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The fractal dimension of nuclear chromatin as a prognostic factor in acute precursor B lymphoblastic leukemia

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Abstract. The fractal nature of the DNA arrangement has been postulated to be a common feature of all cell nuclei. We investigated the prognostic importance of the fractal dimension (FD) of chromatin in blasts of patients with acute precursor B lymphoblastic leukemia (B-ALL). In 28 patients, gray scale transformed pseudo-3D images of 100 nuclei (May–Grünwald–Giemsa stained bone marrow smears) were analyzed. FD was determined by the Minkowski–Bouligand method extended to three dimensions. Goodness-of-fit of FD was estimated by the R^2 values in the log-log plots. Whereas FD presented no prognostic relevance, patients with higher R^2 values showed a prolonged survival. White blood cell count (WBC), age and mean fluorescence intensity of CD45 (MFICD45) were all unfavorable prognostic factors in univariate analyses. In a multivariate Cox-regression, R^2, WBC, and MFICD45, entered the final model, which showed to be stable in a bootstrap resampling study. Blasts with lower R^2 values, equivalent to accentuated "coarseness" of the chromatin pattern, which may reflect profound changes of the DNA methylation, indicated a poor prognosis. In conclusion the goodness-of-fit of the Minkowski–Bouligand dimension of chromatin can be regarded as a new and biologically relevant prognostic factor for patients with B-ALL.

Keywords: Morphometry, complexity, texture analysis, fractality, karyometry

1. Introduction

Examination of nuclei of routine histologic or cytologic preparations reveals important information on cell physiology and, furthermore, is of great diagnostic and prognostic importance. In order to overcome the diagnostic insecurity caused by subjective interpretation, quantitative analysis is mandatory [27]. This can be done both by simple morphometric procedures or more sophisticated texture analysis methods [5,12,15–17,22–24,38,39]. Scale-invariant self-similarity, which cannot be described adequately by classic morphometric analysis based on Euclidean geometry, has shown to be an important feature of many biological structures. It can be estimated by the determination of the fractal dimension, which turned out to be an interesting alternative tool in image analysis [1,7,9–11,13,19,21]. Recently it has been postulated that the fractal nature of the DNA arrangement could be a common feature of all cell nuclei of higher organisms [21], thus underlining the importance for the fractal analysis of chromatin structures. Investigations on the prognostic relevance of the fractal geometry of nuclei in neoplasms are rare, however [38]. In our study we tried to find out whether the measurement of the fractal dimension of the chromatin structure in nuclei of leukemic blasts would be of prognostic value for patients suffering from acute precursor B lymphoblastic leukemia (B-ALL).

2. Patients and methods

All patients with newly diagnosed B-ALL treated at our Institution between August 2002 and Septem-

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ber 2004 entered the investigation. The diagnosis was based on peripheral blood counts, bone marrow cytology, cytogenetics and immunophenotyping by flow cytometry of bone marrow aspirates (whole blood lysis technique). Antigenic expression was detected using triple combinations of monoclonal anibodies. Data acquisition was performed on a FACSCallibur flow cytometer using CellQuest^{TM} and Paint-A-Gate^{TM} softwares (Becton Dickinson). The expression of each antigen was recorded as mean fluorescence intensity (MFI). Random pictures from at least 100 nuclei per patient of routinely May-Grünwald–Giemsa stained bone marrow slides were captured by a Kontron Zeiss KS-300 system (bmp-format; 0.1 μm/pixel spatial resolution; 1.25 numerical aperture, 100$\times$ oil immersion objective). Nuclei were interactively segmented. The images were converted to grayscale format with levels of luminance ranging between 0 and 255 (Figs 1a and 2a). We created pseudo-3D images, where the x and y coordinates represent the position of the objects and the z coordinate the respective grey levels (Figs 1b and 2b). The fractal dimension (FD) was determined using the Minkowski–Bouligand method [9] extended to three dimensions. In brief we calculated the fractal area which is estimated by the volume$/2\varepsilon$ (being ε the radius,varying between 1 and 30 pixels) of the non-planar structuring element in form of a ball [9]. The linear regression was calculated in a log–log plot (area versus ε) containing 30 points (Figs 1c and 2c). The goodness of fit was estimated by the R^2 value of the regression between the real and the estimated values. Furthermore, we tested with the Kolmogorov–Smirnov test, whether the residuals followed a normal distribution, calculating for each patient the percentage of cells with normally distributed residuals (P). Finally the prognostic relevance of all these parame-

