Scientific Research Publishing

Potential of Hematopoietic Stem Cells and Mitotic Activity in Peripheral Lymphocytes to Predict Life Expectancy of Patients with Metastatic Non-Small Cell Lung Cancer after Conventional Therapy

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Abstract

To prevent potential overtreatment of metastatic non-small cell lung cancer, the individual parameters of circulating hematopoietic stem cells (HSCs) (percentage of $CD133^+$ HSCs, $CD34^+$ HSCs, and mitotic activity in the circulating lymphocyte fraction) were measured before conventional cytotoxic therapy in 35 patients by flow cytometry and then compared retrospectively with their individual survival periods. The plot of dependence of the $CD133^+$ HSC × mitotic activity product versus $CD34^+$ HSC revealed the prognostic properties during the survival period (range 0.3 - 124 months). Discrimination of patients with an expected survival shorter than 12 months was possible based on the positions of individual points on the plot, with a sensitivity and specificity of ~100 each and a diagnostic odds ratio of 1250. The evaluation of individual lymphoproliferative resources before cytotoxic treatment may be useful for the optimal therapeutic compromise between the desired inhibition of malignant target cells and the life-threatening depression of lymphocytopoiesis.

Keywords

Lung Cancer, Circulating Hematopoietic Stem Cell, Lymphocyte, Prediction, Survival Period

1. Introduction

Upon receiving a diagnosis of a fatal illness, such as metastatic cancer, a reasonable question is how much time the patient has left to live. The answer is important for patients with fatal non-small cell lung cancer (NSCLC) because of the high probability that subsequent anticancer treatment may bring about a poorer result compared with palliative therapy or even the absence of treatment [1] [2] [3] [4] [5]. The survival rate and median survival can be used to determine the potential benefits of therapy but cannot predict what will happen for an individual patient, as they are based on previous outcomes of large numbers of patients. Comorbidities are also associated with inferior overall survival, but a single patient may survive for as long as several months or years [6].

The performance status (PS) of patients is significantly associated with the cumulative survival, defined by the survival rate and median survival. Even after adjustment for performance status, some patients show much longer or shorter survival than expected [7] [8]. Recent studies showed that a high neutrophil-to-lymphocyte ratio in the blood is a useful prognostic factor for poor overall survival of patients with lung cancer [9] [10], but this does not solve the problem of individual prediction, because the survival period of patients with a similar neutrophil-to-lymphocyte ratio after conventional treatment is highly variable over the range of 1 - 60 months [11]. Therefore, accurate prediction of life expectancy in terminally ill patients continues to be challenging.

The current rationale for the volume of cytotoxic therapy does not take into account functional deterioration of the hematopoietic tissue proliferative resource caused by the previous cancer progression, even though a similar deterioration is the main cause of premature aging and reduced life expectancy even in the absence of a tumor [12]. The lowest level of permissible clinical lymphocytopenia (0.5×10^9/L) induced by treatment [13] on the one hand and the success of conventional cytotoxic therapy on the other hand are not compatible with stimulation of anticancer immunity, and the safety of the procedure is often simply ignored. Therefore, practical assessment of individual lymphocytopoietic resources in the clinic is required to achieve optimal therapeutic compromise between the desired inhibition of malignant target cells and myelodepression, which accelerates the onset of life-threatening cachexia [14]. Recent data indicate that the health condition and level of fitness of individual patients markedly influence the morphogenic function of circulating stem cells of bone marrow origin (HSCs), which migrate to many different tissues, including those that are malignant, and stimulate proliferation, regeneration, and repair [15] [16] [17] [18]. These conflicts with the traditional doctrine of antitumor immunity, but can only explain the association between the benefits of traditional cytotoxic therapy and its inevitable hemo/immunosuppressive activity [14] [18] [19]. We investigated whether some signs of stem cell activity in peripheral blood have individual prognostic properties. Here, the CD133^{+} HSC, CD34^{+} HSC, and common mitotic activities (Mt) in the circulating lymphocyte fraction were in-

vestigated before treatment and compared post-mortem with the individual survival of patients with advanced NSCLC.

