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ARTICLE

Prognostic impact of tumor budding in endometrial carcinoma within distinct molecular subgroups

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Abstract

Tumor budding is a robust prognostic parameter in several tumor entities but is rarely investigated in endometrial carcinoma. We applied the recently standardized counting method from the International Tumor Budding Consensus Conference for colorectal cancer (ITBCC) on a cohort of 255 endometrial carcinomas with known molecular profiles according to The Cancer Genome Atlas (TCGA) subgroups. Our investigation aims to clarify the potential prognostic role of tumor budding in endometrial carcinoma in contrast to other known prognostic factors, including molecular factors. In addition, the microcystic elongated and fragmented (MELF) pattern and tumor budding were compared with respect to their potential as markers for epithelial–mesenchymal transition (EMT). Tumor budding was found in $n = 67$ (26.3%) tumors, with a very low mean of 0.7 buds per ×20 HE field. Tumor budding was significantly associated with depth of invasion, nodal status, lymphatic invasion (each $p < 0.001$), grading ($p = 0.004$), and vascular invasion ($p = 0.01$). Tumor budding showed moderate inter-observer-variability with prognostic stratification irrespective of the observer (κ-value = 0.448). In multivariate analysis, tumor budding served as a significant independent prognosticator for worse outcomes in overall and recurrence-free survival (HR 2.376 and 2.736, $p < 0.001$), but not when the TCGA subgroups entered into the analysis. In consequence, dependency had to be clarified in the subgroup analysis for Polymerase E mutated (POLEmut), mismatch repair deficient (MMRdef), nonspecific mutation profile (NSMP), and P53 aberrant (P53abn) endometrial carcinomas. A particular impact was identified in the intermediate prognostic groups of NSMP and MMRdef carcinomas. Tumor budding outperformed the MELF pattern in single and combined prognostic information. In conclusion, the presence of tumor budding alone is a promising, robust, and easy-to-apply prognostic parameter in endometrial carcinoma. In a morpho-molecular approach, it exerts its prognostic potential in the most clinically relevant subgroups of endometrial carcinoma and serves as a good biomarker for EMT.

Introduction

Since the introduction of The Cancer Genome Atlas (TCGA) on endometrial carcinoma (EMCA) in 2014, the important question has been widely discussed of whether the two-tiered Bokhman system of type I and type II endometrial cancer should be expanded to a four-tiered system based on molecular profiling [1–3]. POLE-mutated cases are of particular interest as their excellent prognosis allows patients to possibly avoid aggressive treatments [4–6]. On the other hand, serous-like carcinoma with evidence of the TP53 mutation represents a more aggressive disease, with over 40% percent of patients dying within 5 years [7]. However, the cases of mismatch repair deficient (MMRdef) carcinomas and, moreover, the nonspecific mutation profile endometrial carcinoma (NSMP-EMCA)—outlined as copy number variation in the TCGA

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analysis—lead to a therapeutic conundrum in two thirds of endometrial cancer cases with intermediate prognosis [7].

Therefore, novel biomarkers should be investigated that could improve the stratification of classical EMCA. Recently, tumor budding evolved to be a highly prognostic marker in many solid tumors, such as those of the lung, pancreas, esophagus, and colorectum [8–12]. Particularly, the latter shows many morphological similarities with EMCA in terms of gland formation, resulting in similar grading systems, "dirty" necrosis as well as molecular/genetic molecular genetic aberrations such as MMR-deficient tumors, carcinomas stratified according to copy number variants, and even the existence of POLE-mutated carcinomas [13]. However, tumor budding in EMCA has so far been rarely investigated in EMCA [14–17]. Han et al. associated "extensive" tumor budding as a side parameter for occult lymph node metastasis based on endometrial biopsy specimens within a small cohort without defining an assessment method or cut-offs for tumor budding estimation [15]. Koyuncuoglu et al. and Park et al. defined tumor budding similarly to colorectal cancer as $n < 5$ cells aside the cohesive tumor part and chose a cut-off of five tumor buds per 20 HPF hotspots as the analytical criteria. Both studies found effects on worse overall survival given the presence of high tumor bud counts and an association with other pathological parameters like depth of invasion, grading, and nodal status [16, 17]. However, until now, an analysis of tumor budding in the context of the molecular background of EMCA has never been performed.

An advantage of tumor budding in comparison with other pattern-based approaches like the microcystic elongated and fragmented (MELF) pattern lays in its more quantitative rather than qualitative evaluation [11, 18]. Of note, tumor budding measurement has recently been standardized for colorectal cancer during an International Tumor Budding Consensus Conference (ITBCC) [19].

In this study, we aim to clarify the role of tumor budding in EMCA in a large, single-center cohort using the approach of a standardized tumor budding measurement. The effect of tumor budding on the outcome will be compared with the classical prognostic parameters as well as to the newer molecular TCGA subgroups in endometrial cancer.

