Fractal dimension of chromatin is an independent factor for survival in melanoma
Valcinir Bedin*, Randall Adam*, Gilles Landman**, Bianca C.S. de Sá**, Konradin Metze K*
*Department of Pathology – University of Campinas – Campinas, SP - Brazil
**Department of Pathology – Hospital A C Camargo – São Paulo – SP- Brazil

§Corresponding author

e-mail addresses:
VB: drbedin@uol.com.br
RA: randalladam@yahoo.com.br
GL: glandman@terra.com.br
BS: bianca.sa@terra.com.br
KM: kmetze@fcm.unicamp.br

Abstract

Background: Prognostic factors in malignant melanoma are currently based on clinical data and morphologic examination. These clinicopathologic features, although robust and reproducible prognostic markers, cannot accurately predict the clinical outcome for a single patient.

Examination of nuclei in histological or cytological preparations reveals important information on cell physiology and, furthermore, is of great diagnostic and prognostic importance.
Previous studies demonstrated that fractal characteristics are of prognostic importance in neoplasias.

The aim of this study was to investigate whether the fractal dimension of nuclear chromatin measured in routine histological preparations of malignant melanomas could be a prognostic factor for survival.

Methods: We examined 71 primary cutaneous and metastatic melanoma specimens with at least 1mm thickness, from patients with a 5 year minimum follow up. Nuclear area, form factor and fractal dimension of chromatin texture were obtained from digitalized images of H&E stained of tissue micro array section. Clark’s level, tumor thickness and mitotic rate were also determined.

Results: The median follow-up was 104 months. Tumor thickness, Clark’s level, mitotic rate, nuclear area and fractal dimension were significant at worse prognostic factor in univariate Cox regressions. When performing multivariate Cox regression, stratified for the presence or absence of metastases at diagnosis, only the Clark level and fractal dimension of the chromatin were included as independent prognostic factors in the final model.

Conclusion: In general, a more aggressive behaviour is usually found in genetically unstable neoplasias with a higher number of
genetic or epigenetic changes, which on the other hand, provoke a more complex chromatin rearrangement. The increased nuclear fractal dimension found in more aggressive melanomas is the mathematical equivalent of a chromatin architecture with higher complexity. We would like to conclude that there is strong evidence that the fractal dimension of the nuclear chromatin texture might be a new and promising variable in prognostic models of malignant melanomas.

Background

Malignant melanoma is the most lethal tumor of the skin with a constantly and rapidly increasing incidence in the last decades [1].

Prognostic factors are currently based on clinical data and morphologic examination (including variables such as tumor thickness, mitotic rate, etc.) [1,2,3].

These clinical-pathologic features, although robust and reproducible prognostic markers, cannot accurately predict the clinical outcome for a single patient. Therefore a search for new
markers is desirable so that patients at higher risk for relapse could be identified and submitted to early therapeutic intervention.

For this purpose traditional or new immunohistochemical markers, gene expression arrays, array comparative genomic hybridization and mutational profiling have been applied. [1,4,5] Most of these techniques are sophisticated and expensive, requiring specially equipped laboratories with a trained staff.

Examination of nuclei in histological or cytological preparations reveals important information on cell physiology and, furthermore, is of great diagnostic and prognostic importance [6,7,8,9].

Neoplastic growth induces important modifications not only of the DNA, but also of the composition and distribution of the histone and non-histone nuclear proteins thus provoking alterations of the distribution of heterochromatin in the nucleus. Physiological or pathological changes of the cell are accompanied by changes of the nuclear texture [10,11,12]. Chromatin texture features can even be used as prognostic markers in neoplasias.

The resources available in commercial softwares are usually restricted to basic morphometric parameters such as diameter, area, perimeter [13], which can not "measure" morphological features such as texture of nuclei in a sufficient manner.

Recently it has been shown that Fractal Geometry can be successfully applied to nuclear chromatin. [14,15]. The fractal concept describes complex irregular structures characterizing its self similarity in a
scale-invariant manner. In other words, a fractal shows very similar features when examined at different scales.

Previous studies demonstrated that fractal characteristics are of prognostic importance in neoplasias [16,17,18,19,20,21].

The aim of this study was to investigate whether the fractal dimension of nuclear chromatin measured in routine histological preparations of malignant melanomas could be a prognostic factor for survival.

**Methods**

**Study subjects**

We selected patients with superficial spreading cutaneous melanoma, diagnosed and treated at the Hospital AC Camargo Center for Cancer Research and Treatment. between 1994 and 2000. Criteria of inclusion were:

1. tumor size at least 1 mm
2. paraffin blocks available for the construction of the tissue micro array.
3. detailed and complete clinical follow-up for at least 60 months in survivors
All cases were reviewed according to the protocol established by the Brazilian Melanoma Group and the Brazilian Society of Pathology by a dermatopathologist (GL).

