Professional article/Strokovni prispevek

IMAGE DNA ANALYSIS OF BONE MARROW MEGAKARYOCYTES IN DIFFERENTIAL DIAGNOSIS OF THROMBOCYTOSIS

SLIKOVNA ANALIZA DNK MEGAKARIOCITOV KOSTNEGA MOZGA V DIFERENCIALNI DIAGNOZI TROMBOCITOZE

Joško Vučković, Rok Pavlič, Mateja Grat, Ivan Žuran, Štefka Krivec

Interni oddelek, Splošna bolnišnica, Oblakova 5, 3000 Celje

Arrived 2004-02-26, accepted 2004-03-17; ZDRAV VESTN 2004; 73: Suppl. I: 23–5

Key words: *cytofotometry; Feulgen; nuclear DNA; megakaryocyte ploidy; primary and secondary thrombocytosis*

Ključne besede: *citofotometrija; Feulgen; jedrna DNK; ploidnost megakariocitov; primarna in sekundarna trombocitoza*

Abstract – Background. *Megakaryocyte (Mk) nuclear DNA content was measured by image analysis using new S-Form software. Feulgen-stained bone marrow aspirates from 11 patients with reactive thrombocytosis and 12 patients with clonal thrombocytosis of chronic myeloproliferative disorders (CMPDs) were analysed. Ploidy of 100 Mk was calculated for each patient and the fractions of hyperploid cells were compared. The presence of more than 5% of Mk with nuclear ploidy > 96N or ≥ 2% of Mk with nuclear ploidy > 128N in patients with thrombocytosis strongly suggests CMPD. On the other hand, the absence of hyperploid cells > 128N or the presence of a small fraction (less than 5%) of moderately hyperploid cells (96–128 N) indicates reactive thrombocytosis. Image cytometry analysis therefore appears to add valuable information to conventional cytological examination in the diagnostic assesment of thrombocytosis.*

Conclusions. *S-Form software image analysis is much faster and more reliable than microdensitometric DNA analysis, which we previously used for cytometric studies. We believe the method of image DNA analysis has the potential for widespread clinical use in the differential diagnosis of megakaryocyte disorders.*

Izvleček – Izhodišča. *Število, velikost in ploidnost megakariocitov so odločilni kazalci za diagnozo mieloproliferativnih bolezni (MPB). Pri reaktivnih stanjih najdemo občasno v kostnem mozgu podobne spremembe, ki jih z dostopnimi metodami težko razlikujemo od tistih pri MPB. Z novo metodo slikovne citometrije smo določali razliko v ploidnosti megakariocitov pri nekaterih MPB in reaktivnih trombocitozah.*

Metode. *Z metodo slikovne citofotometrije in novim računalniškim programom S-Form smo analizirali aspirate kostnega mozga 11 bolnikov z reaktivno trombocitozo in 12 bolnikov s klonalno trombocitozo pri MPB. Za barvanje smo uporabili modificirano Feulgenovo metodo. Pri vsakem bolniku smo analizirali po 100 megakariocitov in izračunali delež hiperploidnih celic čez 96N in 128N.*

Rezultati. *Prisotnost > 5% megakariocitov s ploidnostjo več kot 96N ali ≥ 2% megakariocitov s ploidnostjo več kot 128N pri bolniku s trombocitozo govori v prid MPB. Nasprotno odsotnost hiperploidnih celic > 128N ali prisotnost malega deleža (manj kot 5%) celic s ploidnostjo med 96N in 128N kaže na reaktivno trombocitozo.*

Zaključki. *Slikovna analiza DNK megakariocitov z računalniškim programom S-Form je koristna in praktična metoda za razlikovanje primarnih in reaktivnih trombocitoz. Menimo, da bo pomemben dodatek pri citološki diagnostiki posameznih megakariocitnih motenj.*

Introduction

The regulation of platelet production is centred around changes in megakaryocyte proliferation and maturation. Megakaryocyte number, size and ploidy rise in response to increased platelet demand, as seen in thrombocytopenia and reactive thrombocytosis (RT), or as a result of the autonomous, clonal proliferation of chronic myeloproliferative disorders (CMPDs) (1–5). However, megakaryocyte hyperplasia in reactive states doesn't reach the degree that is suggested to be a feature of the CMPD (5).

Chronic myeloproliferative disorders represent a group of monoclonal disorders characterized by the overproduction of one or more of the formed elements of blood in the absence of a definable stimulus, bone marrow hypercellularity with fibrosis of different degrees and morphological changes dominantly in megakaryocytic cell line (5).