Material and methods

Patient cohort

A total of $N = 255$ tumor samples from patients were gathered retrospectively, selected by the availability of tumor tissue with the diagnosis of EMCA after surgery from patients who gave consent between 2004 and 2015 to the use of their tissue for research. The University Hospital of Bern is a tertiary referral clinical; the patients in the cohort are therefore representative of high-risk tumors and patients. All patient characteristics are displayed in Table 1. The cohort is representative of the tertiary referral clinic setting, meaning a higher-risk patient cohort. One patient with

Table 1 Patient characteristics.

Feature	Freq *N* (%) or mean (range)
Patient age ($n = 255$)	66.0 (33–92)
BMI ($n = 249$)	30.6 (16–60)
Menopausal status ($n = 255$)	
Pre menopausal	12 (4.7%)
Post menopausal	231 (90.6%)
Peri menopausal	12 (4.7%)
Endometrioid	212 (83.1%)
Serous	12 (4.7%)
Mucinous	3 (1.2%)
Histological subtype ($n = 255$)	
Clear cell carcinoma	7 (2.7%)
Villoglandular	12 (4.7%)
Other	9 (3.5%)
pT1a	123 (48.2%)
pT1b	70 (27.5%)
pT2	33 (12.9%)
T-category	
pT3a	14 (5.5%)
pT3b	13 (5.1%)
pT4	2 (0.8%)
cN0	47 (18.4%)
cN1	1 (0.4%)
N-category	
pN0	163 (63.9%)
pN1	44 (17.3%)
None	48 (18.8%)
Pathological lymph node examination	
Sentinel only	14 (5.5%)
Dissection	193 (75.7%)
Sentinel only	3.1 (1–6)
Number of lymph nodes	
Dissection	32.2 (2–85)
G1	92 (36.1%)
Tumor grade	
G2	107 (42.0%)
G3	56 (22.0%)
L0	197 (77.3%)
Lymphatic invasion	
L1	58 (22.7%)
V0	201 (78.8%)
Venous invasion	
V1	54 (21.2%)
Present	48 (18.8%)
MELF pattern	
Not present	207 (81.2%)
POLEmut	10 (3.9%)
MMRdef	81 (31.8%)
TCGA subgroups	
NSMP	132 (51.8%)
P53abn	32 (12.5%)

clinical evidence of lymph node metastasis had parallel distant metastasis and was not selected for lymphadenectomy but was included as a cN1 patient. In three of the 255 resections we had to refer to the prior curettage material for the major tumor component. A centralized reevaluation was performed according to the 4th edition of the WHO classification of tumors of the female reproductive organs [20] for subtypes and grading and the 8th edition of the TNM-classification [21] including lympho-vascular status.

Tissue microarray construction

To assure homogenous immunohistochemical testing conditions for the complete cohort, a next-generation tissue microarray (ngTMA) was created according to principles published previously [22]. In brief, punch biopsies 0.6 mm in diameter were assembled in triplicate of the tumor center and invasive front from all $n = 255$ EMCA and transferred into recipient blocks. All steps were controlled visually using digitized whole slide images using the Panoramic Flash Scanner BD250 (3DHistech, Budapest, Hungary) and TM Grandmaster (3DHistech, Budapest, Hungary).

Immunohistochemistry

Routine protocols for immunohistochemistry were used (Table 2). According to accreditation, protocols met the criteria of certified round robin tests (QUIP, Germany, and NORDICQ, Denmark) and international recommendations for these specific antibodies were followed [23–27]. Internal on-slide controls were present as per the ngTMA approach with a variety of cancers on each slide.

POLE mutational status and TCGA stratification

The POLE mutational status was based on hotspot-Sanger sequencing of exons 9–14 as published previously [4]. A very conservative approach excluding variants of unknown significance was applied, in accordance with recent publications from whole-exome sequencing data on POLE mutations [5, 6]. All POLE mutations included in this study were reevaluated as pathogenic using the novel POLE risk score, which is based on six specific molecular criteria. In detail, the ten POLE-mutated cases comprised 7x P286R, 2x S297F, and 1x V411L mutations, which all have a POLE risk score of 4–6 [5].

Further stratification into separate TCGA subgroups was performed with MMR-protein immunohistochemistry and p53 immunohistochemistry as described above and applied to the tissue microarray slides. Mismatch repair deficiency (MMRdef) was defined as loss of nuclear staining in at least one out of the four proteins MLH1, MSH2, MSH6, or PMS2 as described previously [26–28]. Aberrant P53 protein expression (p53abn) in terms of a mutational pattern was interpreted, if either complete loss of nuclear protein expression or strong homogenous overexpression was encountered as proposed by Kobel et al. [23] as the optimal surrogate marker for true p53 mutations. The limitations of ngTMA immunohistochemistry evaluation were balanced with analysis in triplicate and case controls of borderline cases on whole slide images during a separate multi-centric cohort with access to the corresponding mutational status of selected cases [6].