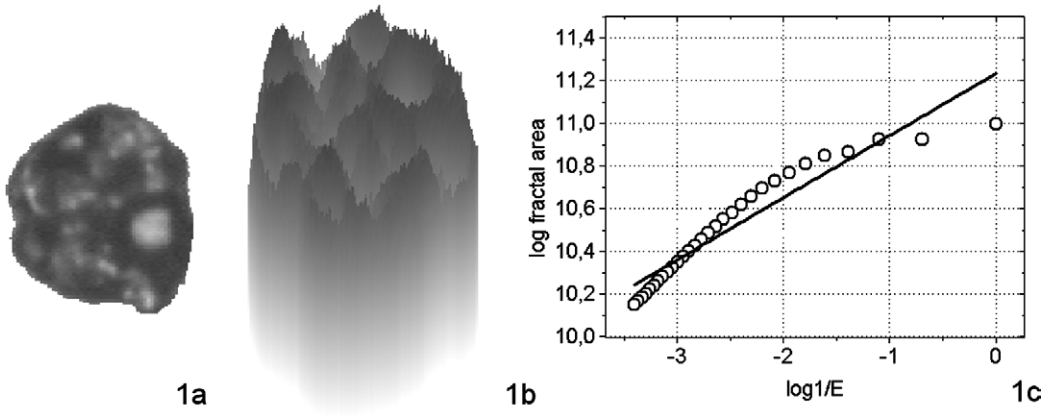

Fig. 1. Leukemic blast (a). Accentuated coarseness of the surface in the pseudo-3D representation of the chromatin (b) and concavity of the regression line in the log–log-plot ($R^2 = 0.904$; (c)).

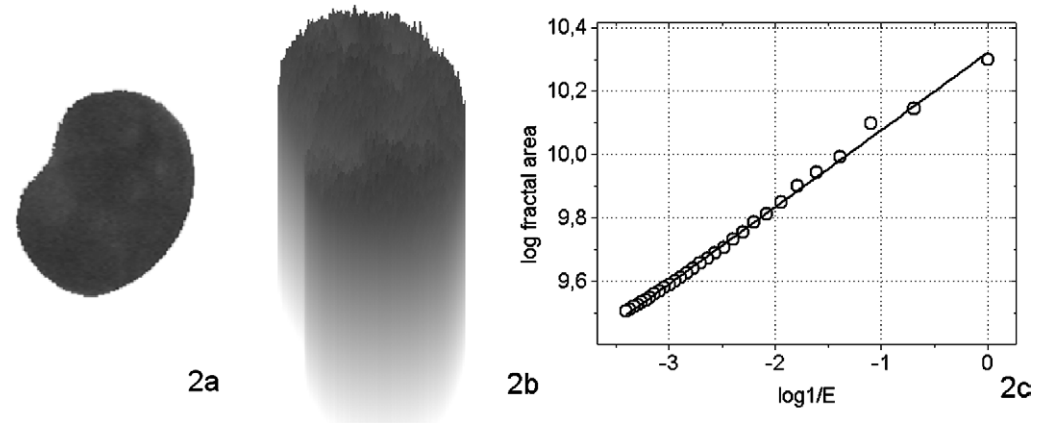

Fig. 2. Leukemic blast (a) with relatively smooth surface in the pseudo-3D representation of the chromatin (a) and good approximation of the curve by a linear regression in the log–log-plot ($R^2 = 0.997$; (c)).

ters was analyzed in univariate and multivariate Cox regressions ($p = 0.05$ for input and $p = 0.1$ for output, backward conditional step-wise selection), comparing them with established prognostic factors such as age, white blood cell count and MFI of CD45 (MFICD45) [37]. The internal stability of the final Cox model was tested on 100 newly created data sets by bootstrap resampling with replacement [25,31,34]. SPSS 8.0 and WinStat softwares were used for calculations.

