Published in final edited form as:

Histopathology. 2017 January ; 70(2): 264–272. doi:10.1111/his.13060.

Breslow density is a novel prognostic feature in cutaneous malignant melanoma

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Abstract

Aims—In 1970, Breslow described his eponymously named thickness measurement. No-one has sought to enhance Breslow thickness (BT). This purpose of this study was to demonstrate proof of concept that the density of melanoma cells at the position where Breslow thickness is measured is a morphological prognostic biomarker, which we name Breslow density (BD). The hypothesis was that BD has prognostic value for overall survival (OS) and is independent of BT.

Methods and results—We analysed 100 cutaneous melanomas and followed REMARK guidelines. BD was the estimated percentage dermal area occupied by melanoma cells in a specified location. BT and BD had a strong correlation ($p = 2.1 \times 10^{-11}$) but despite this they were independent prognostic factors for OS in Cox regression (BD: HR 1.03, $p=0.001849$ and BT: HR 1.09, $p=0.000146$). This was corroborated by an independent effect on melanoma specific survival. We assessed whether BT and BD could be combined into a Breslow score. A prognostic index based on Cox regression coefficients was used and this showed a marginal improvement in predicted 5-year survival compared to BT alone (area under curve 94.8% v 96.7%).

Conclusions—We show proof of concept that BD represents a novel morphological prognostic biomarker that is independent of BT and that there is potential to combine these into a Breslow score. Larger studies are needed to validate BD, but the simplicity of this biomarker makes it a strong candidate for translation to clinical practice.

Keywords

Melanoma; REMARK; skin; cancer; biomarker; prognosis

Introduction

In 1970 Breslow described a measurement for melanoma invasion, now known as the Breslow thickness (BT)¹, and demonstrated that this correlated with risk of metastasis. In particular, he found that no melanoma with a thickness of less than 0.76 mm developed

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Conflict of interest

The authors declare no conflicts of interest.

further disease. Although this study was small, comprising only 98 patients, the broad findings have been reproduced many times during the subsequent decades, with the result that BT is now regarded as a robust evidence-based data item and has been the foundation for the T stage parameter in successive versions of the AJCC TNM staging system². Several other morphological features in the primary melanoma have shown prognostic value with varying degrees of support from the literature. Ulcer and mitotic rate are established prognostic factors, recognised by their central role for AJCC staging². Clark level is also important, but it has been superseded as a major determinant in AJCC staging by mitotic rate. The prognostic value of other morphological features, such as tumour infiltrating lymphocytes^{3–6} and regression^{7–12}, is controversial due to conflicting reports in the literature.

In his original article, Breslow was seeking a simple surrogate for tumour volume¹, which seems reasonable if one assumes that the total dermal invasive burden would be the feature with strongest prognostic impact. One can envisage a sequence of increasing complexity for estimating invasive burden ranging from BT at the simplest, to measuring density of cells at the position where BT is measured, to measuring cross-sectional area of invasive cells in the whole section where BT is measured and finally measuring total invasive volume across all sections. However, the difficulty of achieving these measurements in day to day practice increases as we move across this spectrum from BT to total invasive volume. For example, the latter would require the whole melanoma to be embedded in every case, assessment of tumour cross sectional area in each slice and knowledge of the thickness of each slice to compute total tumour volume. This would be time consuming to perform. One reason the BT is powerful is that it so simple to measure and is reproducible. Surprisingly, the easiest of the aforementioned BT enhancements for estimating invasive burden, namely measuring the density of melanoma cells at the position where BT is measured, has never been assessed. It seems reasonable that of two melanomas with the same BT but differing density of cells at the position of measurement, the one with fewer invasive cells will have a better prognosis. Thus, we propose a new measurement, the “Breslow density” (BD), to capture this hitherto unmeasured variable. This purpose of this study was to describe a method to measure BD by making an estimate of melanoma cell area in a specified location to demonstrate proof of concept that this would be a viable, simple and reproducible morphological prognostic biomarker and to determine whether detailed future evaluation is warranted. The primary hypothesis was that BD has prognostic value for overall survival (OS) and that it is independent of BT. A secondary hypothesis was to also analyse melanoma specific survival (MSS). Finally, we aimed to show proof of concept that BD might be used in combination with BT to yield a Breslow score (BS).