Tumor budding and MELF pattern assessment

Evaluation of tumor buds was performed as described by the ITBCC [19]. In brief, it should be screened for the most relevant tumor slide at low magnification and next a hotspot in ×20 HPF analyzed. A tumor bud was defined as a cluster of 1–4 tumor cells detached from the cohesive tumor part. The evaluation took place on whole slide images from scans with the panoramic Scanner Flash BD250 (3DHistech, Budapest, Hungary) and displayed with CaseViewer (3D Histech, Budapest, Hungary). A ×20 HPF with an area of 1.2 mm^2 was evaluated in the hotspot region of the tumor. MELF pattern was evaluated according to early descriptions in the literature and described as present or not [29]. Evaluation of the tumor budding and MELF pattern was performed by TR, LC, and EB.

Statistics

Associations of tumor budding counts and categorical patient characteristics were analyzed using the nonparametric Wilcoxon test or Kruskal–Wallis test, where appropriate.

Table 2 Antibodies and immunohistochemistry conditions.

Target	Clone	Provider	Dilution	Pretreatment	Stainer
p53	Do-7	Dako	1:800	EDTA at 95 °C for 20 min	Leica Bond
MLH1	M1	Roche Diagnostics	Ready to use	EDTA at 100 °C for 32 min	Ventana Benchmark
MSH2	G219-1129	Roche Diagnostics	Ready to use	EDTA at 100 °C for 40 min	Ventana Benchmark
MSH6	SP93	Roche Diagnostics	Ready to use	EDTA at 100 °C for 64 min	Ventana Benchmark
PMS2	A16-4	Roche Diagnostics	Ready to use	EDTA at 100 °C for 92 min	Ventana Benchmark

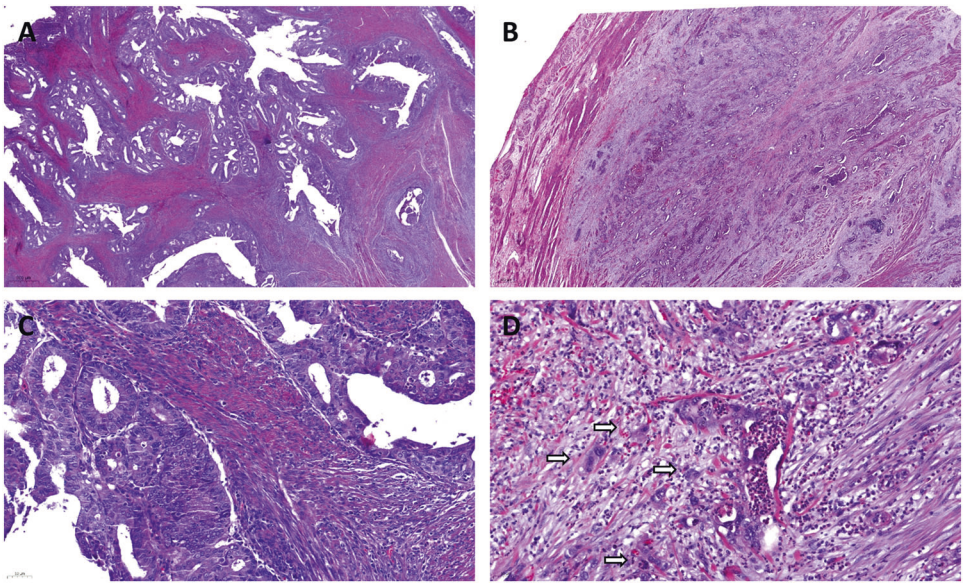

Fig. 1 Different levels of tumor budding in EMCA. Cases with no tumor budding visible in HE staining **a** [×4] **c** [×20] in contrast to a case with abundant tumor budding (arrows) comparable with colorectal cancer **b** [×4] **d** [×20]. Clearly negative cases for tumor budding show an expansile pushing invasive margin (**a**, **c**).

Inter-observer agreement was measured with Pearson's correlation, R^2-values, and Fleiss Kappa statistics. Student's t tests were used differences of means. Univariate survival analysis was performed using Kaplan–Meier curves with log-rank tests, while Cox proportional hazards regression analysis was used for multivariable analysis. Hazard ratios (HR) and 95% confidence intervals (CI) were used to determine effect size. Known confounding factors were included in the analysis. All analyses were two-sided, and results were considered statistically significant at $p < 0.05$.