2. Methods

2.1. Patients

The study population consisted of 35 patients with advanced non-small cell lung bronchioloalveolar carcinoma (T3, N2.3, M0) who received conventional chemoradiotherapy (60 Gy in 30 daily fractions, dose-fractionation standard for stage III NSCLC) [20] and platinum-based chemotherapy after palliative surgery at the Russian Research Center of Radiology and Surgical Technologies in St. Petersburg, Russia. The cumulative 1- and 5-year survival rates of patients with lung cancer were 37% and 11%, respectively. The institutional ethics committee approved this study, and all patients provided informed consent.

2.2. Mortality Rates

Survival curves for each subgroup were approximated using exponential curve fitting in Excel [21] with the following Equation (1):

$$S_t = Ae^{-kt} + Be^{-\lambda t} + C. \tag{1}$$

where S_t is the proportion of surviving patients at any point during the 10-year period, t is the time (months) after the beginning of therapy, k and λ are the exponential mortality rates per month, A and B are the proportions of patients who died corresponding with a monthly mortality rate $= k$ or λ, and C is the proportion of patients who did not die during the extended 10-year follow-up period.

2.3. Blood Samples for Flow Cytometry

A parallel study of blood samples was conducted 1 - 2 days before the start of chemoradiotherapy in 35 patients with lung cancer (average age 66.2 ± 1.1 years, 13 women, 22 men) who provided written informed consent. A separate group of 16 adult volunteers without any malignancies (average age 61 years, 7 women, 9 men) was included to obtain control (baseline) data.

Immediately after collection of peripheral blood (10 mL), the mononuclear cell fraction (MNC) was isolated by classical Ficoll density separation [22], omitting the final step of magnetic cell enrichment. Viability was assessed using the trypan blue exclusion test.

Cells from two equal portions of the fresh MNC fraction were stained for flow cytometric analysis. The nucleic acid stain Hoechst 33342 (bis-benzimidazole fluorochrome; Sigma-Aldrich, St. Louis, MO, USA) was used for cell cycle analysis, which was performed as described previously [23] with slight modifications. First, MNCs were resuspended at a density of 10^6/mL in pre-warmed (37˚C) Dulbecco's Modified Eagle's Medium supplemented with 2% heat-inactivated fetal calf serum (Gibco BRL, Grand Island, NY, USA) and 10 mM HEPES (Gibco

BRL). Hoechst 33,342 was added to a final concentration of 5 μg/mL, and the cells were placed in a water bath at 37˚C for 120 minutes. Tubes were mixed gently every 20 minutes during incubation. The cells were then centrifuged (483 relative centrifugal force for 6 minutes at 4˚C in a precooled rotor), resuspended in cold Hank's Balanced Salt Solution/2% fetal calf serum/10 mM HEPES at a concentration of $1 - 2 \times 10^7$/mL, and kept at 4˚C until analysis.

The phenotypes of the circulating cells were evaluated using standard protocols for cell staining. MNCs were prepared for conventional dual-color immunophenotyping using fluorescent allophycocyanin-conjugated anti-CD133/2 (MiltenyiBiotec, Bergisch, Gladbach, Germany) and fluorescein isothiocyanate-conjugated anti-CD34 (BD Bioscience Pharmingen, San Jose, CA, USA) monoclonal antibodies. Isotype-matched irrelevant monoclonal antibodies (Becton Dickinson, San Jose, CA) were used as negative controls.

