ANTICANCER RESEARCH *40*: 2695-2699 (2020)
doi:10.21873/anticanres.14240

Tumour Budding Is an Independent Predictive Factor of Cutaneous Squamous-cell Carcinoma Aggressiveness

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Abstract. *Background/Aim: Tumour budding (TB), i.e. the presence of groups of ≤5 tumour cells ahead of the invasive tumour front, is a pathological feature associated with an aggressive outcome in several cancer types. The aim of this study was to assess the value of TB as an independent prognostic factor of cutaneous squamous cell carcinomas (cSCC). Materials and Methods: We studied 25 cases of aggressive cSCC (defined as tumours that developed local recurrences and/or metastases after adequate excision) and 27 cases of non-aggressive cSCC. TB was expressed as the mean number of tumour buds in 5 adjacent high-power fields (HPF). Results: Statistical analysis showed that TB is an independent predictive factor of cSCC aggressiveness. When the cut-off value of 0.8 buds/HPF was considered, the positive and negative predictive values for cSCC aggressiveness reached 77.3% and 75.0%, respectively. Conclusion: As with other cancer types, TB appears to be a new independent pathological factor of aggressiveness of cSCC, providing a new tool to predict cSCC outcome, similar to other already established features associated with an adverse outcome (such as tumour size).*

Tumour budding (TB) is the process during which single neoplastic cells, or small groups of cells, detach from the main tumour mass in the invasive front and infiltrate the surrounding connective tissue. This feature is associated with the acquisition of a mesenchymal phenotype by neoplastic cells, which enables them to infiltrate the connective tissue surrounding the neoplasm, penetrate into blood and/or lymphatic vessels, and thereby reach distant tissues where the cells may settle down and engender a distant metastasis (1-4). TB has been recognized as a feature of adverse outcome in a variety of human solid cancer types, including colon (2-4), oesophagus (5), pancreatic duct (6), lung (7), gallbladder (8), head and neck (9), tongue (10), oral cavity (11), external auditory canal (12), breast (13), endometrium (14), stomach (15) and larynx (16). The inclusion of TB as a prognostic factor in the pathological reports of some of these carcinomas has been suggested (2, 3, 10). Concerning cutaneous SCC (cSCC), microscopic features known so far to portend an adverse prognostic significance include macroscopic (horizontal) size, microscopic thickness, Clark level of invasion/involvement of deep tissues beyond the subcutaneous fat, poor differentiation and intravascular and perineural invasion (17-24). In a previous study, we found that TB was also significantly associated with aggressive outcome in cSCC (25). Subsequent studies confirmed the prognostic significance of TB in cSCC, namely by predicting their metastatic spread (26-29). In the present study, we extended the results obtained in our initial study (25) by comparing the prognostic value of TB with that of other recognized microscopic prognostic features of cSCC. The findings of the present study suggest that TB is an independent prognostic factor of cSCC, showing a closer correlation with tumour aggressiveness compared to other adverse prognostic features.

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Key Words: Tumour budding, squamous-cell carcinoma, skin, aggressiveness.

Materials and Methods

Study design. A retrospective pathologic study was performed on biopsy or excision specimens of a total of 52 cSCC that had been obtained from a group of organ-transplant recipients followed in our specialized outpatient Dermatology clinic. Of these, 27 cases had an aggressive course, defined as the development of local

recurrences following complete surgical excision and/or regional or distant metastases. A total of 25 non-aggressive cSCC were also included in the study; they had been excised from the same group of patients during the six months preceding or following excision of the aggressive cSCC, and were matched (as much as possible) for anatomical location. These non-aggressive cSCC had not developed recurrences or metastases during the same follow-up period as that of their aggressive counterparts.

