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ORIGINAL ARTICLE

Prognostic Significance of Lacunarity in Preoperative Biopsy of Colorectal Cancer

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Abstract

The quantity and quality of preoperative material in colorectal cancer is often limiting factor in determination of risk factors and therapy planning. The most important negative prognostic factors are intravascular and perineural invasion, as well as tumor budding. Usually, the only parameter available in preoperative biopsy is tumor budding. However, the growing body of evidence suggests that cancer differentiation based on the poorly differentiated clusters has better prognostic value. The limiting factor in applying of these new parameters is reproducible, simple, cheap and fast method of their determination. In this paper we investigated the prognostic value of lacunarity, determined in preoperative biopsy. Lacunarity is a measure of spatial heterogeneity (inhomogeneity) in an image. It quantifies how objects fill the space, and enables analysis of gaps distribution, homogeneity of gaps, and presence of structures. It was shown that lacunarity and the total number of buds could be combined in a model which clearly divides colorectal cancer patients in low, medium and high risk subgroups. The paper also points out that the quantitative numerical methods are superior to semiquantitative methods, and that individual methods should be combined using algorithms to obtain a more accurate prediction. Because the study described is designed as a pilot study, verification is needed on a larger sample of patients from independent researchers.

Keywords Colorectal cancer · Preoperative biopsy · Intratumoral budding · Lacunarity · Prognosis · Recursive partitioning · Image analysis

Introduction

Single cells detached from the tumor glands as an invasive parameter were first recognized by Japanese and American authors more than 40 years ago [1–3]. They have investigated detached tumor cells in the invasive tumor margin. The result of these studies was the definition of tumor budding as the finding of single tumor cell or small group up to five cells separated from the tumor invasive front [4].

These papers were followed by a large number of studies, which mainly went in two directions. One direction was to investigate the molecular properties of these cells, which led to the concept of epithelial-mesenchymal transition (EMT) [5, 6]. These malignant epithelial cells adopt mesenchymal characteristics and thus enhanced invasiveness. Another direction of research is the attempts to suggest the optimal method of tumor budding quantification [3, 7–11]. Different groups proposed budding assessment methods, with quantitative criteria, which usually subdivide patients in two or more prognostic subgroups [3, 9, 11–13].

All proposed methods have been re-evaluated repeatedly, and most have been shown not to be fully optimal. There are problems related to staining methods (hematoxylin-eosin versus cytokeratin), size and layout of the field, optimal sample cutting thickness, the experience of the pathologist [8, 14–21]. Consequently, Lugli et al, proposed a method for determining the degree of tumor budding that attempts to resolve the above problems. It is based on counting buds in the invasive tumor margin (hot spot) on hematoxylin-eosin (HE) stained samples. The results could be subdivided in three score-based groups, with the recommendation of recording also the absolute bud number [22].

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Most of the cited papers, as well as Lugli's, discuss budding in the invasive tumor margin, that is, peritumoral budding. However, as one of his conclusions, Lugli also cites the importance of recognizing intratumoral budding as a prognostic factor associated with lymph node metastases [22]. Tumor budding in preoperative biopsy, i.e. intratumoral budding, is an indicator of metastasis to the lymph nodes and distant organs, and associated with resistance to neoadjuvant chemotherapy [23].

The most important step in diagnostic process of colorectal carcinoma is to obtain adequate tissue sample for establishing pathohistological diagnosis. The tissue is usually taken during colonoscopy, it is limited in size, and sometimes in quality. Reliable diagnosis could be established if the material is adequate. However, there are some limitations: (a) the tissue is taken from the superficial part of the tumor; (b) it represents only a minor part of the tumor; (c) it is usually mechanically changed due to process of sampling. Many important parameters in the planning of treatment are limited, and the final profiling is based on resected postoperative specimens.

It is well known that the most important negative prognostic factors of colon cancer are lymphatic and vascular invasion, perineural invasion, and number of buds [24–28]. Among these factors, in preoperative biopsy, only tumor budding is usually available [23, 29–31], and as such, important part of a histopathological assessment. In addition, the standard procedure for determining adenocarcinoma differentiation grade uses the percentage of the tumor mainly made of tumor glands [24].

With this parameter, the highest percentage of cancers are moderately differentiated (G2), and, as such, not sufficiently accurate to determine prognosis. Therefore, determining the degree of differentiation by poorly differentiation clusters (PDC) counting is suggested as a substitute, which can be also applied to preoperative biopsy [12, 32–34]. While tumor budding was defined as a single cancer cell, or cancer cells clusters with less than 5 cancer cells, PDC were defined as cancer cells clusters comprising 5 or more cells infiltrating the stroma without gland formation. However, PDC counting as well as tumor budding has not yet come into routine use.