2.4. Flow Cytometry

The LSR Fortessa flow cytometer (Becton Dickinson) was adjusted for immunofluorescence before measurements using the BD Cytometer Setup and Tracking Beads kits. The lymphocyte fraction was separated by gating on forward and side light scatter dot plots, excluding cellular debris. A red laser (640 nm, 40 mW) was used for detection of CD133+ cells, a blue laser (480 nm, 50 mW) was used for CD34+ cells, and an ultraviolet (UV) laser (355 nm, 20 mW) was used for the detection of cells labeled with Hoechst 33,342.

2.5. Flow Cytometric Analysis

A minimum of 500,000 total events were recorded twice when CD34^{+} and CD133^{+} cells were detected in the lymphocyte fraction. The percentage of positive cells was calculated by subtracting the value of the appropriate isotype control. A dot plot of double (simultaneous) emission of Hoechst 33342 in blue (x-axis) and red (y-axis) wavelengths was used for separation of events (G0 + G1), S, and (G2 + M) (Mt) phases, as previously described [22] [23]. The major double (blue and red)-negative emitting population in the lower left corner of the plot represents cells in the (G0 + G1) stages of the cell cycle. The populations in the center and upper right corner of the dot plot represent the double emitting cells in the S and mitotic phases (Mt) of the cell cycle, respectively.

The Mt data were used to calculate the reproductive activity (RA) for each patient: (RA = percentage of Mt phase events × percentage of CD 133-positive cells in the pool of lymphocytes).

2.6. Kinetic and Statistical Analyses

The goal was to identify specific features in cell parameters prior to commencement of treatment, which may reliably distinguish patients with different survival periods. We defined the functions that best described the trends in the parameters according to survival by searching for appropriate equations [21]. Kinetic

analysis of survival (Equation (1)) allowed separation into only two subgroups with different survival periods according to the exponential regression lines. For patients within each of the two subgroups, the interrelationships among Mt, CD133+ HSC, CD34+ HSC, RA, and survival values were analyzed automatically using Excel program. The trends of plotted curves were described adequately using non-linear approximations. As the coefficient determination R^2 is a statistical measure of the goodness of fit of the regression line to the data, we assessed its maximal value. Satisfactory R^2 values were confirmed using Equation (2) [24]:

$$t = \sqrt{R^2 \times (n-2)/(1-R^2)}. \tag{2}$$

Diagnostic odds ratios (DORs) [25], Student's t test, standard deviation (SD), standard errors (SEs), and probabilities were used to compare mean values, as appropriate. Fisher's exact test was used to explore statistical significance for the non-parametric variables. In all analyses, $p \leq 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Analysis of Survival Curve

According to **Figure 1** and Equation (1), current survival S_t was calculated as follows:
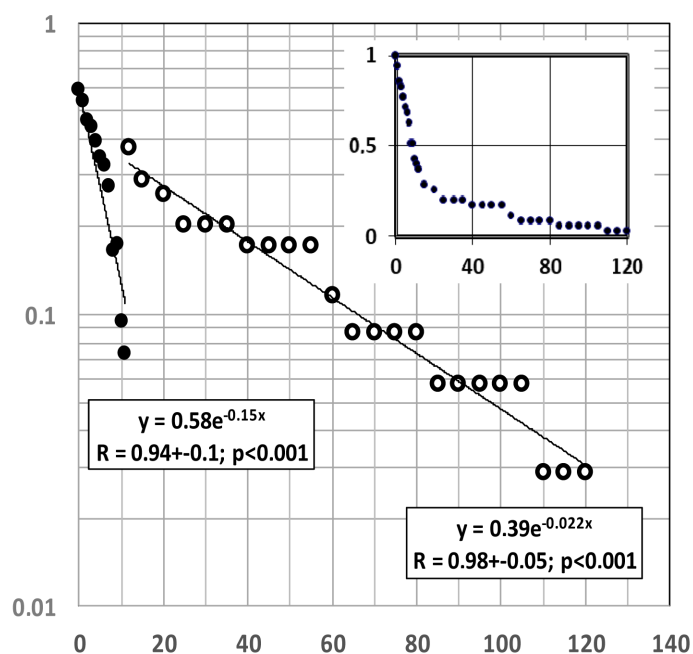
Figure 1. Exponential analysis of survival curves. Abscissa: survival, months. Ordinate: survival, relative units. A linear curve is presented in the right upper corner of the plot. The equations for separate exponents are shown in the boxes below. Left: subgroup with short survival (subgroup A) with a mortality rate = k = 0.15/month. Right: subgroup with long survival (subgroup B + C) with a mortality rate = λ = 0.022/month.