Methods

Patients

Tissues were selected from the archives of the University Hospitals of Leicester Cellular Pathology department. A favourable opinion from an NHS ethics committee was obtained for tissue use. A search of melanoma cases from January 1st 2004 was performed and patients with cutaneous melanoma, showing invasive disease and diagnosed in Leicestershire

were sequentially selected until a total of 100 was reached. Selection was stopped at this point because there were sufficient events for the primary analysis of OS (35 deaths) to analyse two covariates, BD and BT alongside an interaction term, using the rule of thumb that approximately 10 events per covariate are required. We excluded 5 patients: slide stain too faded (n=1), slides not in file (n=2) and metastasis at diagnosis (n=2). Patients living outside Leicestershire were not part of the target population because follow-up could not be accurately ascertained for patients receiving care in other regions. Clinical stage was according to AJCC version 72.

Breslow Density

Breslow density was measured at the invasive area where BT was measured with the same formalin-fixed paraffin-embedded sections as used by the reporting histopathologist. The BD was measured according to a protocol, as follows, and graphically summarised in Figure 1A. First, the location containing the deepest cells, and hence the position where BT had been measured, was identified. Second, assuming the axis perpendicular to the epidermis is vertical and the axis parallel is horizontal, a rectangular virtual window was created at low power. In the horizontal axis the window was limited by the crudely estimated width of a x10 field (2.1 mm with Olympus BX45 microscope using Plan x10/0.25 objective), this estimate being achieved by switching between x10 and lower power objectives as required. In the vertical axis this was limited superiorly by the epidermal basal layer and inferiorly by the deepest melanoma cell. The window was positioned such that a vertical line bisecting it passed through the deepest melanoma cell. Third, at low power the estimated window was adjusted in the horizontal axis such that it maximised the dermal area occupied melanoma cells while still containing the deepest melanoma cell. Fourth, the field of view was centred on the estimated window from the previous step the view was switched from low power to the x10 objective. Histological landmarks that defined the two horizontal edges of the x10 field (e.g. a specific rete tip, hair follicle, tumour / inflammatory cell cluster or sweat duct) were identified and the field was switched back to low power. A precise rectangular window was mentally re-constructed from the horizontal landmarks, the basal layer of the epidermis and the deepest cell. In this way the full window could be assessed at scanning power. We found in practice that being able visualise the whole rectangle at once made scoring much easier. However, with very thick melanomas the field was not seen even at low power so vertical scanning was required to see the whole rectangular area. Horizontal landmarks were easy find in every case. The percentage area of dermal stromal tissue occupied by melanoma cells was recorded as the BD to the nearest 5%, but with precision to nearest 1% for scores below 5% and above 95%. It thus follows from the above protocol that the window was always created at a fixed location (a position that contained the deepest melanoma cell), its horizontal dimension was always the same for every case (the width of the x10 field, 2.1 mm for our microscope) and its vertical dimension varied from case to case, being the same as the BT. Importantly however, all tumours with the same BT had the same window area, allowing for meaningful evaluation of BD relative to BT, as shown Figure 1C and D. It is important to note that while the method seems cumbersome to describe, it was very easy to execute once the position of BT measurement was identified, typically taking less than a minute and often only a matter of seconds.

Statistical analyses

Statistical analyses were all performed in R version 3.2.013. Survival analysis was performed using the “survival” package¹⁴. BT, measured in mm, and BD, measured as percentage, were analysed as continuous variables. For correlation, Pearson’s correlation coefficient was used. Time to event analysis was performed with 2 outcomes, melanoma specific survival (MSS) and overall survival (OS). For both, the date of diagnosis was taken as the date of primary sample accession in the pathology laboratory. For MSS, death from melanoma was considered as failure, while for OS death from any cause was considered as failure. For MSS, death from another cause was regarded as censoring. Survival was analysed by the Kaplan Meier method with BD and BT split at the median to create two groups and the log rank test was used to compare survival curves. For Kaplan Meier analysis of MSS, there were 55 cases in the low BD score group with 4 events and 45 in the high score group with 15 events. For BT there 49 cases in the thin BT group with 1 event and 51 in the thick BT group with 18 events. For Kaplan Meier analysis of OS, there were 55 cases in the low BD score group with 10 events and 45 in the high score group with 25 events. For BT there 49 cases in the thin BT group with 8 events and 51 in the thick BT group with 27 events. Univariable and multivariable hazard ratios were determined using the Cox proportional hazards method. BT and BD were used as covariates. The median follow up was 87 months. The proportionality assumption was checked by examining plots of scaled Schoenfeld residuals against transformed time and with a goodness of fit test in the R survival package. Proportionality was not violated. This study adhered to REMARK guidelines¹⁵, see Table 1.