Lacunarity

Word lacunarity comes from the Latin word *lacuna*, which means slit or lake [35]. In geometry, the term lacunarity describes the way in which a formation fills a space, whereby formations with more slits, or larger slits, also have a greater slackness. It is primarily considered as a measure of spatial heterogeneity (inhomogeneity) in an image. It quantifies how data (objects/pixels) fill the space, and enables analysis of gaps distribution, homogeneity of gaps, and presence of structures [36]. The important property of lacunarity is its sensitivity to clustering (aggregation) of structural elements [36]. In this way the complexity of the stained tissue could be quantified [37]. In the literature, the Greek letters Λ or λ are used as symbols for lacunarity.

Lacunarity is impossible to calculate manually, so it is determined using different computational methods. It should be emphasized that there is no one standard method, but several procedures have been developed for assessing and interpreting lacunae. The most common method uses a process called box counting [35]. Similar to looking at a microscopic slides at different magnification levels, the box counting algorithm looks at a digital image at different resolution levels to determine how certain features change when resizing an element used for image inspection. The number of pixels in each box is counted, and the mean (μ) and standard deviation (σ) of pixels per box are counted. The result could be expressed in a different ways, and the version of result used in this paper is called the CV^2, where CV denotes coefficient of variation [35, 38]. In another words,

$$\text{Lacunarity}(\Lambda) = (\sigma/\mu)^2$$

Analysis of the lacunarity of glandular elements in tumors has shown a significant difference in comparison to healthy tissue [39]. It has also been shown that the lacunarity of histological elements can be used to predict tumor susceptibility to chemotherapy [37, 40]. The lacunarity of the epithelial elements of intestinal adenocarcinoma has so far been analyzed solely as a diagnostic tool, that is, the role of lacunarity in discriminating healthy and neoplastic tissue [41]. However, the significance of this factor in the analysis of the biological behaviour of colorectal carcinoma, according to available data, has not been analyzed.

Patients and Methods

Patient Selection

The number of patients included in the study was 105. They were selected from the archive of the Department of Pathology and Cytology (University Hospital Dubrava, Zagreb, Croatia), and represent patients surgically treated for primary colorectal adenocarcinoma in the period from January 1 2009 till June 30 2012. Only patients with matched preoperative biopsy and resected post-operative material were included. Patients were excluded in the case of: (a) preoperative biopsy material was not available or in insufficient quantity for IHC staining; (b) inflammatory bowel disease present; (c) familial adenomatous polyposis; (d) neoadjuvant chemoradiotherapy; (e) survival data missing. Clinical data were obtained from the hospital database system, and included age (years) at

diagnosis, gender, tumor location. Survival data were obtained from the Croatian National Cancer Registry.

Slide Selection and Staining

All biopsies were fixed in 10% buffered formalin, paraffin-embedded and archived at the Department of Pathology and Cytology (University Hospital Dubrava, Zagreb, Croatia). Routine HE slides of preoperative biopsies and post-operative resected material were revised by two independent pathologists experienced in gastrointestinal pathology. Slides were stained with primary antibody for pan-cytokeratin (clone AE1/AE3, DAKO, Glostrup, Denmark, product number M3515, dilution 1:75).

Staining protocol:

1 Incubation time 30 min at 22 °C in DAKO autostainer link 48 + with epitope retrieval HIER PT link 20 min at 97 °C.
2 DAKO Polymer Conjugate Envision K5007, 30 min at 22 °C.
3 DAKO Chromogen DAB K5007, 10 min at 22 °C.
4 HE counterstaining.

Budding Counting in Preoperative Biopsy

Preoperative biopsies were reviewed for evidence of intratumoral budding using two methods. The first was modified Nakamura's method by Giger at al. [31], based on the three-point scale: 0 – no budding at magnifications × 40 and × 100; 1 (low-grade budding) – no budding at magnification × 40, but visible at × 100; 2 (high-grade budding) – budding visible at magnification × 40. The second method was method recommended by Lugli et al. [22], also based on three-point scale: 1 (low budding) 0–4 buds on magnification × 200 per 0.785 mm^2; 2 (intermediate budding) 5–9 buds on magnification × 200 per 0.785 mm^2; 3 (high-budding) ≥ 10 buds on magnification × 200 per 0.785 mm^2. Also, the absolute bud number was recorded for each specimen. All countings were made on CK AE1/AE3 stained slides.

Histopathological Staging of Resected Postoperative Material

The American Joint Committee of Cancer (8th Edition) criteria were used for pathological staging of resected material: histological type, histological grade, intravascular and perineural invasion, and pTNM [24].

Determination of PDC in Preoperative Biopsy

In preoperative biopsy, PDC was measured on the three-point scale: G1–0 PDC on magnification × 200; G2–1–2 PDC on magnification × 200; G3–3 and more PDC on magnification × 200 [32].

Image Acquisition and Analysis

Preoperative biopsy tumor area on CK AE1/AE3 stained slides was acquired using OLYMPUS B41 microscope (OLYMPUS, Tokyo, Japan), with OLYMPUS DP71 camera (OLYMPUS, Tokyo, Japan). Magnifications used were × 4, ×10, × 20, and × 40. Selected pictures were stored in JPEG format (2040 × 1536 pixels).