Results

Quantitative aspects of tumor budding in EMCA

In our cohort the average number of tumor buds per ×20/HPF hotspot was very low across all patients, with a mean of 0.7 per ×20 high-power field, which is substantially lower than that in colorectal cancer cohorts. For example, mean tumor budding values of 15.8 were reported by Rieger et al. [30] and medians of 14 by Horcic [31] in a ×10 high-power field approach at ×40 magnification, which would even correspond to 7.97 and 2.14 corrected for the single ×20 field size of 1.2 mm^2 as applied in our study. Examples of tumor budding assessment in standard H&E staining are presented in Fig. 1. Of note, the number of EMCA cases showing no tumor bud was high ($n = 188$, 73.7%), which again is contrasted by the values of Horcic et al., with only 4 out of 105 colorectal cancers with no buds (3.8%) [31]. To highlight the distribution of tumor bud counts across the cohort, we depict the cumulative percentages of tumor budding counts in Fig. 2 for the complete cohort and molecular subtypes. In consequence, a biological cut-off of

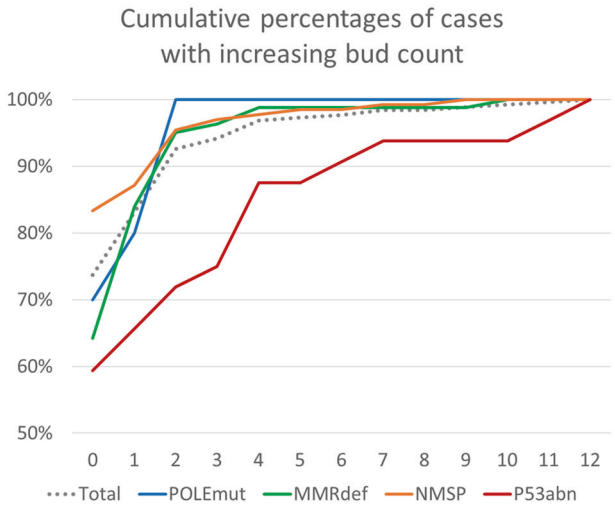

Fig. 2 Plot of cumulative percentages of cases according to tumor bud counts. Most cases of EMCA do not show any tumor buds. Cut-off determination was therefore chosen between no versus any buds. Of note, a cut-off above two would have excluded all POLEmut cases and a cut-off of five would have over-weighted p53abn cases.

no versus any tumor budding was further used for survival analysis, as the proposed stratification system for colorectal cancer [19] would not be meaningful. This also assured that no preselection towards a specific molecular subtype was introduced via a higher cut-off selection.

Inter-observer agreement of tumor budding assessment in EMCA

Tumor budding was rated by two independent pathologists (TR and LC). Regarding exact quantification the values correlated significantly between both observers (Pearson's $R = 0.727$, $R^2 = 0.529$, $p < 0.001$). With respect to the

applied cut-off a moderate inter-observer agreement was measured (κ-value = 0.448, $p < 0.001$). Examples of non-concordant cases are displayed in supplemental Fig. 1. The further analysis relies on consensus values after agreement. However, initial prognostic stratification was reached for both observers independently.

Tumor budding as a single prognostic parameter in EMCA

Tumor budding is significantly associated with all investigated clinicopathological features of the TNM system (Table 3). Of note, an increase in mean budding counts was detected from stage T1 to T4, as well as from G1 to G3 cases.

The prognostic effect of tumor budding regarded in univariate analysis leads to a significantly higher HR of 2.376 and 2.736 applying the cut-off of none versus any buds for overall and recurrence-free survival ($p < 0.001$ each).

In a quantitative view each additional tumor bud per ×20 HPF increases the risk for overall death 1.13 fold ($p = 0.020$), which tends to account for recurrence-free survival as well, with a similar HR of 1.12 ($p = 0.053$) for each additional tumor bud. This strong prognostic effect of tumor budding in EMCA can also be displayed in the corresponding survival curves (Fig. 3). Of note, this stratification applies very well to the clinically challenging low-stage tumors of pT1a and pT1b tumors without lymph node involvement. In detail, overall survival was significantly lower, if tumor budding was present in this subgroup (log-rank, $p = 0.002$). However, recurrence-free survival could not be stratified, as less events were noted (log-rank, $p = 0.12$).

Table 3 Association of budding counts with clinicopathological features.

Feature	ITBCC (mean)	*P* value
T stage		
T1	0.6	<0.0001
T2	0.5	
T3	1.6	
T4	4	
Grading		
G1	0.3	0.0036
G2	0.6	
G3	1.5	
MELF pattern		
0	0.56	0.001
1	1.3	
N stage		
N0	0.6	0.0004
N1	1.4	
M stage		
M0	0.7	0.0292
M1	1.5	
L stage		
L0	0.5	<0.0001
L1	1.4	
V stage		
V0	0.6	0.0134
V1	1.1	
Recurrence		
None	0.6	0.0105
Yes	0.9	

Tumor budding in tumors with marked heterogeneity

Regarding heterorgeneity $n = 44$ EMCAs were analyzed in more detail. Besides the hotspot assessment across the whole tumor, tumor budding was assessed in the heterogenous counterpart. Seven serous carcinomas showed a minor component of endometrioid or clear cell carcinoma. Herein, the two cases with tumor budding present showed more pronounced budding in the serous component. The three cases of clear cell carcinoma with adjacent minor endometrioid component were tumor budding negative. Thirty-two endometrioid adenocarcinomas showed minor elements not justifying a different subtype like squamous morules and mucinous cell components in <5% of the tumor. Only seven cases showed tumor budding. In detail, tumor budding was either more expressed in the major endometrioid component ($n = 3$) or vice versa ($n = 3$) and one case showed an identical tumor bud count in both parts. The last two cases (outlined as others in Table 1) comprised a carcinosarcoma and one endometrioid adenocarcinoma with neuroendocrine carcinoma component with no budding differences. For the further study purpose, the highest tumor budding count was used irrespective of heterogeneity.

