUMOR BUDDING IN SPORADIC COLORECTAL CANCER STAGE II PATIENTS WITH A LOW NUMBER OF LYMPH NODES EXAMINED

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SUMMARY – Stage II colorectal cancer (CRC) is a major therapeutic challenge because it is not easy to decide whether patients will benefit from adjuvant chemotherapy or not. This study was designed as a retrospective prognostic study combining standard histopathologic parameters with tumor budding and microsatellite instability. The study included 89 patients on elective treatment for stage II primary colorectal adenocarcinoma from January 2011 to December 2015. Study results indicated that the prognosis of patients with stage II CRC depended on the combination of three factors, (in the order of importance): number of lymph nodes examined; total number of buds *per* 0.785 mm^2 (≥5.5); and positive lymphovascular invasion. There is increasing evidence that tumor biology and non-anatomic characteristics are important in the prognosis and treatment of CRC. One of them is tumor budding which is not yet an integral part of the AJCC staging system. A low number of the lymph nodes examined is associated with high-risk patients. All patients without an adequate number of lymph nodes examined (less than 8 lymph nodes) should *a priori* be considered a very high-risk group, with a very low survival rate, and chemotherapy should be used.

Keywords: *Colorectal cancer; Stage II; Tumor budding; Lymph nodes; Prognosis*

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer mortality worldwide[1]. Staging of the CRC describes the extent of cancer and how far it has spread. Consequently, it is an essential information for rational therapy planning[1]. Stage II of CRC is a major therapeutic challenge. It is not easy to decide whether the patient will benefit from adjuvant chemotherapy or not[2]. In stage I, the main therapy is surgical removal of the tumor, without chemotherapy. In stage III, with lymph node metastases, patients definitely benefit from chemotherapy. In stage II, the majority of patients remain disease-free after 5 years but in approximately 25% of patients the disease returns[2]. Routine use of chemotherapy in stage II is not mandatory as it improves 5-year survival by only 2%-5%[2]. Adverse effects of chemotherapy are serious, and it should be

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used only in situations when we are reasonably confident in its usefulness[2].

Adenocarcinomas of the colon and rectum stage II, according to the extent of the tumor (T), extent of spread to the lymph nodes (N), and presence of metastasis (M) (TNM) staging by the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC), are not such a heterogeneous group (pT3/4, pN0, pM0) compared to other stages. There still is a large difference in 5-year survival among stage II subgroups IIA, IIB and IIC compared with stage IIIA. According to the Surveillance, Epidemiology, and End Results (SEERS) data, the 5-year survival of stage II patients varies from 66% in stage IIA to 37% in stage IIC, which is significantly less than stage IIIA in which lymph nodes are positive, and 5-year survival is 73.1%[3].

There are many studies trying to identify high-risk stage II patients using different prognostic factors. Some of the factors identified are morphological features such as pT4, grade 3 tumors, fewer than 12 lymph nodes examined by pathologists, and lymphovascular/perineural invasion[1-6]. Various biochemical and molecular markers are also used, e.g., blood levels of carcinoembryonic antigen (CEA), microsatellite instability, and gene profiling[6-9].

Several studies have shown that the number of lymph nodes collected has an important impact on survival due to a more accurate stage and better determination of the prognosis[10]. At least 12 lymph nodes are required for proper staging of radical colon and rectal resection in patients undergoing surgery[11].

Sometimes after surgery, either due to the patient anatomic characteristics, inappropriate surgical technique, or a smaller number of lymph nodes collected by the pathologist, we do not have a minimum of 12 lymph nodes examined, which can lead to incorrect determination of pN stage, which can affect the choice of therapy and ultimately survival[12]. In such cases, it is necessary to look at the broader picture in addition to the standard components of histopathologic findings, currently used but not yet included as part of a standard pathologic report, such as tumor budding.

Tumor budding can be defined as the finding of a single tumor cell or a small group of up to five cells separated from the invasive front of the tumor[13]. It is a well-established negative prognostic factor in CRC[13-15]. Despite the clear value of tumor budding as a prognostic factor, it is not yet an integral part of pathologic findings in patients with CRC. This is due to several reasons, such as the lack of consensus on the definition of tumor budding, heterogeneity in the methodology for determining tumor budding, as well as quantification of tumor budding itself[16].

Lymphovascular invasion (LVI) is the finding of cancer cells within endothelium-lined lymphatic or venous structures[17]. Invasion of lymphatic and blood vessels is a negative prognostic sign and is found in 10%-85% of CRC, depending on the stage[18].

