Spontaneous apoptosis in chronic lymphocytic leukemia is not an independent prognostic factor for stability of disease when compared with combined AgNOR and TTM scores

To the Editor,

It has been shown that AgNOR staining can provide useful information for diagnosis in cytology of the oral cavity [22], body fluids [3,18], hematologic smears [10,11,14–17] or lymph node aspiration cytology [12]. We would like to draw attention of the readers towards an easy AgNOR evaluation which has shown to be very useful as prognostic marker in chronic lymphoid leukemia (CLL) patients. This disease shows considerable variability of its clinical presentation and evolution. Patients in stable phase may, after months or years, change to a progressive phase. Whereas initially no treatment is warranted, chemotherapy will be necessary in the latter. In a previous exploratory study [17] we suggested that the percentage of CLL cells in peripheral blood with AgNOR clusters at first diagnosis might be an independent prognostic marker for the duration of the stable phase, besides the total tumor mass (TTM). An index created by summing up both values showed to be a more powerful prognostic factor than other common clinical and laboratory parameters [17]. In order to validate this model, we decided to repeat this study with other CLL patients. Since the apoptosis rate has shown to be of prognostic importance for various neoplasias and since resistance to apoptosis could be involved in the disease progression of B-CLL, [19], we also investigated the spontaneous apoptosis rate of CLL cells in culture.

Unselected patients with newly diagnosed B-CLL entered the study. Counting of AgNOR clusters and the determination of TTM were done as previously described [10,16,17]. AgNOR staining of cytological preparations from acute leukaemias allows the differentiation of clusters (aggregations of precipitations within a common matrix in the nucleolus) and dots (small singular precipitations without a matrix). CLL clusters showed a longest chord of 2.07 μm (95% CI 1.85–2.4 μm) and compact nucleoli, 1.07 μm (95% CI 0.95–1.2 μm) (Fig. 1) [16].

Apoptosis rate was measured at diagnosis by flow cytometry as the percentage of annexin V positive cells after a 48 hours culture as described previously [19]. Pearson’s correlations were calculated between the variables. Univariate Cox regression analyses were performed to examine the relationship between the treatment-free period and percentage of annexin V positive cells, TTM, percentage of cells with AgNOR clusters in peripheral lymphocytes and the Index (= percentage of cells with AgNOR clusters + TTM). For all further analyses the Index values were logarithmized, in order to get a good approximation to the normal distribution. Then we tried to find out, whether comparing all these parameters in a multivariate Cox-regression, the Index would again be the strongest variable, as postulated previously [17]. Finally we tested the strongest variable from this regression together with the parameter “annexin V positive cells”. In order to estimate the stability of the models we applied the Cox regression to 200 new data sets created by bootstrap resampling [17]. For all calculations SPSS 8.0 software was used.

During the study period 32 patients were analyzed. The mean observation time was 18 months and during this period 22 patients fulfilled the criteria for start of chemotherapy. Median time of the stable phase was 13 months in the Kaplan–Meier curve. Pearson’s correlations demonstrated statistically significant inverse correlations between the apoptosis rate and TTM (r = −0.47), the AgNOR score (r = −0.40) and the (logarithmized) Index (r = −0.52). In univariate Cox analyses TTM (Exp(B) = 1.087; CI 95% 1.027 to 1.15; p = 0.004), percentage of cells with AgNOR clusters (Exp(B) = 1.137; CI 95% 1.014 to 1.274; p = 0.027) and the Index (logIndex: Exp(B) = 5.84; CI 95% 1.83 to 18.6; p = 0.003) were unfavourable predictive variables. In a multivariate Cox regression only the logIndex remained in the final model. In the stability test by bootstrap resampling the variable log Index was included in 69.5% of all models, whereas TTM in only 33% and the AgNOR score in only 29.5% of the cases. Spontaneous apoptosis rate was a favourable prognostic factor for treatment-free period in the un-
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Fig. 1. AgNOR stained CLL lymphocytes (100× objective magnification). On the top lymphocyte with one cluster, on the bottom lymphocyte with 2 dots.

The variate Cox model (Exp(β) = 0.9373; CI 95% 0.8898 to 0.9873; p = 0.015). Analysing it together with the logIndex in a multivariate Cox model, only the logIndex remained as an independent prognostic parameter. This was confirmed in a bootstrap resampling study where the logIndex was included in the final model in 91% of the cases, whereas the “spontaneous apoptosis” in only 12.5%.

CLL is characterized by a slow and progressive accumulation of lymphocytes arrested in G0 [19]. In vivo, clonal B CLL cells present a decreased susceptibility to undergo apoptosis. However, they enter spontaneous apoptosis when cultured in vitro. It is postulated that perhaps due to accumulation of genetic abnormalities [19], spontaneous apoptosis is less frequent in advanced stage patients, thus provoking a prolonged lifespan of the CLL cells. Therefore, a high spontaneous apoptosis rate, as measured by the percentage of annexin V positive cells in culture, was expected to be a favourable prognostic factor for the stable phase in CLL, what we could show in our study. To our surprise, however, this variable was no longer an independent prognostic factor, when compared simultaneously together with the prognostic index in the multivariate Cox model. Our predictive model demonstrated a high internal stability, because the Index entered in more than 90% of the bootstrap models. Thus in this validation study we are confirming the results of our previous exploratory study [17].

There are different approaches to prognostic factors in pathology of neoplasias. We can investigate genomic alterations by molecular biology [1,2,6,26], nuclear or cytoplasmic protein expression by immunohistochemistry [8,9,25] analyze DNA by image or flow cytometry [12,21], perform morphometry and texture analysis [13,20,24] or analyze interphase nucleolar organizer regions (AgNORs) [4]. The AgNOR technique is a very quick, easy and cheap method when compared with the others. There are some controversies on the evaluation of the silver precipitations. Whereas the morphometric approach by measuring the silver-stained nuclear area has been emphasized [4], an easier evaluation by counting dots and clusters has also shown to be efficient in cytology [5,7,10–12,14–19]. This procedure shows good reproducibility [10] and is robust against variations during the staining procedure [14]. Since morphometry is not required, special equipment for image analysis is not necessary. In case of doubt, the size of the precipitations can be simply and rapidly measured using an eyepiece graticule.

It has been shown that the AgNOR area (per nuclear area) correlates well with the cell duplication time and is therefore an important physiologic variable [4], but various studies have also stressed the importance of the AgNOR cluster as an important cell kinetic parameter [5,7,10–12,14–19]. In CLL, the peripheral lymphoid cells with an AgNOR cluster most likely represent the circulating proliferative fraction. In this disease the percentage of CLL cells with clusters correlates with lymphocyte doubling time and therefore it is easy to understand that this variable is an independent prognostic factor for the duration of the stable phase in CLL [10,16,17]. In summary, we think that AgNOR staining can provide important prognostic information in daily routine cytology. The peculiar AgNOR morphology should be taken into consideration for diagnostic and prognostic studies in order to improve the clinical value of the AgNOR technique.

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