Lacunarity Analysis

The lacunarity analysis was performed by ImageJ program (www.imagej.nih.gov/ij/download.html). It is a freely available image analysis program developed by the National Institutes of Health (NIH) of the United States of America [42]. The program is written in Java programming language and its capabilities can be extended through various plug-ins [42]. One such add-on is FracLac, which enables the analysis of lacunarity (Figs. 1 and 2) [35].

Data Analysis

All obtained data were organized in a data matrix and analysed using data analysis software R, version 3.5 [43]. Counted data and scores are presented as numbers and percentages, while continuous data are presented as means, standard deviations (SD) and medians [44].

Prognostic significance of lacunarity was analysed using recursive partitioning implemented as rpart module in the programming language R [43, 45]. The name rpart is the acronym for Recursive PARTitioning, and this module is the most used application for construction of survival trees, a method which enables identification and comparison of prognostic factors in a simple and straightforward manner [46, 47]. This method begins the analysis with all patients included and divides them in prognostic subgroups. The final result is expressed as a survival tree, which contains decision nodes, and terminal nodes or leaves [46]. Each decision node contains variable which is used to subdivide patients in two subgroups with maximum difference in hazard ratios. This process is repeated until no further improvement in subdivision is possible, and terminal nodes are reached. Patients in the first decision node (node 1) has hazard ratio of 1. The hazard ratio for patients in other nodes is expressed in comparison to this value. The advantage of the recursive partitioning is clearly established the hierarchy of variables, that is, this method lists the variables by their importance for prognosis [47].

Additionally, the difference in survival between patients in terminal nodes was analyzed using log-rank test and presented as survival curves based on Kaplan-Meier survival estimate