$$S_t = 0.58e^{-0.15t} + 0.39e^{-0.022t} + 0.03.$$

where C represents one individual who showed survival for > 60 months.

Semi-logarithmic analyses showed that all patients could be retrospectively divided into two subgroups: subgroup A with short survival (0.58, n = 21) and a mortality rate ($k = 0.15$ per month) and subgroup (B + C) with long survival (0.39 + 0.03 = 0.42, n = 14) with a low mortality rate ($\lambda \leq 0.022$ per month). After dividing the patients according to survival period, subgroup A and subgroup (B + C) were characterized by survival periods of <12 months and ≥12 months, respectively.

3.2. Cytokinetic Parameters

Figure 2 shows the differences in the cell parameters of peripheral lymphocytes with time from cytometric analysis to death (*i.e.*, survival period).

Decreases in CD34$^+$ HSC, mitotic (Mt) cells and RA proportions were strongly confirmed with p < 0.001 (all approximating equations are shown in the plot).

The relationship between the product of Mt × CD133$^+$ (RA) and the CD34$^+$ proportions is shown in **Figure 3**.

The second-order polynomial approximation indicates that dependency RA from CD34$^+$ (double common line in **Figure 3**) has left and right branches (p < 0.001). The left and right branches (dashed lines on the plot by power function) were confirmed separately (p = 0.003 and p = 0.013, respectively). The RA values in the right branch were positively related to the CD34$^+$ expression level and corresponded to patients surviving ≥ 12 months. The left branch represents RA values inversely related to the CD34$^+$ level among patients surviving < 12
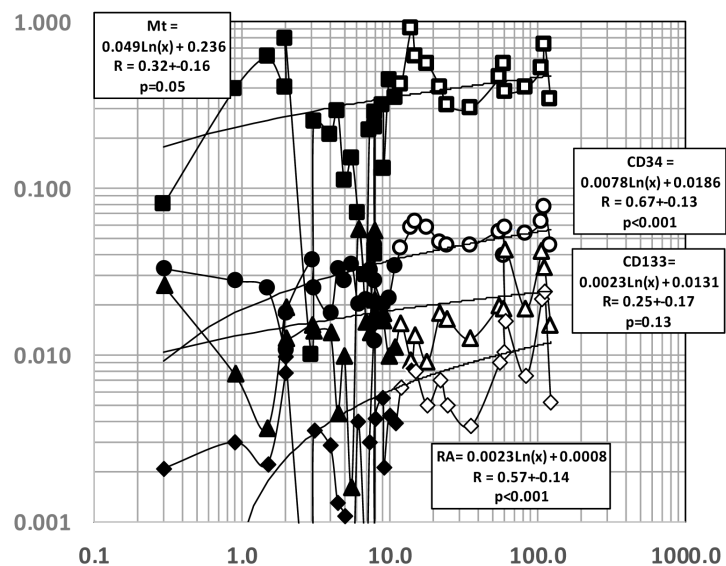
Figure 2. Dependence of cell parameters on the actual survival period from diagnosis/analysis to death. Abscissa: survival period, months. Ordinate: % mitoses (Mt; squares), CD34$^+$ HSC (circles), CD133$^+$ HSC (triangles), PA (rhombuses) in the pool of lymphocytes.
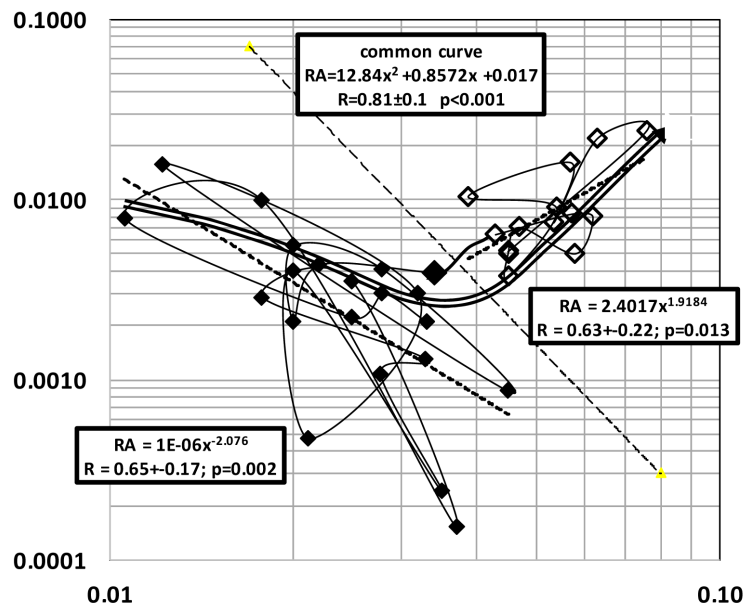
Figure 3. Dependence of regenerative activity (RA = Mt × CD 133$^+$ HSC) from CD34$^+$ HSC in patients with long and short survival periods. Abscissa: % CD34$^+$ HSC. Ordinate: RA in the lymphocyte fraction. The broken line on the plot divides the patients according to prognosis. Black symbols represent subgroup A (survival < 12 months) and white symbols represent subgroup (B + C) (survival ≥ 12 months). See text for details.