3. Results

Twenty eight patients fulfilled the criteria of inclusion. The mean value of the Minkowski–Bouligand FD was 2.265 with a range between 2.238 and 2.285. The R^2 values, as a measure of goodness-of-fit, ranged between 0.945 and 0.986 (mean 0.967). The P values varied considerably: the minimum was 2.0% and the maximum 100%, with a mean value of 72.4%. R^2 was significantly correlated with FD ($r = -0.59; p = 0.001$) and P ($r = 0.69; p < 0.0001$). In the univariate Cox-regression, FD was not statistically relevant for survival ($p = 0.15$). However, the R^2 values proved to be a statistically significant favorable prognostic variable ($B = -45.5947; p = 0.049; R = -0.1616$) but this was not the case for the variable P ($p = 0.12$). White blood cell count ($p = 0.0212; R = 0.2346; B = 0.0133$), age ($p = 0.0194; R = 0.2331; B = 0.413$) and MFICD45 ($B = 0.725; p = 0.0127; R = 0.2911$) were all of unfavorable prognostic relevance in univariate Cox regressions. In the multivariate Cox regression, only the variables "R^2" ($p = 0.021$), "white blood cell count" ($p = 0.047$) and "MFI CD45" ($p = 0.026$) entered the final model, which was confirmed by the bootstrap resampling study: R^2 was included in 72%, white blood cell count in 60%, MFICD45 in 74%, but age only in 28% of the models.

4. Discussion

Many cell structural and biochemical features change with increasing aggressiveness of a neoplasia. Therefore we can estimate the outcome of the patient by different techniques. Prognostic factors can be determined by molecular biology, flow cytometric investigations, or image analysis of DNA alterations [4,14, 24,40–42,44]. Furthermore, changes of nuclear, cytoplasmic or mitochondrial protein expression may reflect different biological behaviour of the tumour [20, 42,43]. In contrast to these sophisticated and expensive techniques, relevant prognostic, as well as diagnostic information can also be obtained by morphometric and texture analyses of whole tissue sections or individual cells [1–3,8,24,26,28–36]. These methods are based on simple, reproducible and unexpensive staining methods, or, as in our case, can be done on routine slides from the files, thus permitting retrospective studies without any additional costs.

An important aspect of texture analysis is the determination of the fractality, since this variable shows important aspects of the complexity of a structure not revealed by classical morphometry based on Euclidean geometry.

In some studies the fractal dimension is measured after binarization of the image [7]. But, since we are dealing with contiuous gray value transitions of the chromatin, the segmentation process would be somewhat arbitrary. Moreover, a categorization reduces the information content [27]. In our case 256 gray values, equivalent to 8 bits/pixel, would be reduced to a black and white image, equivalent to 1 bit/pixel. In order to use the whole information, we decided not to binarize, but rather to apply the Minkowski–Bouligand method [9] extended to the pseudo-3D images, as has been done by other researchers [10].

The main question, whether a given structure should be considered as fractal, is linked to its scaling characteristics, which follow power laws. Therefore the fractal dimension of a given image relies essentially on the linear regression of the log–log plot [9,13]. The goodness-of fit of the linear regression is an important, but not the only criterion for fractality [13]. Moreover, we must draw attention to the residuals, which should scatter around the regression line following a normal distribution. If these assumptions do not hold, the value of the calculated FD cannot be supported [13]. In our study many cells did not fulfil these criteria. Therefore we should interpret the calculated FDs with great caution.

There are several methods to determine the fractal dimension, but all of them, including the box-counting technique and the Minkowski–Bouligand dimension, show limitations for technical reasons [9], such as the digitalization of the image. The precision of the Minkowski–Bouligand dimension is poor, since the dots never lie straight on the linear regression line, but, form a more or less pronounced concavity, as seen in Fig. 1c. This effect is due to the presence of local maxima and minima in the image [9] and increases with their number. Therefore nuclei with higher FD

show worse goodness-of-fit, since a larger number of local maxima and minima increases both the complexity (FD) and the expression of the concavity. Reasonable goodness-of-fit, equivalent to high R^2 values, and normally distributed, randomly scattered residuals, is therefore expected to be present only in nuclei with few local maxima and minima. Therefore the goodness-of-fit may be interpreted as a measure of roughness of the surface of the pseudo-3D image of the nucleus.