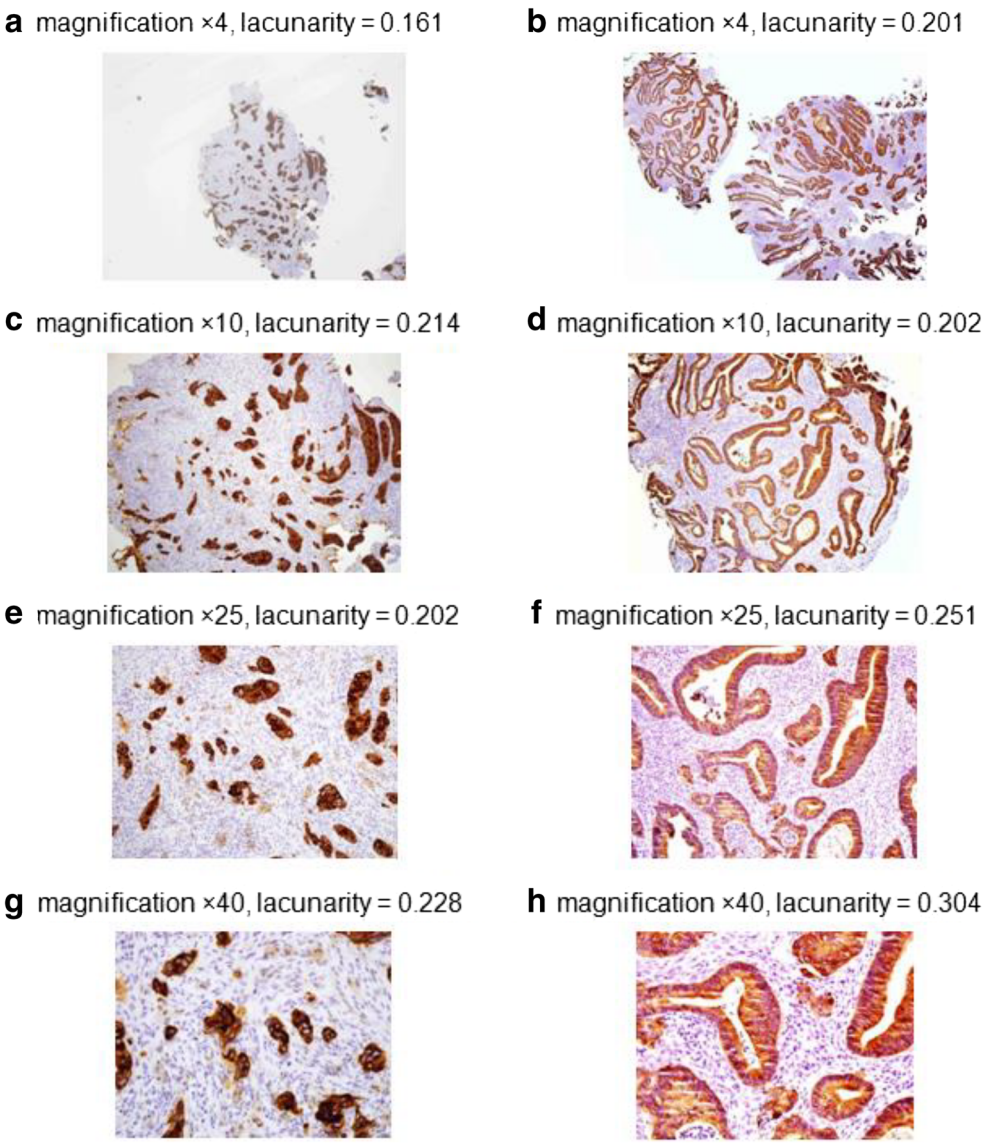

Fig. 1 Two examples of preoperative colorectal cancer slides (CK AE1/AE3 stained) with low (1A, 1C, 1E, 1G) and high lacunarity values (1B, 1D, 1F, 1H) at magnifications × 4, ×10, × 20 and × 40.

[44]. This part of analysis was based on survival module in programming language R [43, 48]. Censored data are shown only for the tails of survival curves, because this information is important for the estimation of follow-up maturity. Data were considered statistically significant if the P value of the log-rank test was ≤ 0.05 [45].

Results

Characteristics of patients and parameter of the preoperative biopsy, matched with post-surgical resection, are given in Table 1. Different budding parameters and lacunarity are shown as Table 2.

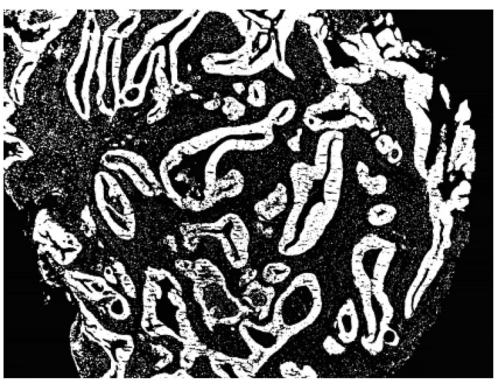

Fig. 2 The example of binary image (black and white), which is automatically created by ImageJ software before lacunarity measurement.

Table 1 Patients (n = 105) and pTNM parameters.

Age (years)	66.9 ± 10.60 (mean ± SD), 69 (median)
Gender	Female − 42 (40%), Male − 63 (60%)
Histological type	Non-mucinous − 96 (91.4%), Mucinous − 9 (6.9%)
Localisation	Rectum − 51 (48.6%), Left colon − 27 (25.7%), Right colon − 27 (25.7%)
pT	pT1–4 (3.8%), pT2–10 (9.5%), pT3–84 (80.0%), pT4a − 6 (5.7%), pT4b − 1 (0.98%)
pN	PN0–42 (40.0%), pN1a − 17 (16.2%), pN1b − 18 (17.1%), pN1c − 1(0.98%), pN2a − 14 (13.3%), pN2b − 13 (12.4%)
No. lymph nodes collected	14.1 ± 8.22 (mea ± SD), 13 (median)
Metastasis	1a − 5 (4.8%), 1b − 0 (0.0%), 1c − 4 (3.8%)
Lymphovascular invasion	Positive − 45 (42.9%), Negative − 60 (57.1%)
Perineural invasion	Positive − 37 (35.2%), Negative − 68 (64.8%)
Grade	Grade 1–2 (1.9%), Grade 2–83 (79.0%), Grade 3 − 1 (10.4%), Missing − 9 (8.6%)
Censor	Censored − 56 (53.3%), Noncensored − 49 (46.7%)
Follow-up (months)	19.9 ± 33.9 (mean ± SD), 73.3 (median)

Table 2 Budding and lacunarity parameters in the preoperative biopsy in CK AE1/AE3 stained slides.

Budding (Nakamura) [11]	Score 0–22 (21.0%), Score 1–38 (36.2%), Score 2–45 (42.8%)
Tumor bud count [22]	4.5 ± 5.54 (mean ± SD), 2 (median)
Tumor budding score (Lugli) [22]	Score 1–66 (62.9%), Score 2–15 (14.3%), Score 3–19 (18.1%), Missing (4.8%)
PDC [32]	Score 1–23 (21.9%), Score 2–39 (37.1%), Score 3–38 (36.2%), Missing − 5 (4.8%)
Lacunarity (× 4)	0.23 ± 0.046 (mean ± SD), 0.22 (median)
Lacunarity (× 10)	0.27 ± 0.074 (mean ± SD), 0.25 (median)
Lacunarity (× 20)	0.31 ± 0.093 (mean ± SD), 0.29 (median)
Lacunarity (× 40)	0.36 ± 0.139 (mean ± SD), 0.32 (median)

Prognostic Value of Lacunarity

Prognostic value of lacunarity (at magnifications × 4, × 10, ×20, × 40) was analysed using recursive partitioning. Data used for analysis were patients age and gender, tumor localisation, histological type and grade, and all parameters from Table 2.