months. The positions of the RA values on the plot inside each branch area are irregular and unpredictable. Such instability in the RA value reflects the turbulence of the kinetics shown in **Figure 2**. The mean CD34$^+$ HSC proportion and the mean RA value in the left branch were lower than those in the right branch (**Table 1**).

Remarkably, the turbulence of the points inside each of the two branches in **Figure 3** was not similar. The coefficients of variation (CV) for four parameters (CD34$^+$ HSC, Mt, CD133$^+$ HSC, and RA) were higher for the patients surviving < 12 months compared with ≥12 months (**Table 1**). The difference (p = 0.014, Fisher's exact test) indicated greater turbulence in bone marrow reproductive potency during the last year of the life. Therefore, the predictive power of the plot in **Figure 3** is limited by the differences between subgroups A and (B + C). The mean parameters of both subgroups revealed abnormally high lymphocytopoiesis activity compared with those of healthy controls (**Table 2**). Those who lived longer (≥12 months) had the greatest increases in Mt (~8-fold), RA (~5-fold), CD34$^+$ HSC (~2-fold) and no difference of CV according Fisher's exact test (p = 0,22). Patients with shorter survival (<12 months) showed moderate increases in Mt (~4-fold), RA (~2-fold) and tendency to rise of CV (Fisher's exact test, p = 0.07). A lower CD133$^+$ HSC value was characteristic of both subgroups (~0.5 and 0.6 relative to the control, respectively), but was more reliable (p = 0.006) for patients with shorter survival.

The markedly increased probability of death in subgroup A (surviving < 12 months, n = 21/35) was associated with reduced CD133$^+$, CD34$^+$, Mt, and RA

Table 1. Mean cell parameter values in subgroups A and (B + C).