Although thrombocytosis is most commonly found in essential thrombocythemia (ET) it is also often present in other CMPDs, especially in polycythemia vera (PV) and chronic idiopathic myelofibrosis (CIMF) (5). Significant diagnostic overlap could exist between the clonal thrombocytosis of CMPD and RT at presentation (6). Despite much study no pathognomonic cytogenetic abnormality has been identified in PV, IMF or ET patients. Cytological and pathological characteristics are not always diagnostic and could be misleading (5, 6). Therefore, a relevant method for defining clonal proliferation would be useful in the clinical diagnosis of these megakaryocyte disorders. Impaired expression of the thrombopoietin receptor Mpl has been recently identified in the platelets and megakaryocytes of PV patients and some ET patients. Similar observations have been made for granulocyte PRV-1 mRNA, but these tests have not yet gained a wide clinical acceptance (7, 8).
A precise analysis of megakaryocyte ploidy profile is another promising approach (3, 9, 10). Hyperploidy and aneuploidy are well-recognized features of malignant processes and different methods of image and flow cytometry analysis to quantify nuclear DNA have been tested in different malignancies (11–17). Analysis of megakaryocyte ploidy by DNA cytometry is a potentially useful tool for differentiating CMPD from the reactive states (3, 9). However, this analysis is not widely employed due to technical complexities and a lack of simple and standardized techniques. In order to differentiate between reactive and neoplastic megakaryocyte hyperploidy, a more efficient method using a new software program for image analysis has been tested and the results are presented here.

Patients and methods

A hundred and sixteen patients with thrombocytosis > 800 × 10^9/L were registered and treated in our hospital in the period from April 2002 to September 2003. Bone marrow aspiration and analysis was performed in all cases that could not be defined as reactive thrombocytosis after initial evaluation, as well as in all cases with suspected CMPD. During follow up, reactive thrombocytosis was confirmed in 11 of such patients. Bone marrow smears of all 11 patients with RT and of another 13 patients, in which CMPD was confirmed, were analysed using an image DNA analysis system after Feulgen staining. Merck's DNA staining kit for Feulgen staining was used and the staining procedure was performed as already described (18, 19). With this method the cell nuclei are stained purple. The amount of stain colour developed is directly proportional to the amount of DNA present in the stained nuclei.
The DNA content of 100 randomly selected megakaryocyte nuclei was determined using an image analysis system (Olympus BX50 microscope with Oil-50 objective, Sony ExwaveHAD SSC-DC58AP digital colour video camera, Vamstec S-Form image analysis software). Histograms for ploidy categories (8, 16, 32, 64, 128 > 128N) were done. The fraction of nuclei with DNA content of > 96N and > 128N were calculated for all patients and compared using SPSS software.

Results

Patients with clonal thrombocytosis had a pattern of increased megakaryocyte ploidy compared to patients with reactive thrombocytosis ($p < 0.001$). Typical DNA histograms for CMPD and RT are shown in figures 1 and 2.
All patients with CMPD had more than 5% of cells in the hyperploid > 96N region and at least 2% of cells with more than 128N DNA content. Among the patients with reactive thrombocytosis there were no megakaryocytes with more than 128N DNA content. However, some hyperploid cells (up to 4%) in the region from 96N to 128N were found (Figure 3).