Alterations of the chromatin structure of leukemic blasts occur in parallel with changes of cytoplasmic and membranous protein expression and are therefore regarded to reflect differences of maturation [36]. Since the heterochromatic regions in Giemsa-stained slides are co-localizing with the extended methyl-rich DNA domains [6], changes in the Giemsa texture could be interpreted as modifications of the methylation pattern. In other words, nuclei with accentuated "coarseness" of the Giemsa pattern, which is an unfavorable prognostic feature according to our study, could reflect profound changes of the DNA methylation. This hypothesis is corroborated by the fact that an increased number of DNA methylation changes is found in B-ALL patients with a bad prognosis [18].

In summary, we suggest that the goodness-of-fit of the Minkowski–Bouligand dimension of nuclei in May–Grünwald–Giemsa stained cytologic preparations might quantify remodeling of the DNA methylation pattern and can be regarded as a new and biologically relevant prognostic factor for patients with B-ALL.

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References

[1] R.L. Adam, T.C.G. Corsini, P.V. Silva, M.L. Cintra, N.J. Leite and K. Metze, Fractal dimensions applied to thick contour detection and residues – Comparison of keloids and hypertrophic scars, *Cytometry* Part A **59A** (2004), 63–64.

[2] R.L. Adam, N.J. Leite, R.B. de Carvalho, P.V. Silva and K. Metze, Granulometric residues as a diagnostic tool in cytology, *Cytometry* Part A **59A** (2004), 63.

[3] R.L. Adam, E. Ribeiro, K. Metze, N.J. Leite and I. Lorand-Metze, Morphometric and granulometric features of erythroblasts as a diagnostic tool of hematologic diseases, *Cytometry* Part A **59A** (2004), 46.

[4] N.J. Armstrong and M.A. van de Wiel, Microarray data analysis: from hypotheses to conclusions using gene expression data, *Cellular Oncology* **26** (2004), 279–290.

[5] A. Böcking, J. Stockhausen and D. Meyer-Ebrecht. Towards a single cell cancer diagnosis. Multimodal and monocellular measurements of markers and morphology (5M), *Cellular Oncology* **26** (2004), 73–79.

[6] A. de Capoa, F.R. Febbo, F. Giovannelli, A. Niveleau, G. Zardo, S. Marenzi and P. Caiafa, Reduced levels of poly(ADP-ribosyl)ation result in chromatin compaction and hypermethylation as shown by cell-by-cell computer-assisted quantitative analysis, *FASEB Journal* **13** (1999), 89–93.

[7] D. Chappard, E. Legrand, B. Haettich, G. Chales, B. Auvinet, J.P. Eschard, J.P. Hamelin, M.F. Basle and M. Audran, Fractal dimension of trabecular bone: comparison of three histomorphometric computed techniques for measuring the architectural two-dimensional complexity, *Journal of Pathology* **195** (2001), 515–521.

[8] E.M.M. Cia, M. Trevisan and K. Metze, Argyrophilic nucleolar organizer region (AgNOR) technique: a helpful tool for differential diagnosis in urinary cytology, *Cytopathology* **10** (1999), 30–39.

[9] B. Dubuc, J.F. Quiniou, C. Roques-Carmes, C. Tricot and S.W. Zucker, Evaluating the fractal dimension of profiles, *Physical Reviews A* **39** (1989), 1500–1512.

[10] A.J. Einstein, H.S. Wu, M. Sanchez and J. Gil, Fractal characterization of chromatin appearance for diagnosis in breast cytology, *Journal of Pathology* **185** (1998), 366–381.

[11] A. Gerger, P. Bergthaler and J. Smolle, An automated method for the quantification and fractal analysis of immunostaining, *Cellular Oncology* **26** (2004), 125–134.

[12] M.F. Gilberti, K. Metze and I. Lorand-Metze, Changes of nucleolar organizer regions in granulopoietic precursors during the course of chronic myeloid leukemia, *Annals of Hematology* **71** (1995), 275–279.

[13] G. Gonzato, F. Mulargia and W. Marzocchi. Practical application of fractal analysis: Problems and solutions, *Geophysical Journal International* **132** (1998), 275.