Parameters		CD34	Mt	CD133	RA = Mt × CD133
Group A (survival < 12 months) n = 21	M*	0.0259	0.2562	0.0174	0.0037
	SD	0.0086	0.1952	0.0143	0.0036
	SE	0.0019	0.0426	0.0031	0.0008
	CV	0.333	0.762	0.822	0.980
Group B + C (survival ≥ 12 months) n = 14	M*	0.0532	0.4914	0.0201	0.0098
	SD	0.0100	0.1692	0.0109	0.0063
	SE	0.0027	0.0453	0.0029	0.0017
	CV	0.188	0.344	0.544	0.644
KV < 12/KV ≥ 12		1.8	2.2	1.5	1.5
M < 12/M ≥ 12		0.5	0.52	086	0.38
p		<0.001	0.002	ns	0.003

*M is given as % in the pool of lymphocytes.

Table 2. Mean control cell parameters and changes in parameters in subgroups A and (B + C) relative to the volunteers' control.

Parameters		CD34	Mt	CD133	RA = Mt × CD133
Subjects: Control, n = 16	M	0.0293	0.0616	0.0326	0.00200
	SD	0.0142	0.045	0.016	0.0012
	SE	0.0036	0.0116	0.004	0.00029
	CV	0.48	0.73	0.49	0.6
(M of survival < 12/M of contr.)*		0.88	4.1	0.53	1.85
p		ns	0.004	0.006	0.05
(M of survival ≥ 12/M of contr.)*		1.81	7.95	0.62	4.9
p		<0.001	<0.001	0.02	<0.001

*Calculated using M, SD and SE parameters from Table 1 and Table 2.

values compared with those who survived for ≥12 months (n = 14/35). The median survival in the two subgroups differed by ~7-fold (4.6 months vs. 31.5 months, respectively).

Using the data shown in Figure 3, discrimination of individual patients with a predicted survival < 12 months appeared to be accurate, with a sensitivity and specificity of ~100% each. The DORs for prediction of individual survival < 12 months was calculated based on these data using a 2 × 2 confusion matrix, as described previously [25], yielding a value of 1250 (95% confidence interval 640 - 2450; $\log_{DOR} \pm \log_{SE\ DOR} = 7.13 \pm 0.34$).

4. Discussion

Some individual survival < 12 months has resulted from strong decreasing (~6 - 10-fold) of lymphoid CD34$^+$ HSC and lymphopoietic reproductive activity RA (Figure 2). The rates of any health failure during the life of an organism repeatedly reduces lymphoproliferative resources via the extra use of circulating

cells in regenerative and repair processes [26] [27]. In naturally aged bone marrow the progenitor cells are skewed toward myeloid cell production with an inevitably strong deficit in lymphohematopoietic progenitor and stem cells [28]. The incidence, mortality, and prevalence of 24 major types of malignancy during natural frailty decrease abruptly with age [29].

These results strongly contradict the concept of anticancer immunity but confirm the feeding function of circulating lymphohematopoietic progenitor and stem cells consumed in proliferating malignant and other normal tissues via fusing with target cells, transdifferentiating into them or secreting growth factors (a paracrine proliferative effect of HSCs). Premature frailty and death in acute hematopoietic syndrome, caused by irradiation or uptake of cytotoxic radiomimetics, are the result of early exhaustion of proliferative resources in the hematopoietic system, especially its lymphoid lineage [30] [31]. Thus, cytotoxic systemic therapy not only kills cancer cells but also adversely affects the physiological systems responsible for maintaining the viability of the host organism.

We postulated that excessive deterioration of lymphoproliferative resources by forced HSCs trophic support of cell replacement in highly proliferative malignant tissues may be the main reason for the markedly shorter survival in malignancy, while the rates of frailty and death at the end of life remain similar for malignancy and natural aging [32].