Multivariate analysis of tumor budding and conventional prognostic parameters

In multivariate analysis, tumor budding showed a tendency towards reduced overall survival and was significantly associated with lower recurrence-free survival as an independent prognostic parameter (Tables 4 and 5). Interestingly, only T-stage and N-stage could be validated in both categories as independent prognostic parameters. Grading only fulfilled significance for overall survival.

The MELF pattern was also evaluated in multivariate analysis but needed to replace another parameter as too small subgroups would have led to overfitting of data. The MELF pattern (replacing grading) did not reach significance with $p = 0.566$ and $p = 0.759$ for overall and recurrence-free survival, respectively.

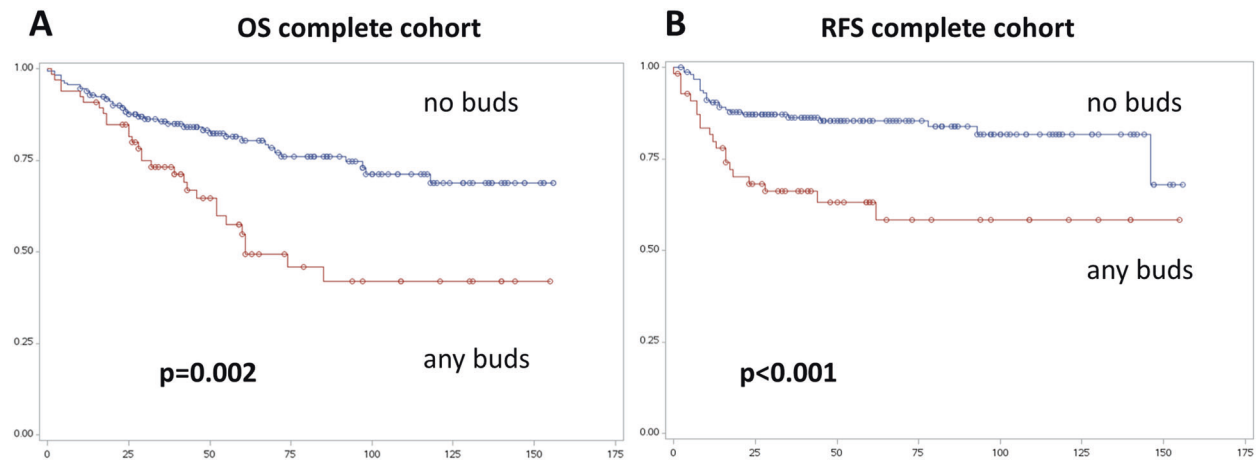

Fig. 3 Prognostication with tumor budding in the complete EC cohort. Kaplan–Meier curves of overall (**a**) and recurrence-free survival (**b**) across the complete cohort of EMCAs without further subtyping with *p* values derived from log-rank tests.

Table 4 Multivariate analysis of overall survival and tumor budding using ITBCC scores (no buds/any buds) in endometrial cancer.

Feature	HR (95% CI)	*P* value
ITBCC		
No buds	1.0	0.0634
Any buds	1.63 (0.9–2.7)	
T stage		
T1–2	1.0	0.0151
T3–4	2.4 (1.2–4.9)	
N stage		
N0	1.0	0.0277
N1	2.03 (1.1–3.8)	
M stage		
M0	1.0	0.1374
M1	1.84 (0.8–4.2)	
L stage		
L0	1.0	0.4897
L1	0.8 (0.4–1.5)	
V stage		
V0	1.0	0.2536
V1	1.37 (0.8–2.3)	
Tumor grading		
G1–2	1.0	0.0194
G3	1.55 (1.1–2.2)	

Table 5 Multivariate analysis of recurrence-free survival and tumor budding using ITBCC scores in endometrial cancer.

Feature	HR (95% CI)	*P* value
ITBCC		
No buds	1.0	0.0329
Any buds	2.0 (1.1–3.8)	
T stage		
T1–2	1.0	0.0875
T3-4	2.16 (0.9–5.2)	
N stage		
N0	1.0	0.0066
N1	3.13 (1.4–7.1)	
M stage		
M0	1.0	0.0041
M1	3.9 (1.5–9.8)	
L stage		
L0	1.0	0.654
L1	0.84 (0.4–1.8)	
V stage		
V0	1.0	0.0936
V1	1.7 (0.9–3.36)	
Grading		
G1–G2	1.0	0.7949
G3	1.06 (0.7–1.8	

Subgroup analysis according to TCGA molecular risk groups

The TCGA subgroups in our cohort showed a significant difference in outcomes in line with our original publication in 2013 where these molecular subgroups were presented (Fig. 4a, b). By analyzing the effect of tumor budding within each of those molecular subgroups, we identified tumor budding as a now dependent parameter with lost significance towards $p = 0.4973$ and no additional stratification effect with a HR of 1.05 (CI 0.9–1.2) in multivariate analysis.