Carcinoembryonic antigen is a glycoprotein mainly secreted by solid tumors. The National Comprehensive Cancer Network (NCCN) recommends CEA as a tumor marker for colon cancer[19]. Although CEA was long thought to have no function, in recent years it has been shown that it can influence the occurrence of metastases by promoting effects on cell adhesion, inhibition of inflammatory response, and inhibition of programmed cell death[20,21].

Microsatellite instability (MSI) is a concept of phenotypic expression observed in patients with sporadic CRC, as well as in patients with Lynch syndrome[22]. MSI arises as a result of dysfunction of the mismatch repair system, which is responsible for microsatellite corrections[22]. Microsatellites consist of repetitive sequences of 1 to 6 nucleotides found throughout the human genome. Microsatellites are mainly found in the coding region of DNA but they can also be found in other regions such as introns or non-coding regions[23]. Regarding the expression of MSI in CRC, three types are distinguished, as follows: high-grade microsatellite instability (MSI-H), low-grade microsatellite instability (MSI-L), and microsatellite-stable tumors (MSS)[24]. MSI-H is a positive prognostic factor in all CRC stages I-IV as compared to MSI-L and MSS, which may influence the modality of therapy, especially in stage II CRC[25].

The aim of our study was construction of a simple prognostic algorithm for identification of high-risk stage II CRC patients who could benefit from adjuvant chemotherapy. Only a few studies have focused on tumor budding and number of lymph nodes harvested. The study was designed as a retrospective prognostic study combining classic demographic and histopathologic parameters with three methods of

tumor budding, MSI, and CEA levels. The data obtained were analyzed by recursive partitioning, a survival analysis method based on machine learning, a branch of artificial intelligence[26,27]. The result of recursive partitioning is a survival tree, which visually describes prognostic subgroups in a simple and straightforward way[26,27].

Patients and Methods

Patient selection

The study included 89 patients. They were selected from the archive of the Department of Pathology of our hospital and represent patients treated for stage II primary colorectal adenocarcinoma in the period from January 2011 to December 2015. Patients were excluded in case of emergency surgery, present inflammatory bowel disease, Lynch syndrome, and missing survival data. Clinical data were obtained from the hospital database system.

Slide selection and staining

All postoperative materials were fixed in 10% buffered formalin. The examination was performed using a 5-mm-thick longitudinal cuts of postoperative material. All slices were embedded in paraffin and stained with hematoxylin-eosin (HE). HE slides were reviewed by two pathologists experienced in gastrointestinal pathology.

Budding counting

Tumor budding (Fig. 1) was assessed using three methods[28]. The first method was total number of buds *per* 0.785 mm^2 [15]. The second was Nakamura's method[29]. It is based on three-point scale: score 0 – no budding at magnifications X40 and X100; score 1 (low-grade budding) – no budding at magnification X40 but visible at X100; and score 2 (high-grade budding) – budding visible at magnification X40. The third method was proposed by Lugli *et al.*[29], and it is also based on three-point scale: score 0 (low budding) with 0-4 buds at magnification X200 *per* 0.785 mm^2; score 1 (intermediate budding) with 5-9 buds at magnification X200 *per* 0.785 mm^2; and score 2 (high-budding) ≥10 buds at magnification X200 *per* 0.785 mm^2.

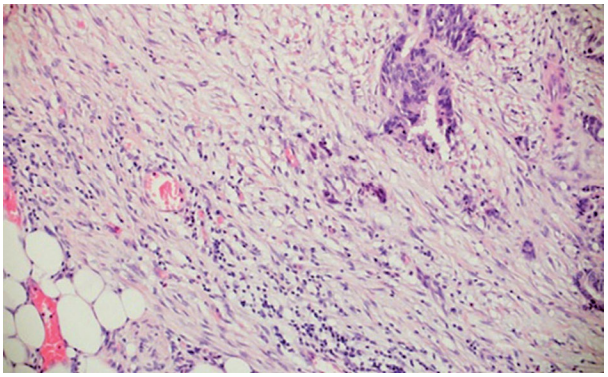

Fig. 1. Presence of tumor budding (hematoxylin-eosin staining, X200).

Histopathologic staging

Histologic type, histologic grade, intravascular and perineural invasion, pT and pN were determined using The American Joint Committee of Cancer (8th Edition) criteria for pathologic staging of resected material[1].