Figure 3 shows nine most informative variables extracted with rpart, and their correlations are shown as Table 3. However, the survival tree suggests that the combination of three variables is sufficient to divide samples in prognostic subgroups (Figs. 4 and 5). The survival tree (Fig. 6) consists of three decision nodes and four terminal nodes (leaves), and the relation between them could be described in the following way:

- Decision node 1, or the starting node, contains 105 patients, and their hazard ratio is 1. The variable used in this node to subdivide patients is lacunarity (Λ) at magnification × 4. The left branch with values of lacunarity > = 0.20 leads to node 2, which contains 82 patients with hazard ratio of 0.80. The right branch with values of lacunarity < 0.20 leads to node 3, which contains 23 patients with hazard ratio of 1.84.
- Decision node 2 could be subdivided in two terminal subgroups, or leaves, using the bud count variable. The left branch, with the bud count < = 3, leads to leaf 1, which contains 51 patients, and their hazard ratio is 0.58. The right branch, with the bud count > 3, leads to leaf 2, which contains 31 patients with the hazard ratio 1.21.
- Decision node 3 could be subdivided in two leaves, or nodes, using the lacunarity (Λ) at magnification × 10. The left branch, with the lacunarity values > = 0.22, leads to leaf 3, which contains 14 patients, and their hazard ratio is 1.18. The right branch, with the lacunarity values < 0.22, leads to leaf 4, which contains 9 patients with the hazard ratio 3.16.

The results of recursive partitioning were further supplemented by survival curves (Kaplan-Meier method) for subgroups defined in each decision node. The difference for subgroups defined by left and right branch of decision node 1 is shown as Fig. 7, and it is statistically significant (log-rank test, P = 0.001). The subgroups defined by left and right branch of node 2 are shown as Fig. 8 (log-rank test, P = 0.010). Figure 9

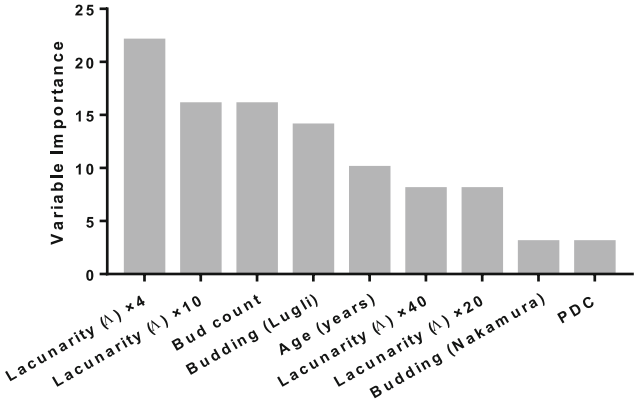

Fig. 3 Variable importance determined by rpart method.

shows that the difference between subgroups of node 3 is also significant (log-rank test, P = 0.043).

Discussion and Conclusions

The quantity and quality of preoperative material in colorectal cancer is often limiting factor in determination of risk factors and therapy planning. The most important negative prognostic factors are intravascular and perineural invasion, and tumor budding [24–28]. Usually, tumor budding is the only available prognostic parameter that can be determine in preoperative biopsy [23, 29–31]. Also, the growing body of evidence suggest that cancer differentiation based on a poorly differentiated clusters has better prognostic value [12, 32–34].The limiting factor in applying of these new parameters is reproducible, simple, cheap and fast method of their determination. Lugli et al [22] proposed budding determination method which can

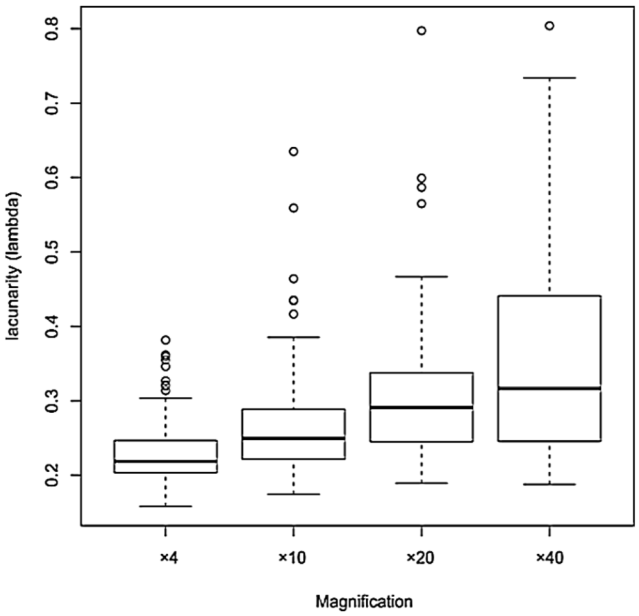

Fig. 4 Box-and-plot description of lacunarity (Λ) at magnifications × 4, ×10, × 20 and × 40.

be applied both on resected specimen and preoperative biopsy. However, the recent papers still use other methods [18, 49–51].Moreover, the new papers suggest combinations of negative prognostic factors as a base for novel histological grading systems of colorectal cancer [48, 52].