[14] M. Grade, H. Becker and B.M. Ghadimi, The impact of molecular pathology in oncology: The clinician's perspective, *Cellular Oncology* **26** (2004), 275–278.

[15] H.Z.W. Grotto, I. Lorand-Metze and K. Metze, Nucleolar organizer regions in normal hematopoiesis: relationship to cellular proliferation and maturation, *Nouvelle Revue Francaise d'Hematologie* **33** (1991), 1–4.

[16] H.Z.W. Grotto, K. Metze and I. Lorand-Metze, Pattern of nucleolar organizer regions in human leukemic-cells, *Analytical Cellular Pathology* **5** (1993), 203–212.

[17] M. Guillaud, D. Cox, A. Malpica, G. Staerkel, J. Matisic, D. van Niekirk, K. Adler-Storthz, N. Poulin, M. Follen and C. MacAulay, Quantitative histopathological analysis of cervical intra-epithelial neoplasia sections: methodological issues, *Cellular Oncology* **26** (2004), 31–43.

[18] M.I. Gutierrez, A.K. Siray, M. Bhargava, U. Ozbek, S. Banavali, M.A. Chaudhary, H.E.I. Soth and K. Bhatia, Concurrent methylation of multiple genes in childhood ALL: correlation with phenotype and molecular subgroup, *Leukemia* **17** (2003), 1845–1850.

[19] A. Huisman, L.S. Ploeger, H.F. Dullens, N. Poulin, W.E. Grizzle and P.J. van Diest, Development of 3D chromatin texture analysis using confocal laser scanning microscopy, *Celular Oncology* **27** (2005), 335–345.

[20] A.J. Kruse, J.P.A. Baak, E.A. Janssen, K.H. Kjellevold, B. Fiane, K. Lovslett, J. Bergh and S. Robboy, Ki67 predicts progression in early CIN: validation of a multivariate progression-risk model, *Cellular Oncology* **26** (2004), 13–20.

[21] D.V. Lebedeva, M.V. Filatova, A.I. Kuklin, A.Kh. Islamov, E. Kentzingerc, R. Pantinaa, B.P. Toperverga and V.V. Isaev-Ivanova, Fractal nature of chromatin organization in interphase chicken erythrocyte nuclei: DNA structure exhibits biphasic fractal properties, *FEBS Letters* **579** (2005), 465–468.

[22] I. Lorand-Metze, M.A. Carvalho and K. Metze, Relationship between morphometric analysis of nucleolar organizer regions and cell proliferation in acute leukemias, *Cytometry* **32** (1998), 51–56.

[23] I. Lorand-Metze and K. Metze, AgNOR clusters as a parameter of cell kinetics in chronic lymphocytic leukaemia, *Journal of Clinical Pathology – Clinical Molecular Pathology* **49** (1996), M357–M360.

[24] I. Lorand-Metze, F.G. Pereira, F.P. Costa and K. Metze, Proliferation in non-Hodgkin's lymphomas and its prognostic value related to staging parameters, *Cellular Oncology* **26** (2004), 63–71.

[25] I. Lorand-Metze, M.P. Pinheiro, E. Ribeiro, E.V. de Paula and K. Metze, Factors influencing survival in myelodysplastic syndromes in a Brazilian population: comparison of FAB and WHO classifications, *Leukemia Research* **28** (2004), 587–594.

[26] T. Mattfeldt, D. Trijic, H.W. Gottfried and H.A. Kestler, Classification of incidental carcinoma of the prostate using learning vector quantization and support vector machines, *Cellular Oncology* **26** (2004), 45–55.

[27] K. Metze and R.L. Adam, Quantification in histopathology – some pitfalls, *Brazilian Journal of Medical and Biological Research* **38** (2005), 141–143.

[28] K. Metze, R.L. Adam, P.V. Silva, R.B. de Carvalho and N.J. Leite, Analysis of chromatin texture by Pinkus' approximate entropy, *Cytometry* Part A **59A** (2004), 63.

[29] K. Metze, V. Bedin, R.L. Adam, M.I. Cintra, E.M. de Souza and N.J. Leite, Parameters derived from the fast Fourier transform are predicitive for the recurrence of basal cell carcinoma, *Cellular Oncology* **27** (2005), 137.

