CHAPTER 6

THE TUMOR PROLIFERATIVE COMPARTMENT IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA
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PEDIATRIC ALL

TUMOR CELL PROLIFERATION AND NEOPLASIA

The transformation of a normal cell into a malignant one requires the stochastic acquisition of multiple genetic alterations (Figure 6, page 32). These involve the inactivation, or loss of function, of negative growth regulatory genes known as tumor suppressor genes, in addition to the activation of growth promoting genes known as oncogenes. The functional interactions of these altered genes and their respective protein products under the influence of other regulatory proteins lead to changes in the cell's ability to respond appropriately to external signals. Ultimately, unchecked cell division and proliferation lead to neoplastic transformation.

PROLIFERATION IN ALL

The measurement of leukemia cell kinetics and proliferation has long been considered to be of importance with the expectation to understand tumor growth patterns, the generation of sub clones, and their chemotherapeutic responses. However, cell kinetics has rarely been investigated for prognostic purposes in ALL, in contrast to many other types of human cancers, in which an association between proliferating activity and clinical outcome has been found. Previous attempts to correlate the pre-treatment leukemia cell kinetics to the clinical
outcome have led to contradictory results (Hoelzer et al., 1988; Chaplin and Gale 1989; Murphy et al., 1977; Scarffe et al., 1980; Dow et al., 1982). The measurement of dynamic kinetic parameters, representing the duration of distinct cell cycle phases, has now become possible by the introduction of labeling techniques with nonradioactive DNA precursors such as Bromodeoxyuridine (BrdUrd) or iododeoxyuridine and flow cytometry. The compartment of proliferating cells is not constant, but in dynamic equilibrium. Some quiescent cells enter the cell cycle, whereas others leave the cell cycle and become quiescent. Although the assessment of the growth fraction in tumor cell population is still complex, antibodies against nuclear antigens specific for cycling cells have been produced, with the anticipation that these studies will provide an accurate estimation of the growth fraction in human cancers.

**Ki-67**

In mitogen-stimulated lymphocytes, the Ki-67 protein was shown to be expressed in all of the non-G0 cells (Gerdes et al., 1984). Ki-67 protein expression starts from the beginning of the first of the stimulated lymphocytes and increases during the S Phase until mitosis, when its expression is maximal. After division, Ki-67 rapidly degrades in the G1 phase, but not to an undetectable level, until the cells re-enters into the S phase and the levels of the antigen increases again (Bruno et al., 1992).

**Ki-67 expression in hematological tumors**

Earlier studies on Ki-67 expression in hematological malignancies have focused on NHLs, which shows a wide range of proliferative indices. Ki-67 expression in
acute leukemia has been studied in children and in adult patients. It was documented that in childhood ALL the Ki-67 expression was quite different from the expression of PCNA. When compared to PCNA, Ki-67 positive cells were apparently smaller. There appears to be a significant correlation between the percentage of Ki-67 positive cells and the fraction of S-phase cells, which were determined by flow cytometry (Tsurusawa et al., 1996).

**Proliferation Cell Nuclear Antigen (PCNA)**

PCNA is a unique nuclear protein inducible in proliferating cells and is a 36kDa nuclear protein that is up regulated in actively proliferating cells from a variety of tissues and species (reviewed by Kelman, 1997). This protein is highly conserved throughout phylogeny, being present in plants, yeasts, and higher eukaryotes. The use of antisense oligonucleotides suggests that PCNA is an essential requirement for DNA synthesis and cell cycle progression and has also been demonstrated as requirement for leading strand synthesis in simian virus 40-virus replication. PCNA functions as a cofactor for DNA polymerase δ in DNA synthesis. Interestingly, in addition to its role in DNA synthesis and cell cycle progression, PCNA also seems to be important in DNA excision and repair mechanisms. Therefore, it is possible that elevated levels of PCNA may not only correlate with increased cell proliferative activity, but may also relate to intrinsic cellular DNA repair and thus resistance to chemotherapy. When cells were stimulated to proliferate, their PCNA levels rapidly increases during the G1 phase and reaches a maximum in the S phase. The amount of PCNA in long-term
quiescent cells is probably negligible, because it is undetectable in human peripheral blood lymphocytes.