The obtained results (Fig. 6) show that the combination of lacunarity and number of buds allow the division of patients into three clearly separated risk subgroups. This division was obtained using rpart method, which expresses the result of the analysis as a survival tree. The interpretation of the survival

Table 3 Spearman's rank order orrelations of lacunarity (Δ) at different magnifications with other five most informative variables (Fig. 3). Bold marked values are statistically significant at level 0.05.

Variable	Lacunarity (Λ) (× 4)	Lacunarity (Λ) (× 10)	Lacunarity (Λ) (× 20)	Lacunarity (Λ) (× 40)
Lacunarity (Λ) (× 4)	1.00	**0.44**	**0.29**	**0.36**
Lacunarity (Λ) (× 10)	**0.44**	1.00	**0.53**	**0.41**
Lacunarity (Λ) (× 20)	**0.29**	0.53	1.00	**0.70**
Lacunarity (Λ) (× 40)	**0.36**	**0.41**	**0.70**	1.00
Total bud count per 0.785 mm^2	-0.17	**-0.32**	**-0.39**	**-0.47**
Budding (Lugli)	**-0.20**	**-0.32**	**-0.40**	**-0.39**
Age (years)	0.10	0.11	0.12	0.12
Budding (Nakamura)	0.06	-0.08	-0.08	-0.01

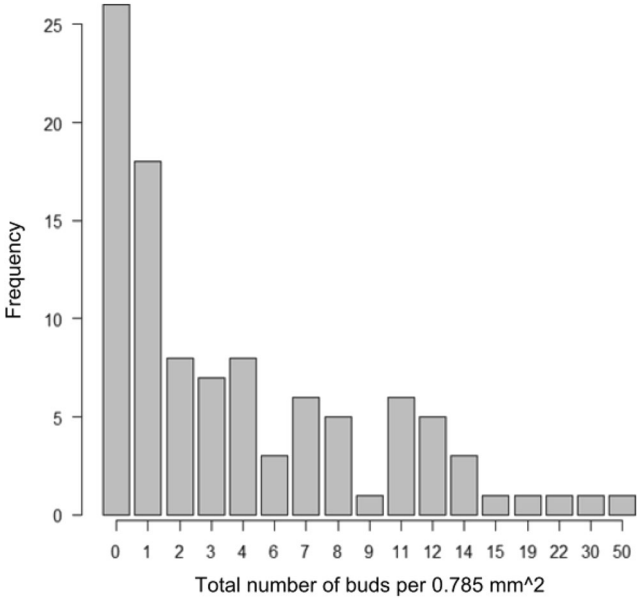

Fig. 5 Distribution of patients by total bud count per 0.785 mm^2.

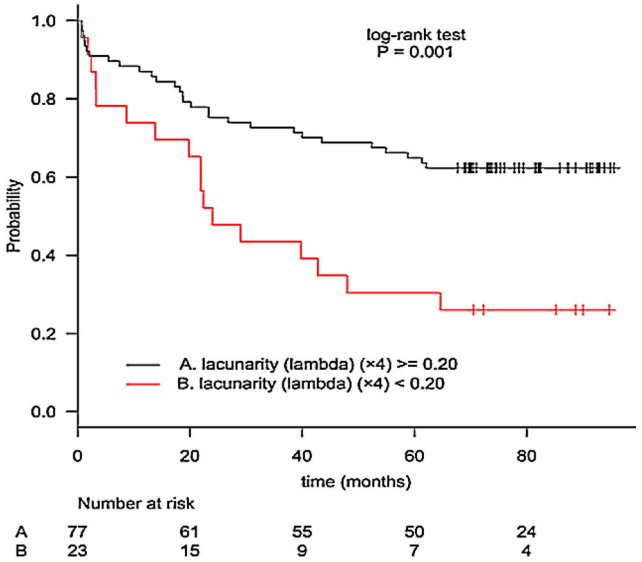

Fig. 7 Difference in patient's survival for the left and right branches of starting decision node (node 1)

tree is relatively straightforward, but two safety measures should be included: (1) The survival tree does not explain the biology of the disease – it connects the information value of variables on the survival of subgroups, expressed as a hazard ratio. However, with cautious interpretation the survival tree can give an important insight into the pathophysiology of the disease; (2) rpart constructs survival tree by dividing starting group in subgroups with maximal difference between them, but which are maximally homogeneous internally. The values in decision nodes should not be interpreted independently and individually, because the survival tree is a functional unity. For example, numerical values in the left and right branches of the decision node 2 (bud count < = 3 and bud count > 3) should be applied only to patients of the left branch of the decision node 1, but not for patients (node 1), or patients in the decision node 2.