Carcinoembryonic antigen

Carcinoembryonic antigen is a polysaccharide-protein complex often used as a biomarker for tumor diagnosis and prognosis[8]. CEA (ng/mL) was determined in patient serum using Human CEA ELISA kit by Novateinbio (https://novateinbio.com/human-elisa-kits/76297-human-cea-elisa-kit.html).

Microsatellite stability/instability

The material is cut into preparations at standard thickness of 4 μm and stained with an immuno-histochemical method, performed on a fully automated device Ventana BenchMark ULTRA platform at the Clinical Department of Pathology and Cytology, Dubrava University Hospital, Zagreb. Microsatellite instability was determined using the following antibodies (MMR IHC panel, Ventana, Roche):

1) MLH1 (MutL Protein Homolog 1, mouse monoclonal antibody), clone M1;
2) MSH2 (MutS Protein Homolog 2, mouse monoclonal antibody), clone G219-1129;
3) MSH6 (MutS Protein Homolog 6, rabbit monoclonal antibody), clone SP93; and
4) PMS2 (Postmeiotic Segregation Increased, mouse monoclonal antibody) clone A16-4.

The 4-primary antibody panel has different staining protocols, according to the manufacturer's instructions. The entire process is automated and deparaffinization is performed using EZ prep and CC1 fluids, and the immunohistochemical reaction is performed in combination with Optiview DAB IHC detection kit and additional reagents Hematoxylin II and Bluing Reagent. The PMS2 antibody requires the OptiView DAB amplification kit to improve signal visibility. Visualization is performed using hydrogen peroxidase with DAB kit.

Microsatellite instability was determined immunohistochemically on operative material and associated endoscopic biopsy. Immunohistochemically, microsatellite-unstable carcinomas were considered as tumors in which there was a loss of expression of at least one of the four markers used (MLH1, MSH2, MSH6 and PMS2). Loss of expression is considered as negativity of all tumor nuclei with a positive internal control (positive nuclei of stromal, inflammatory or non-tumor epithelial cells) (Figs. 2 and 3).

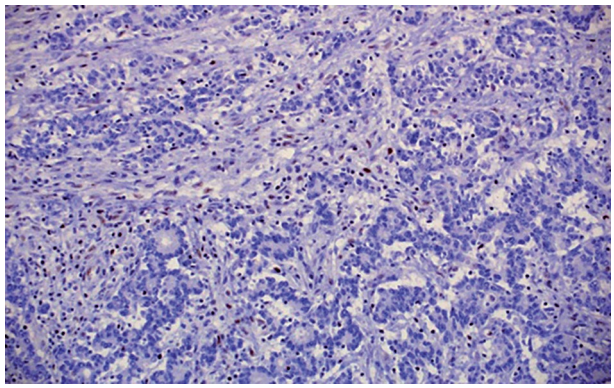

Fig. 2. Negative MLH1, positive inflammatory cells (magnification X200).

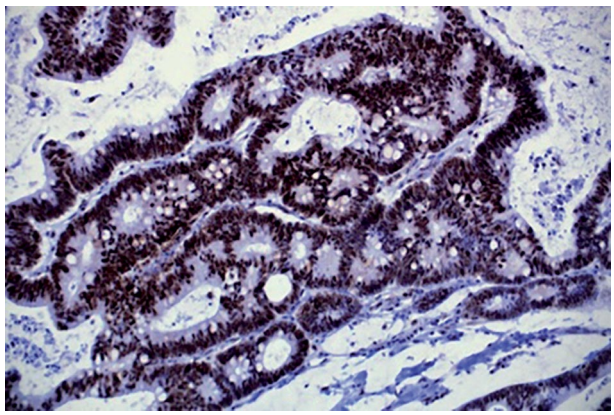

Fig. 3. Positive MLH1 (magnification X200).

Data analysis

Continuous data were presented as means, standard deviations and medians, and plotted using the box-and-whisker methods[31]. Counted data and scores were presented as numbers and percentages, and plotted as bar graphs[31].