Taking this into account, we considered a predominant role of tumor budding in certain TCGA subgroups. Indeed, tumor budding differs significantly in its expression levels between TCGA subgroups (Fig. 2, Table 6), but cases

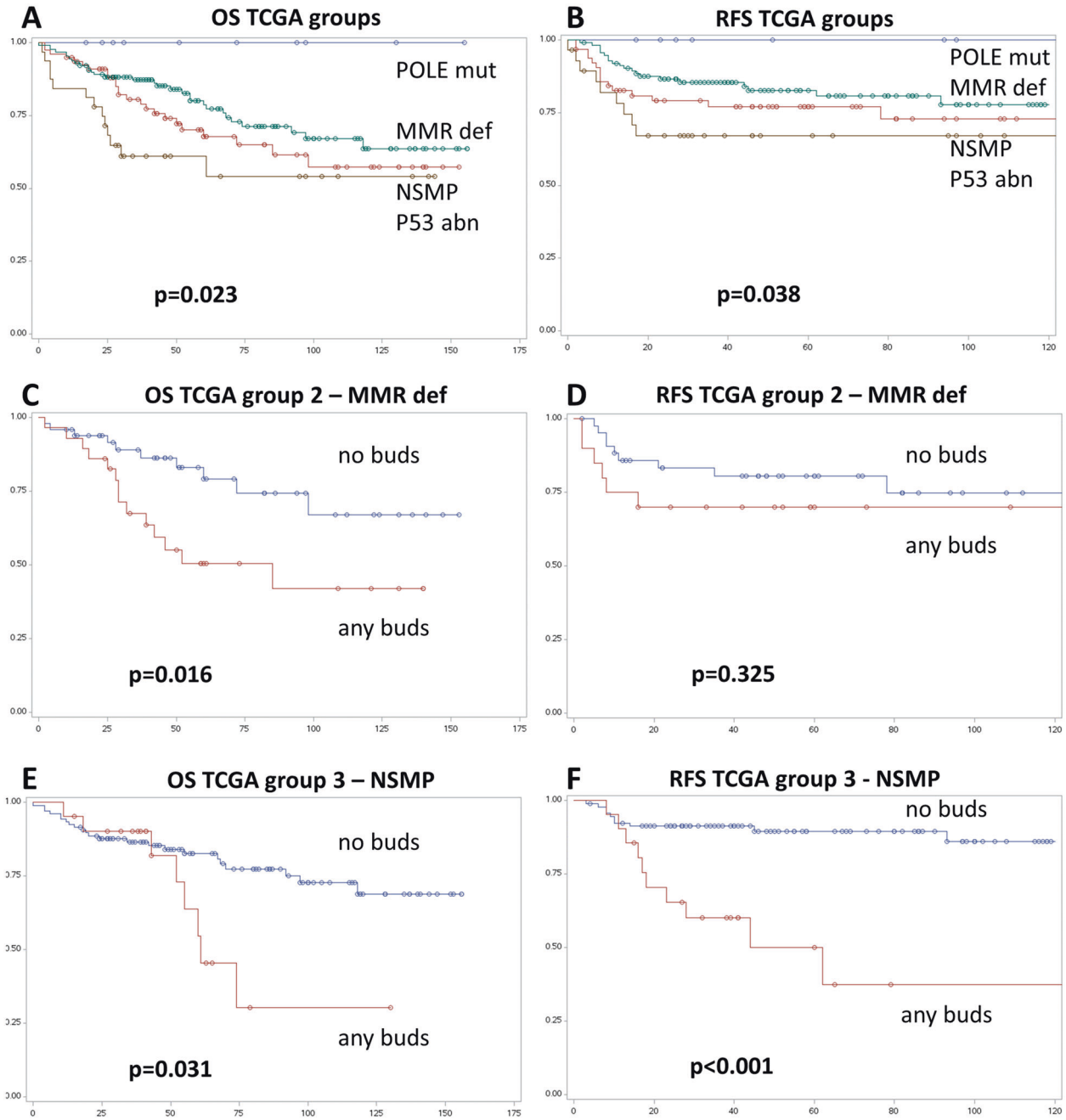

Fig. 4 Prognostication of tumor budding in molecular subtypes of EC. Kaplan–Meier curves of overall (**a**) and recurrence-free survival (**b**) for TCGA subgroups. Groups are defined as POLE mutated (Group 1), MMR-deficient (Group 2), p53 aberrant (Group 4), and NSMP-EMCA (Group 3); the most conventional type of EMCA sometimes referred to as the "triple-negative EMCA". Significances could be reached for the intermediate prognostic groups. MMR-deficient Group 2 showed significant differences in overall survival (**c**) but not recurrence-free survival (**d**). The strongest impact was found for NSMP-EMCA (Group 3) in overall survival (**e**), as well as recurrence-free survival (**f**) (log-rank test).

with tumor budding were found from POLEmut, MMRdef, NSMP, and P53abn EMCAs.