Results

The study evaluated 100 melanomas that were all cutaneous. The baseline features of the cases included in the study are shown in Table 2. The variable of interest in the present study, the BD, was checked for interobserver agreement by two histopathologists who performed independent scoring of a random sample of 20 cases. This showed an intra-class correlation coefficient of 0.96, indicating almost perfect agreement. Aspects of BD scoring are shown in Figure 1.

Breslow density score correlates with Breslow thickness

Our primary aim was to determine whether Breslow density has an independent effect on survival but as a preliminary investigation we sought to determine whether it showed evidence of correlation with Breslow thickness because, if present, this would indicate the importance of multivariable survival analysis to rule out confounding. The two variables are plotted in Figure 2. A scatterplot of BD against log BT was created. A log transformation of BT was done because outlying BT values were high. The BD and BT had a strong correlation (Pearson R^2 0.61, $t = 7.57$, $df = 98$, $p = 2.1 \times 10^{-11}$). This indicated that there was a high possibility that any effect of BD on survival could be confounded by BT.

Breslow Density has an effect on melanoma survival

In order to determine the possible effect of BD on survival, we first performed univariable analysis using Kaplan Meier estimates of the survivor function. BT and BD were assessed

using MSS and OS as time- to-event outcomes. BD and BT were both split at the median into two approximately equal sized groups and the survivor curves were compared using the log rank test. The data are shown in Figure 3. In order to determine whether BD is confounded by BT and also to assess for any interaction between these variables, a Cox proportional hazards regression model was used. Univariable models for BD and BT were fitted and then multivariable models with and without an interaction term. These are summarised in Table 3. They reveal that BD is independent of BT in determining both MSS and OS. The analysis also shows that there is a significant negative interaction for MSS and just short of significant for OS, implying that the effect of BD on survival is reduced at higher values of BT.

The combination of Breslow density and thickness shows a marginal improvement in predicting 5-year survival

In order to determine whether BD and BT could be used to create a “Breslow score” (BS) they were combined. This was achieved by taking a linear combination of BD and BT weighted by the coefficient values from the cox proportional hazards model to yield a prognostic index (PI). The PI thus represented the log of the hazard ratio. For each of MSS and OS, a PI was calculated for two different regression models, one containing BD and BT and one containing BD, BT and the BD.BT interaction term, given that this was significant for MSS and nearly significant for OS. The BD, BT and PIs from the multivariable regression models were used to assess the ability to discriminate between patients surviving up to and including 5 years and those surviving beyond 5 years, with MSS and OS as endpoints. The ROC curves are shown in Figure 4. The combination of BD and BT showed improvement in areas under ROC curves, albeit marginal, indicating the potential for BT and BD to be combined into an overall BS to more accurately classify melanoma outcome. The addition of the interaction term to the regression model was not of value.

Discussion

To our knowledge, no-one has ever sought to enhance BT by determining the density of malignant melanoma cells at the point of BT measurement. Our data shows that although BT and BD have a strong correlation, they remain independent prognostic factors and furthermore have potential to be combined into a single variable, the Breslow score, to discriminate 5-year survival with greater accuracy. The present study show that the combination of BT and BD shows only a marginal improvement over BT alone and further studies are needed to determine the optimal way that BD might be used in practice.