Prognostic subgroups were identified using recursive partitioning, implemented as the rpart module (acronym for Recursive PARTitioning in the R software)[32,33]. This module constructs a survival tree, a visual method which identifies prognostic subgroups[26,27]. The method starts the analysis with all patients included in the study and divides them step-by-step into prognostic subgroups. The final result is expressed as a survival tree, composed of decision nodes and terminal nodes (leaves)[26,27]. Each decision node uses a variable to subdivide patients into two subgroups with the maximum difference in hazard ratios (HR). This process is repeated until further improvements to the subdivision are no longer possible. In this way, the terminal nodes are reached. Patients in the first decision node have a HR of 1. The HR for patients in each node is expressed in comparison to this value. The advantage of recursive partitioning is a clearly established hierarchy of variables by importance, that is, this method lists variables by the content of information related to the prognosis[26,27]. The method is insensitive to missing data, unlike classic statistical methods, and provides reliable and robust conclusions in most clinical situations[34].

The difference in survival between patients in terminal nodes was analyzed by the Gehan-Wilcoxon test and presented as survival curves based on the Kaplan-Meier method[30]. Data were considered statistically significant if the p value of the Gehan-Wilcoxon test was ≤0.05[30]. All graphs with survival curves show the number of patients at risk, an important parameter to assess the maturity of follow-up. Also, the difference

Table 1. Patient characteristics, histopathologic parameters, carcinoembryonic antigen levels, follow-up and censor data, and type of therapy

Variable	Value
No. of patients	89
Gender	
Females, n (%)	28 (31.5)
Males, n (%)	61 (68.5)
Age (years)	
All patients, mean ± SD, median	70.4±10
Females, mean ± SD, median	70.5±7.90, 68
Males, mean ± SD, median	70.4±10.90, 72
Histologic type	
Non-mucinous, n (%)	79 (88.8)
Mucinous, n (%)	10 (11.2)
Localization	
Rectum, n (%)	26 (29.2)
Left colon, n (%)	30 (33.7)
Right colon, n (%)	33 (37,0)
pT	
pT3, n (%)	78 (87.6)
pT4a, n (%)	5 (5.6)
pT4b, n (%)	6 (6.7)
No. lymph nodes collected mean ± SD. median	14.7 ± 7.94, 14
Lymphovascular invasion	
Positive, n (%)	31 (34.3)
Negative, n (%)	58 (65.2)
Perineural invasion	
Positive, n (%)	7 (7.9)
Negative, n (%)	82 (92.1)
Grade	
Grade 1, n (%)	3 (3.4)
Grade 2, n (%)	74 (83.2)
Grade 3, n (%)	2 (2.3)
Grade 4, n (%)	0 (0)
Grade missing data, n (%)	10 (11.1)
Preoperative carcinoembryonic antigen (ng/mL) mean ± SD, median	8.9±29.9, 1.7
Censor	
Complete observations, n (%)	29 (32.6)
Censored observations, n (%)	70 (67.4)
Follow-up (months) mean ± SD. median	69.9 ± 25.84, 72
Type of therapy, n (%)	
Surgery alone, n (%)	75 (84.3)
Surgery and chemotherapy, n (%)	12 (13.5)
Surgery and radiotherapy, n (%)	1 (1.1)
Surgery with chemoradiotherapy, n (%)	1 (1.1)

in HR between the right and left branch of each decision node was estimated using Cox regression.

All data steps were performed using R data analysis software version 3.5.1, using the following module: rpart for survival tree; survival module to calculate the difference in HR between the right and left branch of terminal modes in the survival tree, and the EZR module for Kaplan-Meier curves and the Gehan-Wilcoxon test[35,36].

Results

The study analyzed data on 89 patients with CRC stage II. Their demographic data, CEA, follow-up data, and number of censored data are shown in Table 1. Adjuvant therapies used in the study patients are also shown in Table 1. The majority of patients did not receive any adjuvant therapy (84.3%).

The results of the assessment of tumor budding are shown in Table 2. Three methods were used, as follows: total number of tumor buds *per* 0.785 mm^2 [15], Nakamura's method[29], and the result of tumor budding according to Lugli *et al.*[30]. Results of MSI status[24,25] are also shown in Table 2. MSI was found in 14 (15.7%) patients.