The following histological variables were assessed: tumor thickness, Clark’s level and mitotic rate (number of mitoses per 10 high power fields)

Two core biopsies were obtained from paraffin-embedded tissue of each tumor within the previously identified and marked area. Using a Tissue Microarrayer (Beecher Instruments, Silver Spring, USA ™ ) with a sample needle of 1.0mm, the tissue cores were transferred to a recipient paraffin block, according to the technique described by Kononen et al [22]. A 6 µm section of the master block was stained with H&E for further analysis.

Data collection

Image acquisition was performed with a Leica DC 500 ™ digital camera with high resolution (12 megapixels), and an oil immersion objective (x100). The images were saved without compression in bitmap format 24-bit color. At least 100 randomly selected tumor nuclei were acquired per case by the same operator. Only
non overlapping nuclei with characteristics of melanoma cells were captured, interactively segmented and then converted to a 8 bit gray scale by calculating the luminance.

Finally we measured the following karyometric parameters of each nucleus: nuclear area and circular form factor. The latter represents the ratio between perimeter of a circle with the same area as the nucleus and the actual perimeter of the nucleus itself. We also calculated the fractal dimension of the chromatin according to Sarkar [23].

After normalization of the gray value histogram for each nucleus, a pseudo-three-dimensional image was created with the z axis defined by the gray level of each pixel, thus transforming the hematoxylin-stained chromatin texture into a rough surface. (fig1 A, B)

The fractal dimension (FD) of the surface of the normalized pseudo 3-D images was calculated according to Sarkar with a software developed in our laboratories.

According to Sarkar et al. we filled the space with cubes and counted the number of intersections with the irregular surface. This procedure was repeated with smaller or larger cubes. In a log-log (fig 1 C) diagram for each of these procedures the number of intersections is plotted against the size of the cubes. The fractal dimension can be obtained by the slope of the linear regression line.

Fig 1 C.
Correlations between variables were calculated according to Pearson’s after testing for normality of distribution.

The relations between survival and clinical and morphometric variables were analyzed by univariate Cox regressions.

As event we defined death related directly or indirectly to malignant melanoma. Patients who expired without metastatization entered the study as censored cases.

In a second step, all variables with a p up to 0.05 were included in a multivariate Cox-regression (0.05 for input and P = 0.1 for output, forward conditional step-wise selection) in order to get a prognostic model for the overall survival time.

Categorization of subgroups is not possible in the multivariate Cox model when there are subgroups without event, because in this case, the regression does not converge. When this was the case, we defined each diagnostic category by its quotient calculated between observed and expected events in the log-rank test of the corresponding Kaplan-Meier model, as suggested earlier [24].
Results

The study comprised of 71 patients, 41 women and 30 men.

The median follow-up was 104 months, ranging from 8 to 160 months. 15 patients died due to disseminated disease.

The primary tumor was located in the trunk in 47.1% patients, 44.1% in the extremities in 60 patients and 8.8% in head and neck.

Tumor thickness measured from 1.05 to 17.0 mm (median 2.35 mm) was a significant adverse prognostic factor in the univariate Cox regression (B=0.1623; p= 0.0007).

The Clark level ranged from 2 to 6 and was also a significant adverse prognostic factor (B = 1.1574; p= 0.0011)

At diagnosis patients had a median age of 55 years (range 14 to 89 years). Age was not a prognostic factor in the univariate proportional hazard model (p > 0.10; ).

Eight patients revealed lymph node and 3 patients hematogenic metastases at diagnosis, but these subgroups did not show statistically significant worse survival (p>0.1 in both cases).

The median mitotic rate was 3 per 10 high power fields (ranging between 0 and 52/10 HPF). The mitotic rate was significantly correlated with the tumor thickness (r=0.54; p <0.0001) and revealed to be a negative prognostic factor (B = 0.043. p=0.0095).

The nuclear area (median value : 81.3 micra2; range 33.7 to 139.3) was also correlated with the tumor thickness (r = 0.405; p
=0.00045) and the mitotic rate (r=0.31; p=0.008) and was a significant unfavorable prognostic factor (B=0.0212; p=0.0274).

The form factor (median: 0.706 ranging from 0.573 to 0.807) was negatively correlated with tumor thickness (r=-0.431; p<0.0001) and mitotic rate (r=-0.28; p=0.016), but of no prognostic relevance (p=0.417).

The fractal dimension of the chromatin structure (median: 2.06: ranging from 2.01 to 2.082) was significantly correlated with tumor thickness (r=0.482; p<0.0001) and mitotic rate (r=0.342; p=0.002) and revealed to be of prognostic relevance (B=73.9; p=0.0091).