Lacunarity (Λ) at magnification × 4, the variable used in the starting node (node 0), subdivides patients in low- and high-risk subgroups. This division is not ideal because medium-risk group is distributed between these two subgroups. In the second step, bud count variable is used to precisely separate low-risk patients from medium-risk patients.

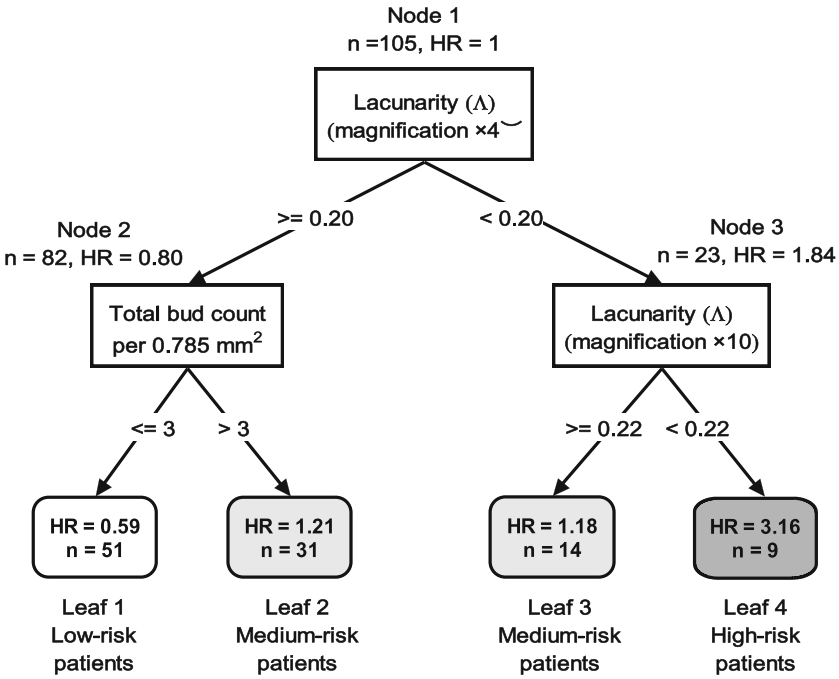

Fig. 6 The survival tree constructed using rpart method identifies three subgroups of patients: low-risk subgroup (white terminal node); medium-risk subgroups (light-gray terminal nodes), high-risk subgroup (dark-gray terminal node).

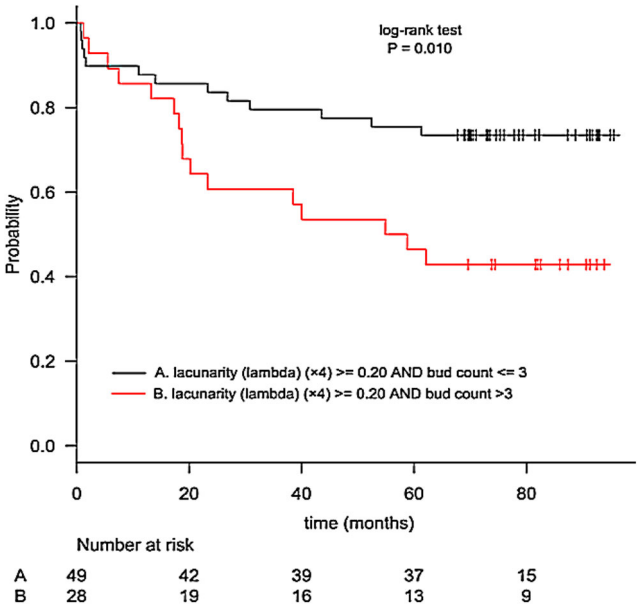

Fig. 8 Difference in patient's survival for the left and right branches of the second decision node (node 2).

Finally, in the third step lacunarity (Λ) at magnification × 20 is used to separate high-risk patients from medium-risk patients. As a conclusion, we may say that lacunarity (Λ) at magnification × 4 estimates global risk, bud count clearly identifies low-risk patients, and lacunarity (Λ) at magnification × 20 points to high risk patients. The combination of these three variables is superior to individual variables because defined risk subgroups are maximally homogenous, with minimal difference between them, measured by the hazard ratio criterion. This is

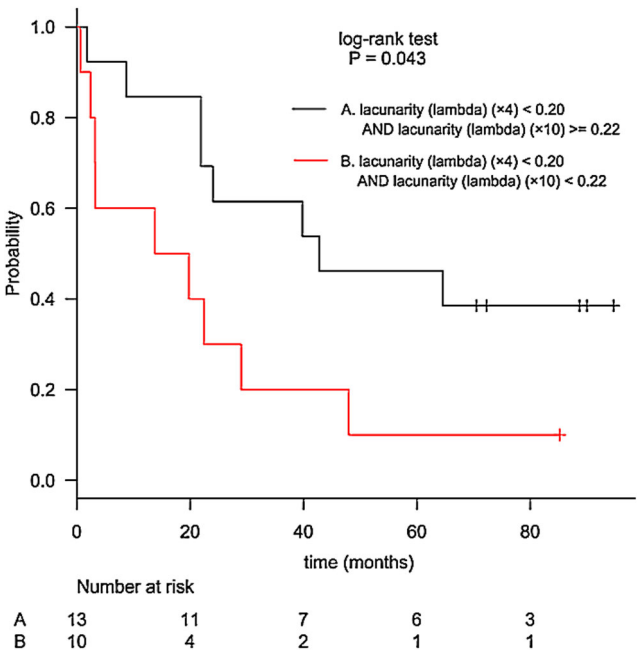

Fig. 9 Difference in patient's survival for the left and right branches of the third decision node (node 3).