Table 2. Budding parameters and microsatellite stability/instability data

Variable	Value
Tumor bud count *per* 0.785 mm^2, mean ± SD median	10.9±5.99, 10.0
Budding (Nakamura)	
Score 0, n (%)	29 (32.0)
Score 1, n (%)	40 (45.0)
Score 2, n (%)	14 (15.7)
Score 3, n (%)	6 (6.7)
Tumor budding score (Lugli)	
Score 0, n (%)	67 (75.3)
Score 1, n (%)	16 (18.0)
Score 2, n (%)	6 (6.7)
Microsatellite stability/instability	
Microsatellite stability	75 (84.3)
Microsatellite instability	14 (15.7)

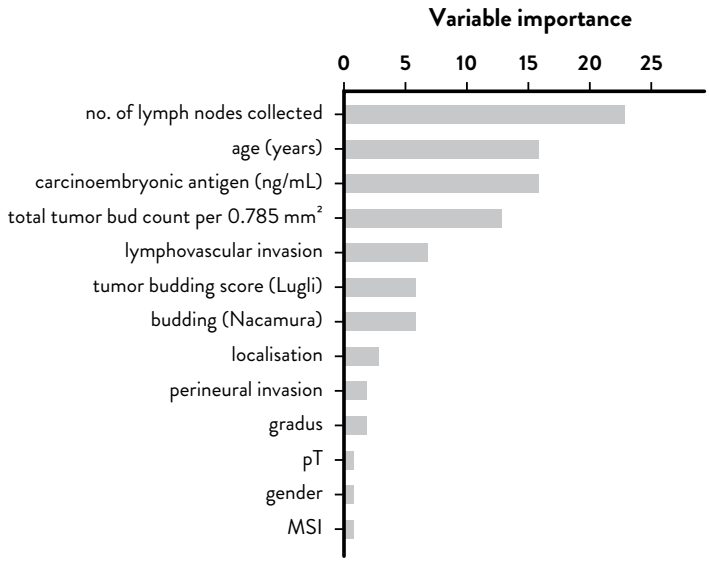

Fig. 4. Importance of individual variables determined by rpart method.

The prognostic values of the variables listed in Tables 1 and 2 were determined using recursive partitioning, a method recommended by the AJCC for the analysis of prognostic studies[26-28]. The importance of individual variables is shown in Figure 4. The four most informative variables were number of examined lymph nodes; age; CEA concentration (serum); and total number of buds *per* 0.785 mm^2. The properties of the most informative variables are shown in Figure 5.

However, it should be noted that the AJCC criteria for prognostic studies require that the prognostic value of a variable must always be evaluated in the context of other variables[26-28]. The results of rpart analysis, which uses all variables, are shown as a survival tree (Fig. 6). The constructed triple has three decision nodes and four terminal nodes (leaves). The decision nodes are shaded gray, and the color of the terminal nodes is white (Fig. 6). The analysis starts with all patients included in the study (N=89), and their HR is 1. Using these variables, patients are subdivided into four terminal nodes. The variables used in the decision nodes are number of lymph nodes collected; total number of buds *per* 0.785 mm^2; and LVI. The importance of variables is determined by their position in the survival tree, i.e., the topmost variable is most informative, the

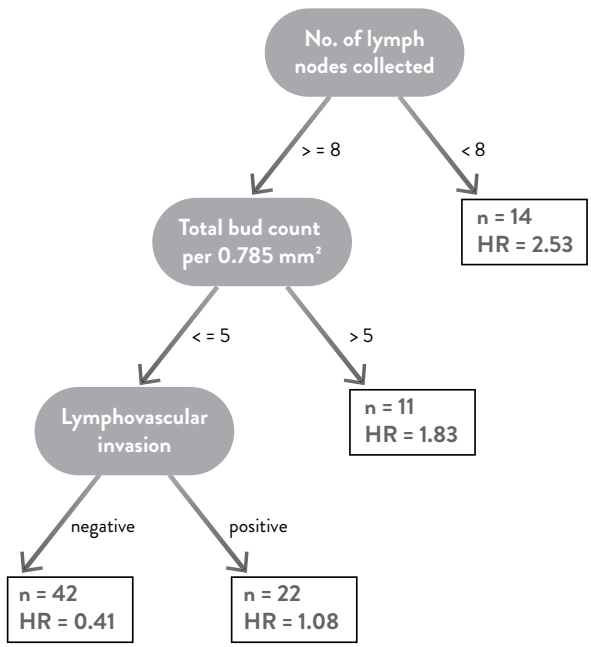

Fig. 5. Survival tree constructed using rpart method with three decision nodes (gray) and four terminal nodes (white). Three variables used in decision nodes were number of examined lymph nodes; total number of buds per 0.785 mm^2; and lymphovascular invasion. Four decision nodes describe identified risk subgroups. Hazard ratio (HR) progressively grows from the left to the right.