Interestingly, the excellent prognosis of POLEmut cases did not allow for stratification of survival statistics. The best results in regression analysis showed that in the NSMP-EMCA subgroup (Fig. 3e, f), but also in MMRdef cases (Fig. 2c, d), a distinct prognostic impact of tumor budding could be seen.

Discussion

Tumor budding in EMCA using colorectal cancer as a template

Tumor budding is most intensively investigated in colorectal cancer. This has led to the recently achieved consensus ITBCC, which, for the first time, has established a

Table 6 Association of budding counts according to ITBCC with TCGA subgroups.

Feature of EC	TCGA group	No. cases	TB mean	TB max
POLEmut	1	10 (3.9%)	0.5*	2
MMRdef	2	81 (31.8%)	0.7§	10
NSMP	3	132 (51.8%)	0.4#	9
P53abn	4	32 (12.6%)	1.9*,§,#	12

Student's t test $p^* = 0.03$; $p^{\S} = 0.04$; $p^{\#} = 0.02$.

standardized approach to the harmonization of studies on tumor budding [19]. The opportunity to compare referenced tumor bud counts can also be used as a cross-entity investigation as in our study. The determination method using conventional HE staining could easily be transferred to EMCA. Even the moderate but significant inter-observer agreement is comparable with colorectal cancer and might be increased with additional immunohistochemical or computational methods [32]. However, the three-tiered cut-off system proposed by the ITBCC consensus does not adequately reflect the tumor biology of EMCA, as we found significantly lower budding counts when comparing EMCA with colon carcinoma, as shown above [30, 31]. According to our data, almost all EMCA patients would fall into the same Bd1 category, defined over a broad range at the lower budding spectrum from 0 up to 4 buds per ×20 HPF. Fortunately, the ITBCC proposes the determination of continuous tumor budding counts [19] aside from the Bd categories, which is statistically preferred by several authors [28, 30]. With respect to EMCA, a baseline category within the ITBCC for the presence of none versus any tumor buds could be suggested, e.g., outlined as Bd0. Of note, also a small percentage of colorectal carcinomas shows no tumor budding [30, 31]. Molecular profiling of differences between completely nonbudding tumors and others is impaired by the absence of this category. A possible anatomical explanation for our generally reduced tumor budding count in EMCA might be the prominent myometrial layer. To our knowledge, no separate data from the different bowel layers exist that could support this hypothesis of different migration resistances of tumor buds in different tissue types. In addition, it is known in EMCA that different infiltration patterns exist, encountering ones with pushing margins or "adenoma-malignum" type patterns with almost no desmoplastic reaction [15, 18, 29]. Therefore, the lower tumor budding counts in EMCA in comparison with colorectal cancer reflect tumor biology and are congruent with the microscopic and macroscopic appearance.

Prognostic value of tumor budding in EMCA

Tumor budding offers the chance of an additional prognostic parameter at almost no cost, as it can be derived from routine H&E staining. The most important prognostic parameters in EMCA are known as depth of invasion, nodal status, grading, and lympho-vascular-invasion: these were applied in the clinical ESMO-ESGO-ESTRO risk stratification summarized after a consensus conference [33]. In our study TNM-classification, grade and tumor budding presented as significant prognostic parameters. Most importantly, in multivariate analysis, combined with these conventional parameters, tumor budding also served as a significant and independent prognostic factor. This could lead to several application scenarios in which tumor budding could have potential utility in clinical decision-making. We identified an important role of tumor budding particularly in low-stage tumors, whereas tumor budding in high stage or heterogenous tumors still warrants further investigations due to our low case numbers. Still, predictors for lymph node metastasis are needed to apply the best surgical and radiotherapeutic treatment, as some recommendations even neglect the prognostic information of the pN category unless sentinel lymph node biopsy is performed. This clinical uncertainty is outlined in recent reviews [34]. Tumor budding might also be actionable in the curettage specimen and provide preoperative risk assessment. In addition, certain high-risk patients with tumor budding might be identified that would otherwise fall in intermediate indecisive ESMO groups. In addition, high prevalence of tumor buds could exclude testing for POLE mutations as an expensive molecular test to prevent over-treatment and open one more time the discussion between molecular and conventional histopathological parameters [35].

Molecular subgroups of EMCA and tumor budding

In recent years the relevance of molecular profiling of EMCA has become increasingly pronounced [5–7]. Starting from the TCGA publication with complex multimodal molecular classification, the group of POLE-mutated EMCAs with their excellent prognosis has gained particular interest [7]. Meanwhile, several groups investigated and confirmed surrogate biomarker stratification translating (1) POLE ultramutated, (2) MSI hypermutated, (3) copy-number low—endometrioid, and (4) copy-number high—serous like into (1) POLEmut, (2) MMRdef, (3) NSMP, and (4) p53abn EMCA, respectively [5, 6, 36–39]. This molecular approach to surrogate markers was followed with our study design and showed excellent risk stratification. Attention is necessary to only include confirmed functionally relevant POLE mutations, e.g., addressed with a high POLE risk score [5] and to put POLE mutation analysis in first place during analytics as rare multiple-classifier carcinomas exist, e.g., with secondary p53 mutation with no obvious further relevance [6].