**PCNA expression in hematological tumors**

The association between PCNA expression and the proliferation of human leukemic cells was first demonstrated by Takasaki et al (1984).

**Figure 48- Variations of the Ki-67 and PCNA content in leukemia cell subpopulations during different phases of the cell cycle**

The difference in the expression levels of PCNA between normal and leukemia cells was first reported by Keim et al (1990). They measured PCNA expression in childhood ALL by two-dimensional polyacrylamide gel electrophoresis. The PCNA levels observed in acute leukemia were intermediate between resting lymphocytes and maximally proliferating lymphoid cells in vitro. Further analysis
of the distribution of PCNA during cell cycle has demonstrated that the PCNA levels were higher in G1 phase, reached a maximum in S-phase, and then decreased slightly in G2/M phase. There appears to be a difference in PCNA expression in different subtypes of the disease.

**Argyrophilic Nucleolar Organizer Regions**

The measurement of silver staining nucleolar-organizing regions (AgNORs) has been studied as a method to compare proliferation and DNA content of cells in dispersed tissue for differential diagnosis and prognosis (Shome et al., 1999). Nucleoli consist of three substructures, fibrillar center, dense fibrillar and granular components. The fibrillar center is the location where the ribosomal RNA transcript is generated and is considered to be the Nucleolar Organizer Regions (NORs). NORs are electron dense regions and can be seen by the light microscope when stained with silver. AGNORs are the result of staining of argyrophilic non histone proteins, comprising mostly of two NOR associated proteins, nucleolin, a 105 kDa protein and numatrin, a 39 kDa protein which are associated with the fibrillar center of the nucleus.

In recent years this method has been widely used in surgical pathology as a cell proliferation marker to distinguish benign from malignant neoplasms and for tumor grading (Trere et al., 1991; Derenzini and Trere, 1991). Studies on hemopoietic cells in interphase have also given important information regarding the proliferative rate. In normal hemopoiesis it has been shown that it is useful to analyze clusters and dots separately. Clusters are groups of silver stained spots within a common matrix corresponding to nucleoli in proliferating cells. Dots are
single silver stained spots and are not associated to a nucleolus. Clusters are only present in proliferating cells and their number is related to the of cell maturation. It has been demonstrated that the number of AgNORs is inversely related to the cell duplication time. There is also a linear correlation between silver stained area per nucleus and the percentage of Ki-67 immunostained nuclei or bromodeoxyuridine labeled cells (Shome et al., 1999). These results suggest that a faster cell cycle is associated with a higher number of AgNORs. In leukemia a correlation between the number of clusters and the peripheral leukocyte count is a well-established parameter of tumor burden (Trere et al., 1991). The leukocyte count however, depends on the proliferative activity as well as the spontaneous death rate of the blasts. In order to explain this phenomenon it would be necessary to analyze the AgNOR pattern together with other cell kinetic parameters.

Therefore, we believe the combined assessment of these dynamic kinetic parameters may allow us to eventually understand the proliferative activity of childhood leukemia cells.

**WORKING HYPOTHESIS**

- Alteration of cell proliferation leads to imbalance in hemopoietic cells and thus can be a causative factor in pediatric ALL.

- Determination of proliferation status in pediatric ALL can give an idea regarding prognosis.
Proliferation status can be evaluated by the combined estimation of PCNA, Ki-67 and Ag NOR in pediatric ALL. Increased proliferation may be closely associated with resistance to therapy.

SPECIFIC AIMS

- To determine the total tumor proliferative compartment in pediatric ALL by determining proliferative index by PCNA expression, Ki-67 expression and by AgNOR staining.
- To evaluate the relationship between the proliferative status and the various clinical parameters.
- Correlation between proliferation and mutant p53 protein, bcl-2 and proapoptotic bax.
- Correlation with overall survival with a five-year follow up study for proliferation associated parameters.

METHODOLOGY

Immunocytochemical analysis

The expression of PCNA (Calbiochem, Oncogene Science Inc. Cambridge, MA, USA) and Ki-67 (Dako A/S, Denmark) proteins was analyzed in all samples by immunocytochemistry using monoclonal antibodies, which detects the respective proteins. The details of the protocol are explained in Appendix 1. Percentage of positive cells was assessed by counting at least 500 cells. A negative control was
run, using PBS instead of the primary antibodies. Evaluation of the results was done by two independent observers.