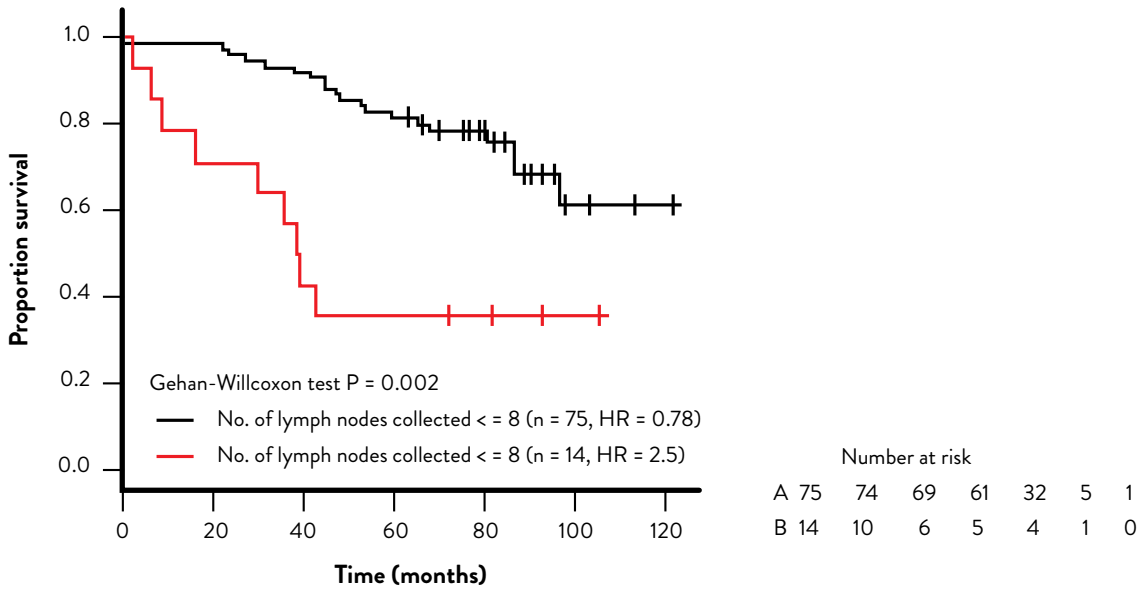

Fig. 6. Difference in patient survival (Kaplan–Meier method) for the left and right branches of the first decision node which uses the number of harvested lymph nodes as a separation criterion.

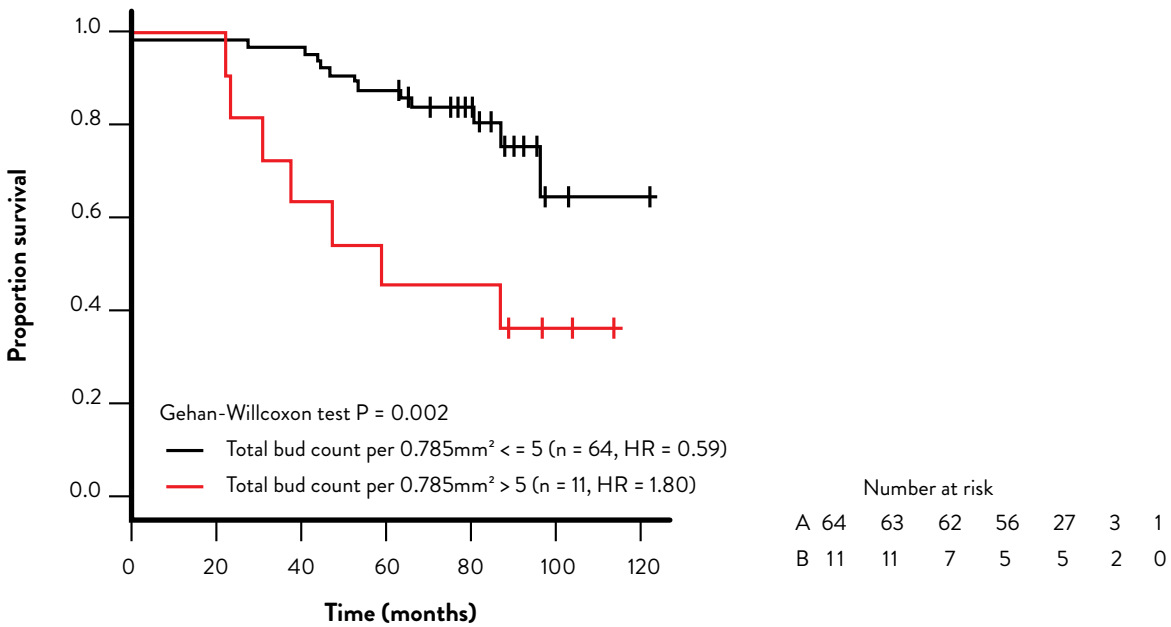

Fig. 7. Difference in patient survival (Kaplan–Meier method) for the left and right branches of the second decision node which uses the total number of buds per 0.784 mm² as a separation criterion.