additionally confirmed by the log-rank test and clearly separated survival curves, as shown in Figs. 7–9.

Pathology is in many aspects subjective and dependent on the experience of the pathologist. Different methods help in objectification, e.g. immunohistochemical analysis as a more complete representation of tumor elements. Lacunarity in our research is a quantitative method independent of the pathologist's experience, apart from selecting the field of the image where the pathologist is important.

Of course, in preoperative colon cancer biopsy, this subjectivity is minimized because the entire area of tumor tissue is covered by a single image. Furthermore, lacunarity as a method involves multiple negative prognostic elements, i.e. buds, poorly differentiated clusters, and their distribution in space.

An important feature of lacunarity is that it quantifies the homogeneity/inhomogeneity of the sample, in a way that high values indicate inhomogeneity and low values indicate greater homogeneity of tumor elements. High homogeneity is associated with a greater number of buds and PDCs, which is a feature of less differentiated tumors. From Fig. 6, it is obvious that patients with a lacunarity value at a magnification × 4 less than 0.20, and a lacunarity value at a magnification × 20 less than 0.22 have an extremely poor prognosis, that is their HR is 3.16.

According to Lugli et al., it is recommended that tumor budding should be counted on HE stained samples, but the strength of the evidence of this recommendation is moderate [22]. This applies to cases of tumors with a pronounced inflammatory infiltrate or severe stromal response. In such case, it is recommended to confirm the number of buds using immunohistochemical cytokeratin staining. Several of the references, cited after these recommendations, advise determining the degree of budding on IHC tissue samples.

In our research, tumor budding was determined on IHC tissue samples to avoid these problems. One of the reasons for recommending HE staining is because it is routine, simplest and cheapest procedure available in all laboratories. However, IHC staining is also practically a routine nowdays, and it is used not only to assist in tumor differentiation but also to identify parameters that are essential for further therapy (e.g. HER2, BRAF, PD-L1, MSI status, etc.)

We used (CK1/AE3), which is one of the most commonly used, priced acceptable, and extends the diagnostic procedure by maximally 1 day. Furthermore, this staining method makes individual tumor cells much easier to identify and significantly increases the measurement reliability (intra- and inter observer agreement) [20, 21]. Given the results obtained, the usefulness of using this staining method justifies the time and resources spent. In addition, IHC staining method unified our measurements because lacunarity and budding were determined on the same samples.

Lacunarity, which has proven to be the most informative variable in the design of a prognostic algorithm, is not

demanding by the time criterion, or by material costs. The lacunarity determination programs (ImageJ and FracLac module) are publicly available and easy to use, so the only prerequisite is a computer system that can capture and store microscopic images. This should not be a problem in most laboratories, since such a system is necessary to measure the quantitative parameters of individual tumors, eg melanoma thickness, size of micrometastases, microinvasive tumors, etc.

Lacunarity includes tumor buds, poorly differentiated clusters, and their arrangement in space. The results presented in Fig. 6 are in accordance with recent paper of Konishi and Wai Kwan Lee[34, 53], which suggest combination of tumor budding and PDC. According to the survival tree (Fig. 6), it has proven to be the most informative variable, so the question arises whether buds and clusters should be combined in defining prognostic index because their combination is clearly superior to the individual variables. Creating algorithms that merge multiple variables is not complicated, and the algorithms expressed as a survival tree are easy to interpret.

The results clearly indicate that a combination of multiple parameters is essential for the classification of patients into valid risk subgroups. Lacunarity (magnification × 4 and × 20) contains information about multiple morphological parameters, so the rpart method singled out these parameters as the most informative. The number of buds is an excellent complement to the lacunarity and is in third place by information value criterion. It is interesting that the PDC shows less information content in determining the prognosis in relation to lacunarity and number of buds, but also in comparison to a number of other parameters (Fig. 1).Our results were obtained on preoperative biopsy which is taken from the superficial part of cancer, in contrast to the vast majority of literature data describing negative prognostic factors in invasive tumor front. This is an obvious advantage of suggested prognostic algorithm.

The paper points that quantitative numerical methods are superior to semiquantitative methods, and that individual methods should be combined using algorithms to obtain a more accurate prediction. Because the study described is designed as a pilot study, verification is needed on a larger sample of patients from independent researchers.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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