Silver staining
This was carried out as described by Smith & Crocker (1988) and Murray et al (1989). Briefly, silver colloidal solution was made up to one volume of 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate. The silver staining was performed for 2hr at room temperature. All the smears were counter stained with neutral red. AgNORs were visualized as distinct silver positive black dots and clusters. One hundred nuclei were assessed by two independent observers and the mean number of dots and clusters per nuclei were calculated separately for each sample. The detailed procedure is described in Appendix 1.

STATISTICAL ANALYSIS
The experimental factors were compared with other clinical and laboratory features at diagnosis by computing the Spearman correlation coefficient for continuous variables (age, leukocyte count, hepatosplenomegaly, platelet number) and its mean by independent t test for comparison of categorical (sex, FAB morphology, peroxidase positivity) variables.
Survival was estimated by the Kaplan Meier survival curves. Terminal event considered was death due to leukemia. The log rank test was used to assess the significance of differences in survival curves.
Multivariate analysis was conducted using the Cox regression (forward stepwise conditional) model to evaluate the relative importance of the analyzed parameters with other prognostic variables.

RESULTS

PCNA expression was seen as intense nuclear immunoreactivity in 53% (71/135) of the leukemic cases with a mean of 22.17±1.72% (Figure 49). Approximately 47% (64/135) the patients were having a low PCNA index [lower than 15% cells (median value) being positive for this antigen]. Ki-67 immunoreactivity was also detected as cells with intense nuclear staining and is found in 54% (80/147) of the total cases with a mean value of 14.14± 0.81%. Patients having ≤ 10% (median value) of cells showing immunoreactivity was considered as having a low Ki-67 index. ALL patients had a mean dot count of 3.11± 0.076 (range = 0.132- 7.34) with a median of 2.67. Similarly, mean cluster count was 2.01± 0.140 (range = 0.452-3.98) with a median of 2.67.

Correlation analysis

PCNA expression was highly correlated with that of bcl-2 expression (r value = 0.3310; p value= 0.0000) and with that of p53 (r value = 0.2570; p value= 0.0026). However, PCNA expression showed only a borderline negative correlation with bax immunoreactivity (r value = -0.1429; p value = 0.09) and no correlation with apoptotic index (p value= 0.3). There was a negative correlation between bax immunoreactivity and PCNA (r value= -0.1429; p value = 0.09), but only of marginal significance. Interestingly, PCNA immunoreactivity showed a
Fig 49A. Ki-67 immunoreactivity in pediatric ALL (Magnification 900x)

Fig 49B. Intense PCNA Immunoreactivity in pediatric ALL (Magnification 900 x)

Fig 49C. Dark AgNOR positivity in pediatric ALL; Arrow Head indicate a cell with two spots (Magnification 1080x)
positive borderline correlation with P-gp expression (r value = 0.2317; p value = 0.074) showing that proliferation and drug resistance go hand in hand for imparting biological aggressiveness of the tumor.

For Ki-67, when looked for association with the other parameters, p53 gave a statistically significant positive correlation (r value = 0.2237; p value = 0.0026), however, apoptosis showed only a negative borderline correlation with Ki-67 expression (r value = -0.1392; p value = 0.092).

Table 18- Correlation matrix for proliferation and apoptosis

<table>
<thead>
<tr>
<th></th>
<th>Ki-67</th>
<th>PCNA</th>
<th>AgNOR-Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R value</td>
<td>P value</td>
<td>R value</td>
</tr>
<tr>
<td>P53</td>
<td>0.2237</td>
<td>0.006*</td>
<td>0.2510</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0.1226</td>
<td>0.1390</td>
<td>0.3310</td>
</tr>
<tr>
<td>Bax</td>
<td>0.088</td>
<td>0.288</td>
<td>0.1429</td>
</tr>
<tr>
<td>PCD</td>
<td>-0.1392</td>
<td>0.09*</td>
<td>-0.088</td>
</tr>
<tr>
<td>P-gp</td>
<td>0.1010</td>
<td>0.268</td>
<td>0.1720</td>
</tr>
<tr>
<td>Ki-67</td>
<td>-</td>
<td>0.2042</td>
<td>0.018*</td>
</tr>
<tr>
<td>PCNA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* represents statistically significant values
# represents marginal significance

Patients with AgNOR count (both dot and cluster) were classified into two groups, one having less than median and the other greater than median. When
anlyzed for correlation with the evaluated parameters, only bcl-2 expression showed a borderline positive correlation with increased dot positivity (r value = 0.1555; p value = 0.06). However, other clinical factors did not show any correlation with AgNOR counts except for an inverse correlation with immediate tumor response to therapy determined as day 7 peripheral blood status (r value = -0.1911; p value = 0.06).