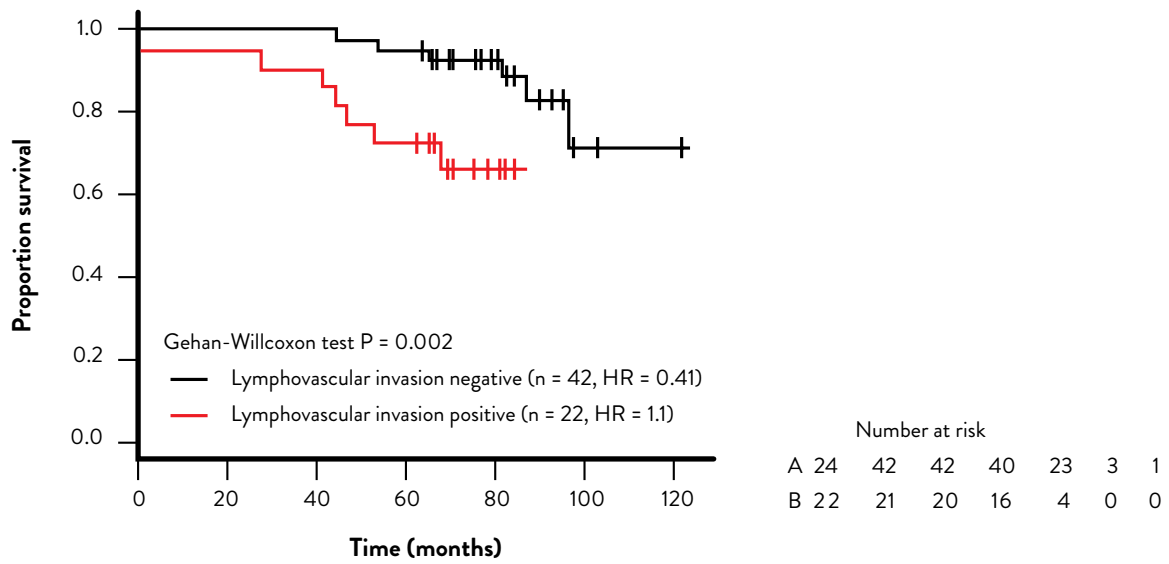

Fig. 8. Difference in patient survival (Kaplan–Meier method) for the left and right branches of the third decision node which uses lymphovascular invasion as a separation criterion.

variable below topmost is the second most informative, and so on.

Three decision nodes divide patients into four terminal nodes. HR increases progressively from the left to the right side of Figure 5. The leftmost terminal node represents low-risk patients (HR=0.41, n=42), and the second represents intermediate risk patients (HR=1.08, n=22). The second terminal node on the right represents high-risk patients (HR=1.8, n=11) and the rightmost terminal node describes extremely high-risk patients (HR=2.5, n=14).

Furthermore, the difference in patient survival for the left and right branches of each decision node are shown in Figures 7-8. They are based on the Kaplan-Meier estimate and Gehan-Wilcoxon test[31]. The HRs and their 95% confidence intervals (CI) for the right branch of each decision node, compared to the left node. These data complement the survival tree (Fig. 6) because the HR ratios in this figure represent the difference compared to all patients included in the study (HR=1).

Discussion

Stage II CRC is a diagnostic, prognostic and treatment challenge. It is not easy to decide whether a patient will benefit from adjuvant chemotherapy or not[2]. There is increasing evidence that tumor biology and non-anatomic characteristics are important in the prognosis and treatment of CRC. Some of them are tumor budding and MSI which are not yet part of the AJCC staging system[1]. The goal of our research was construction of a simple prognostic algorithm based on anatomic and non-anatomic parameters that will identify high-risk stage II CRC patients who will benefit from adjuvant chemotherapy.

The algorithm was obtained using recursive partitioning[26-28], and is visually shown in Figure 5 as a survival tree. This method of analysis is recommended by the AJCC for prognostic studies. The patients included in the study (N=89) were divided by the algorithm into four prognostic categories, i.e., patients at low, medium, high, and very high risk. This detailed division is characteristic of effective prognostic methods, and can significantly facilitate the management of patients with stage II CRC[28].