Assessment of tumour budding (TB) and high-risk microscopic features. Representative haematoxylin-eosin-stained sections of each cSCC were re-examined in a blind fashion as to the outcome of the SCC (aggressive or not) for evaluating the degree of TB, as previously reported (25). Briefly, the sections were first examined at low magnification to assess the most invasive areas of each tumour. The slides were then examined at high magnification (×250) and the number of tumour buds, *i.e.* single cells or groups of up to five tumour cells ahead of the invasive tumour front (Figure 1) in five adjacent microscopic fields was counted. The mean number of tumour buds per high-power field (HPF) for each cSCC was calculated. The macroscopic size (in cm) of the tumours was measured on representative glass slides. The remaining microscopic features were assessed by microscopic examination of the same routinely-stained sections of each case. They included: tumour thickness (in mm), Clark level of invasion (I-V) and degree of differentiation (high/moderate/low).

Statistical analyses. These were done using Matlab, Release 26 (The MathWorks, Inc., Natick, MA, USA). For comparisons between categorical variables, the Fisher's exact test was used. Preliminary statistical analysis using the Fisher's exact test indicated that TB>1 per HPF is indicative of aggressive outcome, however, lowering the cut-off value of TB to 0.8/HPF showed a higher statistical significance. Appropriate regression models were performed for all different budding groupings, including treating it as a continuous variable. The regression model that performed best was the one with 0.8/HPF as the cut-off for the two groups of cSCC. Binomial logistic regression analysis, using the enter method, was performed on selected variables that were included together in the model, to examine the interaction of the multiple categorical variables with the two cSCC subtypes. A p-value of ≤0.05 was considered statistically significant.

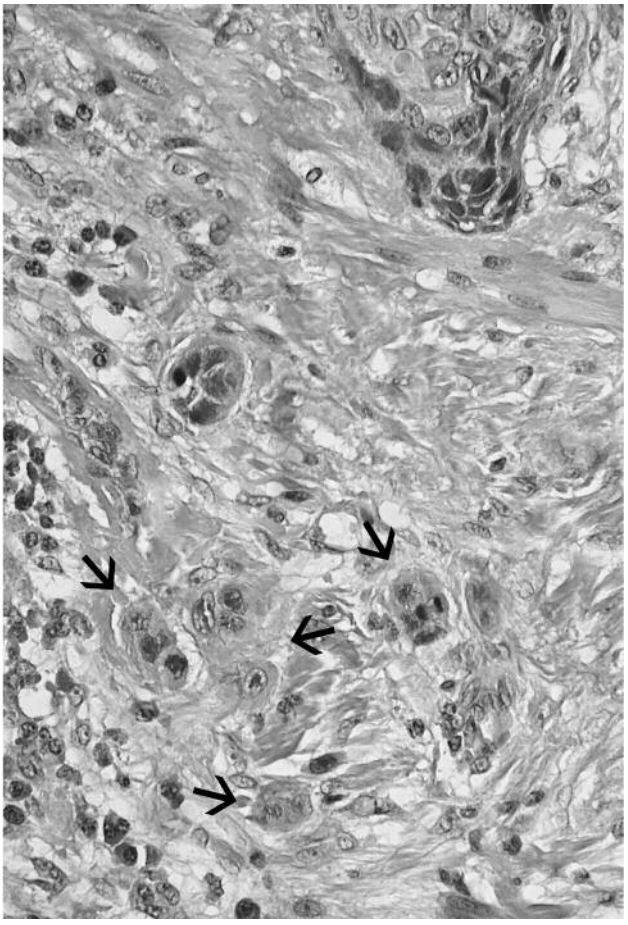

Figure 1. *Tumour budding in a cutaneous squamous-cell carcinoma: several tumour buds, consisting of one to five tumour cells (arrowhs), invade the dermis (haematoxylin-eosin, original magnification ×250).*

Results

Comparison of cSCC classified according to their degree of TB in two groups (≥0.8 and <0.8/HPF) and other high-risk factors showed TB to be an important factor for cSCC aggressiveness prediction (p=0.012). Clark level of invasion was significantly associated with TB (p=0.004 as individual categories I-IV and with a p=0.002 as two larger groups I-III and ≥IV). Similarly, Breslow index (grouped ≤2 mm and >2 mm) was significantly associated with the two TB groups (≥0.8 and <0.8/HPF) (exact p-value=0.002). The degree of differentiation of cSCC was also associated with TB (p=0.008) (Table I).