Figure 50- Scatter Plot of PCNA immunoreactivity with Ki67 expression in pediatric ALL

There was a positive correlation between PCNA immunoreactivity and that of Ki-67. (r value = 0.2042; p value = 0.017).

Parametric t test

Analysis using independent t test was performed to evaluate the differences of mean values of programmed cell death regulatory proteins in patients with different proliferative groups.
Table 19- Mean value of apoptosis regulatory protein expression in low and high PCNA expression status

<table>
<thead>
<tr>
<th></th>
<th>P53 (Mean ± SE)</th>
<th>Bcl-2 (Mean ± SE)</th>
<th>Bax (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA Low</td>
<td>5.59±1.45</td>
<td>5.46±0.97</td>
<td>11.46±1.45</td>
</tr>
<tr>
<td>PCNA High</td>
<td>13.98±2.38</td>
<td>11.18±1.22</td>
<td>9.59±1.39</td>
</tr>
<tr>
<td>P value</td>
<td>0.004</td>
<td>0.000</td>
<td>0.351</td>
</tr>
<tr>
<td>Ki-67 Low</td>
<td>7.01±1.8</td>
<td>6.67±1.05</td>
<td>9.17±1.22</td>
</tr>
<tr>
<td>Ki-67 High</td>
<td>11.9± 1.4</td>
<td>10.94±1.17</td>
<td>10.81±1.39</td>
</tr>
<tr>
<td>P value</td>
<td>0.06</td>
<td>0.008</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Mean value of apoptotic regulatory proteins did not vary much with the AgNOR count.

Figure 51- Mean values for p53, bcl-2 and bax proteins with respect to PCNA expression status in pediatric ALL

The relation between PCNA and the apoptosis regulatory p53 is very clear from this graph. P53 concomitantly increases with PCNA, which again suggests that p53 may be of mutant type. The anti apoptotic bcl-2 is also seen to be increased in PCNA high category. Mean bax value decreased in PCNA high category, but the difference was not statistically significant.
Figure 52- Mean values for p53, bcl-2 and bax proteins with respect to Ki-67 expression status in pediatric ALL

This result was in agreement with that of PCNA. Mean p53 and bcl-2 were significantly increased in samples with high Ki-67 index. (P value < 0.05)

Survival analysis

A 5-year follow up study (60 months) was conducted for analyzing the OS. Patients were divided into two groups according to their median values for the analyzed parameters. The individual influence of the factors on survival is given below in Figures 53, 54 and 55. Overall survival is negatively influenced by the following variables: Ki-67 overexpression, PCNA overexpression and high AgNOR count (Table 20, 21 and 22).
Figure 53- Kaplan Meier survival curve for Ki-67 status in pediatric ALL

Table 20- Survival function (Kaplan Meier) of 144 patients according to Ki-67 status

<table>
<thead>
<tr>
<th>Ki-67 status</th>
<th>No. of patients</th>
<th>No. of events</th>
<th>Survival time (mean±SE) Months</th>
<th>95% confidence interval</th>
<th>% Censored</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>79</td>
<td>42</td>
<td>34±4</td>
<td>27, 41</td>
<td>47</td>
<td>0.0001</td>
</tr>
<tr>
<td>Low</td>
<td>65</td>
<td>14</td>
<td>55±3</td>
<td>48, 61</td>
<td>78</td>
<td></td>
</tr>
</tbody>
</table>
Figure 54- Kaplan Meier curve for five year overall survival for patients with AgNOR count

![Kaplan Meier curve](image)