Even though POLEmut cases did not show tumor bud counts above 2 and even though p53abn cases are significantly enriched with higher bud counts, using tumor budding as a pre-selector for a specific molecular subtype has yet to be validated. The cut-off definition for tumor budding integrated all four molecular subtypes within our study. However, we expected limitations due to the small size of certain subgroups and possible lack of adverse events, which particularly accounted for the POLEmut subgroup comprising only ten patients. Survival stratification of tumor budding was best in the NSMP-EMCA group followed by the MMRdef group, which both represent EMCAs of intermediate risk and compose the largest subgroup. In conclusion, tumor budding can add additional prognostic information on top of the molecular profile of EMCA.

EMT in EMCA and the involvement of tumor buds

Epithelial–mesenchymal-transition (EMT) has been known as an aggressive tumor-biological event for 30 years [40] and represents a notable tumor-biological hallmark. Tumor buds are suggested to be a strong indicator for EMT in many tumor types [41–43]. Prior literature attempted to explain the phenomenon of EMT by the pattern of MELF crypts—the MELF pattern [18, 29, 44]. For instance, the MELF pattern was associated with E-cadherin loss, lowered Ki67, and altered expression of TWIST1 and 2 and β-catenin [15, 45], which all have been described for tumor buds as well [46, 47] and even in endometrial tumor buds [17]. These parallels should not lead to a reflexive assimilation of both distinct histological findings [45]. Of interest, some authors already mentioned casually excessive tumor buds in MELF-pattern EMCA [15]. So far, the MELF pattern is lacking scientific evidence as an independent prognostic factor in multivariate analysis in several studies [15]. One reason for this circumstance was initially described in the first MELF manuscript by Scully et al. [29]. Herein, the prognostic significance is restricted to a MELF pattern with concomitant fibroblastic stroma. The cases of the MELF pattern without stromal response did not worsen prognosis [29]. This should guide our attention towards minor components in the fibroblastic stroma, which could be tumor buds in our opinion. In addition, the MELF pattern as a qualitative but not quantitative descriptor interferes with stage as infiltration deeply in the myometrium is needed for detection; and interferes with grade as crypts are interpreted as glandular structures with better differentiation. These considerations give an explanation for our findings of a strong association between tumor buds and MELF pattern on the one hand but also the superior performance of tumor buds in terms of a prognostic and independent risk stratifier [14–17].

Morpho-molecular profiling of EMCA

In the era of moving towards identifying molecular markers as parameters for risk stratification, here we present easily applicable molecular markers which can be integrated into molecular subgroupings. Taking the TCGA into account, there are strong arguments to separate EMCA into four different entities with different clinical implications. Morphology has been criticized as not being sufficiently reproducible for risk stratification [48]. However, the estimation of tumor buds has been shown to be a robust parameter in several inter-observer studies [30, 31, 49] and is standardized by attempts of the ITBCC [19]. Two previous studies could show a prognostic impact of tumor buds with a similar H&E-based method [16, 17]. Our study confirms these findings. However, slight differences in the cut-off application exists. Han et al. and Park et al. arbitrarily applied a cut-off of $n > 5$ buds per 20 HPF [16, 17]. This does not generate conflicting data, as prognostication with tumor buds regularly follows a continuous scale. In contrast, applying a cut-off of $n > 5$ had inevitably preselected p53abn carcinomas showing the highest bud count. Regarding our data, we applied the natural cut-off of no versus any buds. Hence, a limitation for daily routine could be the certainty to assess really any single tumor bud in an EMCA case. In our approach we did not use immunohistochemistry as an ancillary tool as we decided to rely on the tumor budding definition by the ITBCC. As a consequence, further validation studies to outline inter-observer-variability and possibly easier cut-offs for tumor budding or continuous scale reporting of tumor buds in EMCA are warranted.

In addition, we found the best results in the combination of tumor buds as a classical morphological parameter for the first time with the molecular subtype of EMCA. Tumor budding is a promising prognostic parameter, particularly for the intermediate risk subgroups of NSMP and MMRdef EMCA. These findings underline the molecular differences for EMCAs in their ability to form tumor buds as a tumor-biological phenomenon of EMT. In general, a consequent morpho-molecular approach would allow a reevaluation of the plethora of histological parameters like infiltration patterns, morula formation, lymphocytes, nuclear grade, etc. beyond tumor budding, as well as with a refreshed perspective.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All patients signed the broad consent of the Tissuebank Bern. The study was approved by the Ethics Committee Bern, Switzerland (reference number: 2018–00479).

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