[30] K. Metze, A.C. Chiari, F.L. Andrade and I. Lorand-Metze, Changes in AgNOR configurations during the evolution and treatment of chronic lymphocytic leukemia, *Hematology and Cell Therapy* **41** (1999), 205–210.

[31] K. Metze, A.M. Lobo and I. Lorand-Metze, Nucleolus organizer regions (AgNORs) and total tumor mass are independent prognostic parameters for treatment-free period in chronic lymphocytic leukemia, *International Journal of Cancer* **89** (2000), 440–443.

[32] K. Metze and I. Lorand-Metze, Interpretation of the AgNOR pattern in hematologic cytology, *Acta Haematologica* **89** (1993), 110.

[33] K. Metze and I. Lorand-Metze, Age related decrease of AgNOR activity in acute and chronic lymphocytic leukaemias, *Journal of Clinical Pathology – Molecular Pathology* **52** (1999), 52.

[34] K. Metze, G.B. Oliveira, F.G. Pereira, R.L. Adam and I. Lorand-Metze, Spontaneous apoptosis in chronic lymphocytic leukemia is not an independent prognostic factor for stability of disease when compared with combined AGNOR and TTM scores, *Cellular Oncology* **27** (2005), 199–201.

[35] K. Metze, A.C.S. Piaza, A.A. Piaza, R.L. Adam and N.J. Leite, Texture analysis of agnor stained nuclei in lung cancer, *Cellular Oncology* **27** (2005), 137–138.

[36] K. Metze, R.C. Silva, R.L. Adam, N.J. Leite, F.G. Pereira and I. Lorand-Metze, Relation between chromatin texture and phenotype in acute leukemias, *Cellular Oncology* **27** (2005), 112–113.

[37] A. Nakamura, M. Tsurusawa, A. Kato, T. Taga, Y. Hatae, M. Miyake, J. Mimaya, N. Onodera, A. Watanabe, T. Watanabe, H. Kanegane, T. Matsushita, A. Iwai, N. Hyakuna, K. Gushi, T. Kawakami, I. Sekine, O. Izichi, K. Asami, A. Kikuta, A. Tanaka and T. Fujimoto, Prognostic impact of CD45 antigen expression in high-risk, childhood B-cell precursor acute lymphoblastic leukemia, *Leukemia & Lymphoma* **42** (2001), 393–398.

[38] B. Nielsen, F. Albregtsen, W. Kildal and H.E. Danielsen, Prognostic classification of early ovarian cancer based on very low dimensionality adaptive texture feature vectors from cell nuclei from monolayers and histological sections, *Analytical Cellular Pathology* **23** (2001), 75–88.

[39] G.B. Oliveira, F.G. Pereira, K. Metze and I. Lorand-Metze, Spontaneous apoptosis in chronic lymphocytic leukemia and its relationship to clinical and cell kinetic parameters, *Cytometry* **46** (2001), 329–335.

[40] H. Raatz, A. Böcking and S. Hauptmann, Prognostic impact of DNA-image-cytometry in neuroendocrine (carcinoid) tumours, *Cellular Oncology* **26** (2004), 81–88.

[41] A. Reith and T. Ried, Genes, chromosomes and cancer, *Cellular Oncology* **26** (2004), 167.

[42] E. Ribeiro, C.S.P. Lima, K. Metze and I. Lorand-Metze, Flow cytometric analysis of the expression of Fas/Fasl in bone marrow CD34(+) cells in myelodysplastic syndromes: relation to disease progression, *Leukemia & Lymphoma* **45** (2004), 309–313.

[43] A. Schonherr, M. Bayer and A. Böcking, Diagnostic and prognostic value of Ki67 proliferation fraction in serous effusions, *Cellular Oncology* **26** (2004), 57–62.

[44] M.M. Weiss, E.J. Kuipers, C. Postma, A.M. Snijders, D. Pinkel, S.G.M. Meuwissen, D. Albertson and G.A. Meijer, Genomic alterations in primary gastric adenocarcinomas correlate with clinicopathological characteristics and survival, *Cellular Oncology* **26** (2004), 307–317.