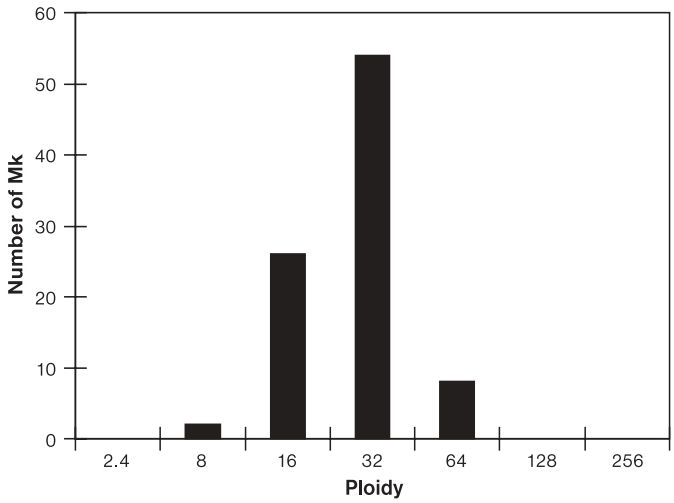

Figure 1. *Megakariocytes (Mk) ploidy distribution in reactive thrombocytosis.*

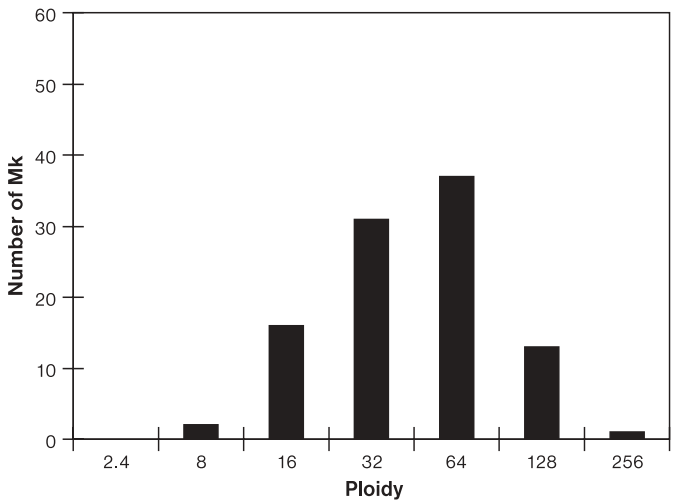

Figure 2. *Chronic myeloproliferative disease.*

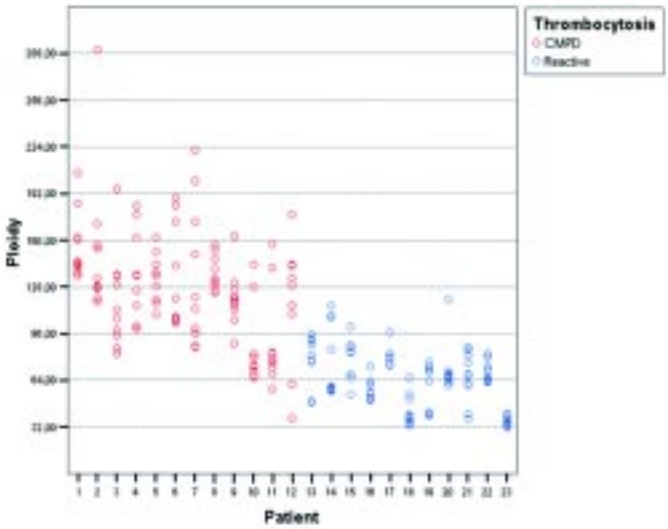

Figure 3. *Megakaryocytes in clonal (CMPD) and reactive thrombocytosis (RT).*

Discussion

Human cells are mostly diploid (2N or 2C), with increased DNA value up to tetraploid value (4N) in the course of the cell cycle. Although some exceptions exist (20), it is generally assumed that the presence of hyperploid cells (more than 5N) in such tissues is a sign of malignancy (11, 12). In contrast, mature megakaryocytes are giant multinuclear cells with a ploidy level dependent on platelet demand (1–3). Therefore the upper level of ploidy for normal megakaryocytes is difficult to determine. It is suggested that normal megakaryocytes do not exceed 64N level of ploidy (3, 9). In clonal proliferation, as seen in CMPD, megakaryocytes with 128N or even higher levels of ploidy are reported (3, 9). A clear cut-off point between RT and CMPD was obtained in our study: all patients with CMPD had more than 5% of cells in the hyperploid > 96N region and at least 2% of cells with more than 128N DNA content, while patients with reactive thrombocytosis had no megakaryocytes with more than 128N DNA content. The technical nature of studying megakaryocyte ploidy is a considerable problem. Microdensitometric studies are time-consuming, requiring more than 2 hours to measure the DNA content of 200 megakaryocytes. Flow cytometric studies are of short duration but the complicated procedure of the megakaryocyte separations required prior to flow cytometry analysis is an obstacle. For both techniques the equipment is expensive and not widely available. New image analysis techniques are shown to be sufficiently precise and reproducible (21). In our experience, image DNA analysis with the new S-Form software and the other easily available equipment we used is practical; may be performed at any time on previously stained smears; and is of acceptable duration, lasting about 30 minutes. According to the preliminary results presented here, this method is a useful aid to cytology in differential diagnosis of megakaryocyte disorders.

Acknowledgement

Zahvaljujemo se Sabini Glavač, dipl. lab. biomed. za odlično tehnično obdelavo preparatov.