In order to establish the independence of the risk factors, a binomial logistic regression analysis was performed to assess the effect of TB (grouped in two groups, *i.e.* ≥0.8 and <0.8 per HPF), macroscopic size (in mm), Clark level (grouped I-III and ≥IV) and Breslow thickness (grouped ≤2 and >2 mm) on predicting the cSCC subtype of the tumours included in the study. The logistic regression model was statistically significant with $\chi^2(2)12.396$ (p=0.002). The final model with TB and size only had a variation measure Nagelkerke R2=0.31 and correctly classified 78.7% of cases. Table II shows the sensitivity and specificity along with all the relevant metrics. Other combinations of risk factors were tested but did not improve the regression model.

TB <0.8/HPF was predictive of non-aggressive cSCC (p=0.013). Increasing values were associated with aggressive cSCC (p=0.025). Size ≥2 cm was also predictive of aggressiveness (p<0.0001). Grouped Clark levels (I-III and ≥IV) and Breslow thickness (grouped ≤2 and >2 mm) did not prove statistically significant as independent variables in distinguishing between cSCC subtypes (aggressive *vs.* non-aggressive).

Table I. *Characteristics and statistical analyses for cSCC.*

Variable	Non-aggressive		Aggressive		Fisher's exact test[a]	Binary logistic linear regression[b]		
	N	%	N	%	*p* (exact,2-sided)	OR	95%CI	*p*-Value
Budding group								
<0.8	19	36.50%	9	17.30%	0.025	6.424	1.477-27.930	0.013
≥0.8	8	15.40%	16	30.80%				
Size group								
≤2	0	0.00%	13	25.50%	0.0001	0.818	0.695-0.964	0.016
>2	27	51.00%	12	23.50%				
Breslow group								
≤2	9	18.80%	8	16.70%	0.764			
>2	14	29.20%	17	35.40%				
Clark level								
I	0	0.00%	0	0.00%	0.244*			
II	2	3.80%	2	3.80%				
III	16	30.80%	10	19.20%				
IV	7	13.50%	6	11.50%				
V	2	3.80%	7	13.50%				
Clark levels grouped								
I-III	18	34.60%	12	23.10%	0.262			
IV-V	9	17.30%	13	25.00%				
Differentiation[c]								
1	24	40.00%	8	13.30%	0.021*			
2	7	11.70%	14	23.30%				
3	2	3.30%	5	8.30%				

[a]Fisher's exact test computed for binary categories of variables *vs*. cSCC types; [b]Regression was performed on selected variables that were included together in the model; [c]: 1: high, 2: moderate, 3: low; *χ^2 test. CI: Confidence interval; OR: odds ratio.

Discussion

Tumour budding (TB) is nowadays regarded as a pathologic feature predictive of poor outcome in several cancer types [reviewed in (1)]. Indeed, even though the methods of TB counting differ among studies, these have consistently shown that in several cancer types the degree of TB positively correlates with lymphovascular invasion, recurrence, lymph node metastases, distant metastases, poorer patient disease-free and overall survival. Regarding cutaneous tumours, four studies have shown that TB is a prognostic factor of poorer outcome also in cSCC. In our initial study (25), we found that aggressive cSCC (defined as those which were subsequently complicated by local recurrences and/or local or distant metastases) had a significantly higher mean number of TB *vs* non-aggressive cSCC (1.6±1.9 *vs*. 0.4±0.9, $p<0.001$). In that study, as in the present one, we considered tumour buds those made of up to 5 cells, and counted them on 5 adjacent microscopic hot-spot fields at X250 magnification. In a subsequent study, Fujimoto *et al*. (26) classified cSCC as TB-positive (if they had >5 tumour buds/1.23 mm^2 of tissue section) or TB-negative. They found that 93% of cSCC with lymph node metastases were TB-positive compared with 26% of non-metastatic SCC, and concluded that TB is an independent prognostic indicator of lymph node metastasis. In a subsequent study (27), the same group classified «early» (<4 cm macroscopic size) cSCC in grades 1-3, according to the number of tumour buds (1: 0-4, 2: 5-9, 3: >10), and reported