Table 21- Survival function (Kaplan Meier) of 131 patients by AgNOR count

<table>
<thead>
<tr>
<th>Cluster count</th>
<th>No. of patients</th>
<th>No. of events</th>
<th>Survival time (mean±SE) Months</th>
<th>95% confidence interval</th>
<th>% Censored</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>70</td>
<td>34</td>
<td>37±4</td>
<td>29, 44</td>
<td>51</td>
<td>0.017</td>
</tr>
<tr>
<td>Low</td>
<td>61</td>
<td>17</td>
<td>50±4</td>
<td>43, 57</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>
Figure 55- Kaplan Meier curve for five year overall survival for pediatric patients with low and high PCNA status

Table 22- Survival function (Kaplan Meier) of 133 patients according to PCNA status

<table>
<thead>
<tr>
<th>PCNA levels</th>
<th>No. of patients</th>
<th>No. of events</th>
<th>Survival time (mean±SE) Months</th>
<th>95% confidence interval</th>
<th>% Censored</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>71</td>
<td>35</td>
<td>36±4</td>
<td>28, 43</td>
<td>51</td>
<td>0.03</td>
</tr>
<tr>
<td>Low</td>
<td>62</td>
<td>19</td>
<td>49±4</td>
<td>41, 56</td>
<td>69</td>
<td></td>
</tr>
</tbody>
</table>
Multivariate analysis

By multivariate analysis, Cox regression analysis of biological variables involved in proliferation showed that Ki-67 immunoreactivity was the only independent predictor of unfavorable overall survival probability (p value = 0.0005). None of the other variables like, PCNA immunoreactivity (high and low), AgNOR count, both cluster and dot (above and below median values) considered in this model, reached the statistical significance of 5%.

To assess the effect of these analyzed markers on different risk groups, the patients were classified into high-risk and standard risk group by their age and initial leukocyte count.

<table>
<thead>
<tr>
<th>High risk group</th>
<th>Age ≥ 10</th>
<th>WBC count ≥ 50,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard risk group</td>
<td>Age ≤ 10</td>
<td>WBC count ≤ 50,000</td>
</tr>
</tbody>
</table>

Among the high-risk group, none of the proliferative markers were not found to influence OS, whereas, among the standard risk group only Ki-67 index was good in predicting OS (p value = 0.0009) (Table chap 7).

DISCUSSION

Acute leukemia is characterized both in vivo and by the proliferation of leukemic stem cells and by the accumulation of immature blast cells. In an attempt to understand the proliferation of leukemia cells, in vivo cell kinetic studies have been performed in the past primarily by injecting tritiated thymidine into patients. This is now replaced by BrdUrd for incorporation in actively dividing cells.
However, administration of BrdUrd into human patients, especially children needs careful consideration to the unknown toxicity of this base analog. Therefore, for clinical use we have used non-invasive and simpler procedures such as staining with specific antibodies for the determination of proliferating cells. It would be beneficial to the patient to have tools available that could more reliably predict the survival, in addition to the classical prognostic factors.

Ito et al (1995) and Tsurusawa et al (1995) have observed that there is a wide variation in the labeling rates of Ki-67 in acute leukemia. This distinct growth pattern may represent the different sub types of the disease (with different biologic background) and may be the reason for observing the wide range of Ki-67 labeling indices.

White et al (1994) have demonstrated the importance of Ki-67 index in assessing the non-erythroid proliferative activity in lymphomas and leukemia. A number of studies have demonstrated the prognostic potential of Ki-67 labeling index in leukemias (Dictor et al., 1999; Astsaturov et al., 1997; Galand et al., 1995). Multivariate analysis of the usefulness in prognosis has been applied most often for the general proliferation antigen Ki-67 (Dictor et al., 1999). We also obtained similar result, Ki-67 bearing the most significant independent predictor of prognosis in pediatric ALL.