References

1. Pennington DG, Olsen TE. Megakaryocytes in states of altered plate production: cell numbers, size and DNA content. Br J Haematol 1970; 18: 447–63.
2. Corash L, Chen HL, Levin J, Baker G, Lu H, Mok Y. Regulation of thrombopoiesis: effects of the degree of thrombocytopenia on megakaryocyte and platelet volume. Blood 1987; 70: 177–85.
3. Torner A, Friese P, Conklin R, Bales W, Archer L, Harker LA, Burstein SA. Flow cytometric analysis of megakaryocytes from patients with abnormal platelet counts. Blood 1989; 74: 594–601.
4. Buss DH, O Connor ML, Woodruff RD, Richards F, Brockschmidr JK. Bone marrow and peripheral blood findings in patients with extreme thrombocytosis. A report of 63 cases. Arch Pathol Lab Med 1991; 115: 475–80.
5. Michiels JJ, Thiele J. Clinical and pathological criteria for the diagnosis of essential thrombocythemia, polycythemia vera, and idiopathic myelofibrosis (agnogenic myeloid metaplasia). Int J Hematol 2002; 76: 133–45.
6. Douglas KV, Tallman SM, Cripe DL, Peterson LA. Thrombopoietin administered during induction chemotherapy to patients with acute myeloid leukemia induces transient morphologic changes that may resemble chronic myeloproliferative disorders. Am Clin Pat 117: 844–50.
7. Spivak JL. Polycythemia vera: myths, mechanisms and management. Blood 2002; 100: 4272–90.
8. Teofili L, Pierconti F, Di Febo A et al. The expression pattern of c-mpl in megakaryocytes correlates with thrombotic risk in essential thrombocythemia. Blood 2002; 100: 714–7.
9. Woods MJ, Greaves M, Trowbridge EA. Megakaryocyte ploidy in thrombocytosis: Improved microdensitometric measurements with a new image analysis system. Eur J Haematol 1990; 44: 220–6.
10. Mazur EM, Lindquist DL, Alaracon PA, Cohen JL. Evaluation of bone marrow megakaryocyte ploidy distributions in persons with normal and abnormal platelet counts. J Lab Clin Med 1988; 111: 194–202.
11. Bocking A, Giroud F, Reith A. Consensus report of the ESCAP task force on standardization of diagnostic DNA image cytometry. Anal Cell Pathol 1995; 8: 67–74.
12. Bocking A, Adler CP, Common HH, Hilgarth M, Granzen B, Aufferman W. Algorithm for a DNA-Cytophotometric diagnosis and grading of malignancy. Analyt Quant Cytol 1984; 6: 1–8.
13. Forsslund G, Zetterberg A. Ploidy level determinations in high-grade and low-grade malignant variants of prostatic carcinoma. Cancer Res 1990; 50: 4281–5.
14. Vučkovič J, Dubravčič M, Matthews JM, Wickramasinghe SN, Dominis M, Jakšič B. Prognostic value of cytophotometric analysis of DNA in lymph node aspirates from patients with non Hodgkin's lymphoma. J Clin Pathol 1990; 43: 626–9.
15. Pignatelli D, Leitao D, Maia M, Schmidt F. DNA quantification and ploidy patterns in human adrenocortial neoplasms. Endocr Res 1998; 23: 869–74.
16. Antal L, Sapi Z, Szendroi M. The prognostic significance of DNA cytophometry and proliferation index (Ki-67) in giant cell tumors of bone. Int Orthop 1999; 23: 315–9.
17. Nordemar S, Hogmo A, Lindholm J, Tani E, Sjostorm B, Auer G, Munck-Eikland E. The clinical value of image cytometry DNA analysis in distinguishing branchial cleft cysts from cystic metastases of head and neck cancer. Laryngoscope 2002; 112: 1983–7.
18. Feulgen R, Rossenbeck H. Mikroskopisch-chemischer Nachweis einer Nucleinsaure vom Typus der Thymonucleinsaure und die darauf beruhende elektive Farbung von Zellkernen in mikroskopischen Praparaten, Hoppe, Seyler: Z Physiol Chem 1924; 135: 203–48.
19. Giroud F, Montmasson MP. Reevaluation of optimal Feulgen reaction for automated cytology. Analyt Quant Cytol Histol 1991; 13: 253–60.
20. Biesterfeld S, Gerres K, Fischer-Wein G, Bocking A. Polyploidy in non-neoplastic tissues. J Clin Pathol 1994; 47: 38–42.
21. Sanchez L, Reigh M, Biesterfeld S, Chatelain R, Bocking A. Performance of a TV image analysis system as a microdensitometer. Analyt Quant Cytol Histol 1990; 12: 279–84.