Breslow, in his original article, sought a measurement of invasive volume but found that thickness was the best predictor¹. Invasive volume has not been widely studied in cutaneous melanoma. Friedman conducted a small study of 35 melanomas and showed that 200 mm³ was a cut off for good and poor 5 year survival¹⁶. A method for estimating tumour volume from macroscopic specimens using slices of known thickness by the Cavalieri method showed good correlation with BT, but the prognostic value of this estimation was not then assessed¹⁷. More recently, tumour volume was found to be a prognostic factor in 122 melanomas, including cases where low thickness but high volumes correlated with poorer

outcome¹⁸. We speculate that the invasive burden of melanoma cells represented by the total invasive cell count is the best predictor of outcome and total invasive volume is likely to be the best approximation of this. However, its clinical value is limited by the relative difficulty of measurement such that histopathologists may be unwilling to adopt this into routine practice. In contrast, the BD is undoubtedly a poorer estimate of invasive burden but represents the simplest next best step up from BT and would be very easy for pathologists to perform.

This study is limited by its small size, but the case number was purposely chosen in order to have sufficient power to make an initial proof of concept estimate of the value of BD for future study, with the primary outcome of OS. As such, the study size was powered to allow the inclusion of BT, BD and an interaction term in a multivariable analysis of OS, which for preliminary investigation represent the most crucial prognostic predictors. Future studies will need bigger case numbers so that BD can be evaluated against other recognised clinico-pathological features for better evaluation of confounding features and to have greater power to develop a combined BT / BD score. In turn a BS would need to be externally validated in the future for it to be generalised for use. The BD is a semi-quantitative scoring system and as such is subject to all the pitfalls associated with this type of measurement, most notably the inherent element of subjectivity in estimating a percentage score. However, the very high intra-class correlation suggests that subjectivity was not a major issue for the authors. If difficulty did arise, it was for cases with expansile junctional nests (specifically, deciding which cells were invasive) and cases with heavy inflammation (deciding which cells were melanoma), but these issues were not especially taxing for our cases. A more pressing methodological concern was how precise the scoring should be, and in particular whether a score to the nearest 5% (nearest 1% at extreme values) was overly exact. Given that this was just a proof of concept study, the optimal precision for BD scoring will need to be defined moving forward. The likelihood is that pathologists in different centres will achieve poorer scoring agreement than colleagues in the same centre, and so a less precise scoring system may be better in future.

The strength of this study lies in the fact that primary analysis of OS was corroborated by secondary analysis of MSS. Another strength is that the BD is very simple to measure and we find that once the position for measuring BT is established it only takes a matter of seconds to make an estimate of the density of melanoma cells and the BD percentage was reproducible between two raters. It likely that melanoma cell immunostains with or without image analysis would improve BD scoring accuracy. However, the benefit would be offset by the increase in time, cost and (in the case of image analysis) availability of relevant software and hardware directly at microscope. The value of image analysis is something that would require a specific study to evaluate in future, whereas we can see clear immediate benefit for immunostains in cases where melanoma cells are difficult to see, for example cases with very brisk lymphocytic infiltrate.

In conclusion, we have identified a novel enhancement to BT for estimating prognosis and show that BT and BD have potential to be employed in combination. We are currently seeing remarkable improvements in the understanding of melanoma molecular pathology with a resultant flood of potential molecular prognostic biomarkers. Such biomarkers are crucial,

because getting the most accurate prognosis at baseline is fundamental to all further melanoma patient care. However, it is very easy to forget that a simple morphological biomarker has advantages over a molecular biomarker because at once any complex analytic procedures are eliminated and can be replaced by a simple morphological measurement performed prospectively at the microscope by a pathologist as part of routine reporting. In turn this makes it simpler to incorporate a morphological biomarker into daily practice for translation. BD fulfils these criteria for being a simple morphological biomarker and should be prioritised for further development.

Acknowledgements

Gerald Saldanha conceived and designed the study, analysed the data and wrote the manuscript. Hala Rashed scored the Breslow densities for all the cases and contributed to the manuscript. Katarina Flatman obtained patient follow up data and contributed to the manuscript. Kah Wee Teo obtained patient follow up data and contributed to the manuscript. Mark Bamford conceived the study with GS and contributed to the manuscript.

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Figure 1.

Breslow density scoring. **A**, cartoon illustrating how the BD window is formed. See the methods for detailed explanation. **B**, intra-class correlation coefficient. **C**, melanoma with Breslow thickness 0.8 mm and Breslow density 10%. **D**, melanoma with Breslow thickness 0.8 mm and Breslow density 60%.