The results obtained suggest that the prognosis of patients with stage II CRC depends on a combination of three factors. They are (in the order of importance):

1) a small number of examined lymph nodes is associated with very high-risk patients (HR=2.5, n=14). This strongly suggests that reliable assessment of prognosis depends on the size and quality of the tissue sample. All patients without an adequate number of lymph nodes examined (less than 8 lymph nodes) should be considered *a priori* as a very high-risk group, with very low survival rate, as shown in Figs. 5 and 6. They should benefit from chemotherapy;
2) biological aggressiveness of the tumor which is manifested in a high total number of buds *per* 0.785 mm^2 (>5). These patients represent a high-risk subgroup (HR=1.8, n=11), with a low survival rate (Figs. 5 and 7). They should also benefit from chemotherapy;
3) positive LVI in patients with a sufficient number of lymph nodes examined and a small number of total buds is a negative prognostic factor (HR=1.1, n=22). However, HR=1.1 represents the HR obtained when comparing all patients (N=89). The actual negative prognostic effect of positive LVI is better assessed if we compare only the left and right branches of the third node (Figs. 5 and 8). Positive LVI has a HR=4.50 (95% CI 1.29-15.68, p=0.018) compared with patients with negative LVI (Figs. 5 and 8). They should also benefit from chemotherapy; and
4) patients with an adequate number of lymph nodes examined, a small number of buds and negative LVI represent a low-risk subgroup (HR=0.41, n=42). It is important to note that this subgroup contained a significant number of patients, i.e., 42 of 89 patients (Fig. 5). These data suggest that assessment of LVI is important to reliably identify low-risk patients who are unlikely to benefit from chemotherapy.

An obvious advantage of the prognostic algorithm proposed is its simplicity as it requires only HE staining, without image analysis and specialized software. The most complex part is determining the total number of buds, which requires experience and a tolerable working time.

It is important to note that the obtained values of the survival tree are not the optimal values of individual variables, that is, the algorithm should be viewed as a whole.

As an information unit, it is necessary to evaluate the number of lymph nodes examined, total number of buds *per* 0.785 mm^2, and LVI. Using isolated values artificially extracted from survival trees is one of the most common mistakes in using classification algorithms derived from machine learning[28].

The results of this study should be evaluated in a prospective study. However, it is a good starting point for planning a larger study because results obtained from survival trees are very robust and provide reliable and valid conclusions in most clinical situations[37].

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Sažetak

TUMORSKO PUPANJE I MIKROSATELITNA NESTABILNOST U BOLESNIKA SA SPORADIČNIM KARCINOMOM DEBELOG CRIJEVA I REKTUMA II. STADIJA

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Drugi stadij kolorektalnog karcinoma predstavlja značajan terapijski izazov. Nije lako odlučiti hoće li bolesnik imati koristi od adjuvantne kemoterapije ili ne. Ova studija je osmišljena kao retrospektivna studija koja kombinira standardne patohistološke parametre s tumorskim pupanjem i mikrosatelitnom nestabilnošću. U studiju je bilo uključeno 89 bolesnika elektivno liječenih od kolorektalnog adenokarcinoma II. stadija u razdoblju od siječnja 2011. do prosinca 2015. godine. Dobiveni rezultati pokazuju da prognoza bolesnika s kolorektalnim adenokarcinomom II. stadija ovisi o kombinaciji tri čimbenika, a to su (prema važnosti) broj pregledanih limfnih čvorova; ukupan broj pupova na 0,785 mm^2 (≥ 5,5); pozitivna limfovaskularna invazija. Sve je više dokaza da su biologija tumora i neanatomske karakteristike važni u prognozi i liječenju kolorektalnog adenokarcinoma. Jedan od njih je tumorsko pupanje koje još nije sastavni dio sustava AJCC. Mali broj pregledanih limfnih čvorova povezan je s visokorizičnim bolesnicima. Sve bolesnike bez odgovarajućeg broja pregledanih limfnih čvorova (manje od 8 limfnih čvorova) treba *a priori* smatrati vrlo rizičnom skupinom s vrlo niskim postotkom preživljenja te im treba preporučiti kemoterapiju.

Ključne riječi: *Kolorektalni karcinom; II. stupanj; Pupanje tumora; Limfni čvorovi; Prognoza*