Table II. *Sensitivity, Specificity and other relevant metrics.*

Statistic	Value	95%CI
Sensitivity	80.00%	59.30% to 93.17%
Specificity	77.27%	54.63% to 92.18%
Positive likelihood ratio	3.52	1.59 to 7.80
Negative likelihood ratio	0.26	0.11 to 0.59
Positive predictive value	80.00%	64.36% to 89.86%
Negative predictive value	77.27%	60.05% to 88.49%
Accuracy	78.72%	64.34% to 89.30%

Sensitivity, specificity, positive and negative predictive value as well as accuracy are expressed as percentages; Confidence intervals for sensitivity, specificity and accuracy are "exact" Clopper-Pearson confidence intervals; Confidence intervals for the likelihood ratios are calculated using the "Log method"; Confidence intervals for the predictive values are the standard logit confidence intervals.

that 83% of metastasizing cSCC had grades 2 or 3, *vs*. 37.5% of non-metastasizing tumours. In the study by Gonzalez-Guerrero *et al*. (28), which also compared metastatic with non-metastatic cSCC, tumour buds were considered as aggregates of 1-4 tumour cells. The percentage of cases with tumour buds, the number of tumour buds per slide, and the intensity of TB (low: <5 buds, high: ≥5 buds) were assessed. That study found that the presence of tumour buds was a significant risk factor for nodal metastasis and for reduced survival time. Another more recent study compared metastatic with non-metastatic cSCC. Tumour buds were defined as clusters of < 5 cells using a 40x objective lens in the invasive frontal region, and cases with ≥5 foci were considered as TB+. This study found that TB correlated with the metastatic risk of cSCC, along with the presence of an 'immature' fibromyxoid stroma which reflects epithelial-mesenchymal transition (29).

The results of our present study corroborate the findings of these previous studies (25-29), which are concordant in showing a correlation of the degree of TB with the risk of recurrence and/or metastasis of cSCC. Our present results suggest also that TB is an independent prognostic high-risk factor for cSCC and that correlation with aggressiveness seems to be stronger than that of other known factors of aggressiveness, such as tumour depth (Breslow) or Clark level. Indeed, our analysis showed that, if the cut-off value of 0.8 buds/HPF is considered, TB can predict the aggressiveness of cSCC with a positive predictive value of 77.3%, a negative predictive value of 75% and a positive likelihood ratio of 3.41. Remarkably, grouped Clark levels (I-III and ≥IV) and Breslow thickness (grouped ≤2 and >2 mm) failed to reach statistical significance as independent variables in distinguishing aggressive from non-aggressive cSCC subtypes. This may be due to the limited sample size of cSCC studied, as suggested by the wide confidence intervals of the OR.

In conclusion, our findings support the contention that TB is an independent prognostic factor of aggressiveness of cSCC. We advocate therefore, that similarly to other carcinomas, such as those of the colon (3) and the tongue (10), this variable should be included in the pathology reports of cSCC, so as to warn the clinicians about the aggressive potential of the tumour. Prior to that, a consensus needs to be reached as to the clinically most relevant method for counting TB in specific carcinoma type.

Conflicts of Interest

The Authors declare that they have no conflicts of interest regarding this study.

Authors' Contributions

GK and JK reviewed the pathological slides, assessed the pathological features of the tumours and studied and wrote the article. EP performed the statistical analyses.

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Received March 31, 2020
Revised April 7, 2020
Accepted April 8, 2020