In a further step we run a multivariate Cox regression, stratified for the presence or absence of metastases at diagnosis including all variables with p < 0.05 in the univariate models. The final regression included only the variables: Clark level (B=1.0427; p=0.0036) and fractal dimension of the chromatin (B=55.169; p=0.05) as independent prognostic factors.
Discussion and Conclusion

The prognostic relevance of tumor thickness, Clark’s level and the mitotic rate, which are all well known in literature, were confirmed by our study [1]. Yet we could not demonstrate the presence of lymph node or hematogenic metastases as significant prognostic variables, but this is certainly due to the very small number of patients with stage III or IV, so that the statistical test power was too low in order to show significant differences. Nevertheless, in order to eliminate even minor influences of the metastases on the proportional hazard model, we stratified the final Cox regression for the presence of metastases.

The main emphasis of our investigation was however, to elucidate the possibilities of karyometric variables as prognostic factors.

The use of computerized image analysis has contributed to an objective description of melanoma cells and decreased substantially inter-observer variations. Objective measurement of nucleolar organizer regions (AgNORs), proliferating cell nuclear antigen (PCNA) and Ki-67, nuclear DNA content or of karyometric variables such as chromatin compactness or nuclear size and shape have been used for the differential diagnosis between melanomas and several forms of melanocytic nevi [25,26,27].

The karyometric differences between benign and malignant melanocytic lesions reflect the alterations on the genetic and epigenetic level during the transformation from common melanocytic nevi to dysplastic nevi to melanoma.
This process involves dynamic changes in the genome produced by mutations with gain of function of oncogenes or loss of function of tumor suppressor genes, but also during tumor progression, passing from the radial to the vertical growth phase and finally to the metastatic phenotype, many additional alterations of gene expression can be found [1].

During the process of cancer progression genetic mutations interact with chromatin remodeling. It has been postulated that the deregulation of signal transduction pathways will inevitably lead to histone modification and alterations of chromatin organization [28]. Thus we might expect that changes of nuclear shape and size and especially the chromatin distribution would occur in parallel to tumor progression in melanoma.

Our karyometric measurements are in accordance with this hypothesis: with increasing tumor thickness, i.e. vertical growth, the nuclear area enlarged and the form factor decreased, which means that the cells turned to loose their roundness. In the univariate Cox regression, nuclear area was also a significant unfavorable prognostic factor, i.e., the ability to metastasize was higher in melanomas with larger nuclei. This result confirms a previous study, where it had been shown that the mean nuclear volume of primary melanomas with subsequent metastatic course was larger than that of tumors without metastatization [29].

According to Talve et al [30], in aneuploid melanomas thickness correlates with nuclear size, which is also related to a larger
proportion of aneuploid cells and increased genetic instability.

The nuclear size was also positively correlated with the fractal dimension of the chromatin architecture of the nuclei.

Fractality of tumor nuclei in cytologic or histologic slides has shown to be of important prognostic relevance in several neoplasias, such as in oral squamous cell carcinomas [31,32], lymphomas [33], leukemias [34], and multiple myelomas [35].

In our investigation the fractal dimension of the haematoxylin-stained nuclear chromatin showed to be an independent adverse prognostic factor for survival in malignant melanomas. As possible explanation we would like to combine nuclear pathophysiology and chromatin morphology in the following way: In general, a more aggressive behaviour is usually found in genetically unstable neoplasias with a higher number of genetic or epigenetic changes, which on the other hand provoke a more complex chromatin rearrangement. The increased nuclear fractal dimension found in more aggressive melanomas is the mathematical equivalent of a chromatin architecture with higher complexity.

In our study, tumor thickness, nuclear size, mitotic index and fractal dimension were all positively correlated with each other. Nevertheless the stepwise procedure in the multivariate Cox regression selected only the fractal dimension and the Clark level as independent variables which built up the best proportional hazard model explaining the
patients’ survival. This means that the complexity of the chromatin distribution contains relevant prognostic information which is independent of the Clark level.

This investigation is based on a relatively small number of patients and should be followed by confirmatory studies, of course; Nevertheless, we would like to conclude that there is strong evidence that the fractal dimension of the nuclear chromatin texture might be a new and promising variable in prognostic models of malignant melanomas.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

VB conceived the study, participated in its design, data collection and analysis and drafted an critical revision of the manuscript. RLA elaborated the software, participated in the data analysis and critical revision of the manuscript. De SA BCS participated in the data collection and analysis and critical revision of the manuscript. GL made the histopathologic review, participated in the data collection and critical revision of the manuscript. KM participated in the study design, statistically data analysis, draft and revising the manuscript for important intellectual content. All authors read and approved the final manuscript.
Acknowledgements

References


Cybernetics 1994; 24:115-120.


28. Rothhammer T and Bosserhoff AK. Epigenetic events in malignant melanoma. Pigment Cell Res. 20; 92–111


Figures:

**Fig. 1 A** - Segmented of melanoma cell after the gray level transformation

**Fig 1 B** - Pseudo 3-D of the nucleus with the z axis defined by the gray levels of each pixel thus creating a landscape-like surface
Fig. 1 C – Box counting according to Sarkar
Figure 1 B
Figure 1 C

Box Counting, Sarkar Method

Log Box Counting

Log Scale

FD = 2.073  \( R^2 = 0.9987 \)