Morphogenic (feeding) migratory cells include the ancestral and angiogenic $CD133^+$ HSCs, progenitor $CD34^+$ HSCs, young thymus migrating cells such as terminal deoxynucleotidyl transferase-positive prelymphocytes, descendant $CD31^+$ angiogenic T lymphocytes, and other "regulatory" cells [17] [33] [34]. These cells collectively affect overall survival and aging of the organism by influencing the replacement of organ-specific effector cells. The life span of HSCs may vary from 10 to 60 months, depending on previous rate of health failures, predetermining the shortening of telomeres at each HSC mitotic cycle [32] [35]. A decrease in the natural limit of the HSC mitotic capacity leads to reduced numbers of these cells in the circulation, which occurs naturally at a rate of ~1.3%/year in individuals aged 30 - 80 years [36]. Thus, the resource of circulating HSCs is limited not only by a reduction in their numbers in target tissues but by decreased renewal capacity, which is self-limiting at the single-cell level. The mean RA value calculated in our investigation roughly reflected the combination of the two factors.

Long-lasting high RA level during cancer progression results in failure of the lymphocytopoietic system much sooner than during natural aging. This was confirmed by the data shown in Table 2, indicating abnormally high presence of $CD34^+$, Mt, and RA in peripheral blood in both subgroups of patients in comparison with healthy controls. At the same time, patients surviving < 12 months (median survival 4.6 months) had 2 - 2.5-fold lower levels of lymphoproliferative resources compared with patients surviving ≥ 12 months (median survival 31.5 months) (Table 1). These observations indicated the inability of exhausted

lymphopoiesis to prolong the enormously high consumption rate of feeding cells. The decrease in proliferative potential in patients with short survival reflected a much higher degree of "turbulence" in their parameters (**Figure 2**, black symbols; **Table 1**, CV).

Qualitatively, a moderate turbulent phase and strong hyperactivity (2 - 8-fold) of lymphocytopoiesis in NSCLC patients are observed over a long period (~50 months, **Figure 2**), which rapidly exhaust the system during the last year of life [14]. A concomitant worsening of prognosis is reflected in the decreased number of CD133$^+$ HSCs compared with healthy controls (**Table 2**) and in the change from a positive to a negative relationship between CD34$^+$ HSC numbers and RA. As shown in **Figure 3**, poor prognosis is accompanied by the inability of lymphocytopoiesis to maintain high levels of CD34$^+$, CD133$^+$, and Mt production simultaneously, as seen in the long survival period group. The transition from one state of lymphocytopoiesis to another is accompanied by increased "turbulence" (**Figure 2** and CV < 12/CV ≥ 12, M < 12/M ≥ 12 parameters in **Table 1**).

Both subtypes of "turbulence" in **Figure 3** arise from the asymmetrical cell division, which is typical for ideal (steady-state) cell production with a lower CV [37]. As reported previously [30], the "turbulence" of lympho-hematopoiesis and probability of death increase with the rate of cell loss in bone marrow, especially in the most vulnerable pools of lymphocytes and HSCs. Remarkably, the benefits of systemic therapy in terms of survival followed obviously by post-treatment lymphocytopenia which indicate the weakening of the tumor trophic support. The therapy becomes ineffective if the lymphocytopenia drops below its "therapeutic" limit during treatment, or if the basic lymphocytopenia before therapy is lower of this limit [38] [39].

5. Conclusion

Cytotoxic therapy has both advantages and disadvantages, because it not only kills cancer cells but also adversely affects the physiological systems responsible for maintaining the viability of the host organism. The prevalence of one over the other effect depends on the ratio of basic lymphoproliferative potency in the patient and the cumulative dose of systemic hematosuppressive therapy; higher potency is associated with a longer period during which the therapy may be successful, and vice versa. Thus, predicting individual treatment outcomes is not possible, and evaluation of lymphoproliferative resources prior to beginning cytotoxic treatment may be beneficial for reliable discrimination of subgroup of patients with an expected survival shorter than 12 months. According our data, the probability of such discrimination reaches to a sensitivity and specificity of ~100% each. Limited number of patients involved in this investigation suggests the necessity of further test of this approach in clinic.

Acknowledgements

this manuscript have no conflict of interest to declare.

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