Figure 2. Scatterplot of natural log Breslow thickness and Breslow density. This shows a positive relationship.

Breslow Density

5 year survival

MSS	Low BD	98%
	High BD	75%
OS	Low BD	91%
	High BD	64%

Breslow thickness

5 year survival

MSS	Low BT	100%
	High BT	76%
OS	Low BT	92%
	High BT	67%

Figure 3.

Kaplan Meier survival plots of Breslow density. For both BD and BT, the upper Kaplan Meir curve in the melanoma-specific survival and overall survival plots represents the low score / thickness group respectively.

Figure 4.
ROC curves for Breslow thickness, Breslow density and combined thickness + density scores

Table 1
REMARK guideline compliance

		Comments	Location in text
Variables			
Biomarker of interest		BD = Breslow Density (Percentage)	Materials and Methods
Other covariates		BT = Breslow thickness (mm); Prognostic indices from Cox regression (PI)	Materials and Methods
Patients			
	n		
Assessed for eligibility	105	Invasive melanoma, resident in Leicester, identified from hospital pathology database from 1 st January 2004, each case assessed sequentially for inclusion and exclusion criteria	Materials and Methods
Excluded (number)	5	Slide H&E stain too faded (n=1), slides not in file (n=2) and metastasis at diagnosis (n=2)	Materials and Methods
Included	100	Based on adequate power to assess BT, BD and interaction term in Cox PH regression	Materials and Methods and Table 2
Analyses			
Bivariate	100	BD versus natural log BT	Figure 2
Univariable KM	100	BD and BT (outcomes = MSS, OS)	Figure 3
Univariable Cox PH ^a	100	BT and BT (outcomes = MSS, OS)	Table 3
Multivariable Cox PH	100	BT and BT (outcomes = MSS, OS)	Table 3
ROC AUC	200	BD, BT and PI (outcomes = 5-year MSS and OS)	Figure 4

PH, Proportional hazards. ROC, Receiver operator characteristic area under curve. MSS, Melanoma specific survival. OS, Overall survival.

Table 2
Baseline melanoma data

AJCC stage		Site	
IA	30 (30%)	Upper limb	15 (15%)
IB	40 (40%)	Lower limb	32 (32%)
IIA	8 (8%)	Trunk	30 (30%)
IIB	6 (6%)	Head & neck	19 (19%)
IIC	16 (16%)	Acral	4 (4%)
Age at diagnosis		Breslow Density	
Mean	61	Mean	68.2%
Median	62	Median	80.0%
Breslow depth (mm)		Mitotic rate (per mm ²)	
Mean	2.6	Mean	3.9
Median	1.1	Median	1.0
Ulcer		Microscopic satellites	
Present	76 (76%)	Present	3 (3%)
Follow up (months)		Sex	
Mean	84.5	Female	56 (56%)
Median	87		

Table 3
Cox proportional hazards regression for Breslow density and thickness.

	Univariable	Multivariable	Multivariable with interaction term
	HR(95% CI)	HR(95% CI)	HR(95% CI)
MSS^a (n=100)			
BD (%)	1.05 (1.02-1.09) P=0.00399	1.04 (1.01-1.08) P=0.01365	1.07 (1.02-1.11) P=0.00872
BT (mm)	1.11 (1.06-1.16) P=0.00002	1.08 (1.02-1.14) P=0.00628	3.36 (1.59-7.06) P=0.00144
BD.BT interaction	-	-	0.99 (0.98-0.99) P=0.00302
OS^b (n=100)			
BD (%)	1.04 (1.02-1.06) P=0.000225	1.03 (1.01-1.06) P=0.001849	1.04 (1.02-1.07) P=0.0015
BT (mm)	1.11 (1.07-1.16) P=2.5x10 ⁻⁸	1.09 (1.04-1.13) P=0.000146	2.04 (1.07-3.89) P=0.03164
BT.BT interaction	-	-	0.99 (0.99-1.00) P=0.05815

HR, Hazard ratio. MSS, Melanoma-specific survival. OS, Overall survival.