Galand et al (1995) have reported that immunoreaction with the PC10 antibody offers a valid substitute to $^3$H-thymidine pulse-labeling in human lymphocytes for evaluating the classical thymidine-labeling index or TLI, so that correlations eventually found between the latter and any clinical data would also apply to the
PCNA index measured in those conditions. PCNA determination has been found to be of diagnostic and prognostic importance in MDS (Bourantas et al., 1996). There are two populations of PCNA in cells, a replicon-bound and a free nucleoplasmic form and therefore can be affected by cell fixation procedure (Kelman, 1997). A recent study has indicated that PCNA labeling was strongly affected by the cell fixation procedure used: poor with paraformaldehyde, strong and uniform with methanol, and variable with acetone/methanol. Only the methanol fixation demonstrated good correlation between S-phase specificity and cell proliferation marker. Since lymphocytes were fixed in cold ethanol, the results we obtained may be more closer to reality. Comparison between PCNA and Ki-67 labeling showed a linear correlation; but when compared in S-phase fraction, Ki-67 performed better than PCNA (Motta et al., 1999).

Previous studies have indicated the number of interphase AgNORs of marrow cell lymphoblasts, at the time of presentation, was related to the progression of the disease (Trere, 1994). In cancer tissues it has been demonstrated that the quantitative distribution of AgNORs is related to the values obtained using other well-established parameters of cell kinetics such as Ki67 labeling index, BrdUrd labeling index and percentage of S-phase cells determined by flow cytometry (Derenzini, 1991).

Recent reports on AML, suggests that NOR counts can predict complete remission, remission duration, and survival in adult AML patients (Pich et al., 1998). In multivariate analysis also, AgNOR count appeared as an independent
prognostic variable (P = .005). In ANLL also, AgNOR number showed a direct correlation with Ki-67 reactivity of leukemic cells (Shome et al., 1999).

The previous findings have demonstrated that cell proliferation is a reliable prognostic parameter in childhood ALL and indicate the opportunity to routinely add cell kinetics evaluation to the other well established parameters for pre-treatment prognostic definition of ALL. Among the methods used for cell kinetics measurement those methods ought to be preferred which evaluate the rapidity of cell proliferation rather than the number of doubling cells. Indeed, it is the former parameter which indicates more precisely the actual growth rate of neoplastic mass.

The importance of the rapidity of cell leukemic lymphoblast proliferation for the clinical course of ALL can be related to the fact that (1) if the therapeutic efficacy is the same, the length of the remission would be determined by the degree of cell proliferation rate and (2) drug resistance may develop more quickly and rapidly than in slowly proliferating cells.

Ribosomal biogenesis necessary for cell duplication is restricted to a shorter period in rapidly dividing cells than in slowly dividing cells with a consequent greater expression of AgNORs in faster proliferating cells. Interphase AgNORs quantification therefore represents a unique tool to evaluate the rapidity of cell proliferation in routinely processed cytohistological samples. In our study, the five-year overall survival for patients with AgNOR count more than the median value was 45% and those having AgNOR count less than the median values was 67% (p value = 0.01). However using multivariate analysis, AgNOR count was
found to be of less importance when compared to more specific Ki-67 immunoreactivity (p value = 0.0009).

Bockstaele et al (1991) have found that Ki-67 antigen to be absent in majority of cycling cells in normal bone marrow cell suspensions, but the antigen present in the majority of cycling cells in stressed bone marrow and in marrow cells cultured in the presence of hematopoietic factors. This suggest that bone marrow cells, growing and proliferation under steady state conditions and guided by normal physiological control mechanisms, may lose their Ki-67 expression upon exiting the progenitor compartment and entering the differentiating compartment. Since leukemia cell populations in their maturation stages, it can be presumed that relatively well-differentiated leukemia progenitor clones that are still in the cell cycle may lose Ki-67 reactivity.

The present results show that Ki-67 immunoreactivity of leukemic lymphoblasts, at the time of diagnosis, was related to the progression of the disease in the patients studied. The group of patients having higher mean values of Ki-67 immunoreactivity was characterized by a higher number of deaths, and shorter five year overall survival than patients having a lower mean values (42% vs. 75%) (log rank test, p value = 0.001). Survival analysis showed an increased survival for patients with lower PCNA immunoreactivity but it was of a borderline significance (67% vs. 43%) (Log rank test, p value = 0.03). In the series of patients considered by us the WBC count lost its significance in multiple regression analysis. This might have been due either to different therapeutic regimens reducing the prognostic impact of WBC
count or/and to the fact that different methodologies were used. In conclusion, the result of the study identifies Ki-67 as a better prognostic variable for predicting survival among the different parameters analyzed.