BIOLOGICAL PROGNOSTIC MARKERS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Summary: Chronic lymphocytic leukemia (CLL) is the most frequent leukemic disease of adults in the Western world. It is remarkable by an extraordinary heterogeneity of clinical course with overall survival ranging from several months to more than 15 years. Classical staging sytems by Rai and Binet, while readily available and useful for initial assessment of prognosis, are not able to determine individual patient's ongoing clinical course of CLL at the time of diagnosis, especially in early stages. Therefore, newer biological prognostic parameters are currently being clinically evaluated. Mutational status of variable region of immunoglobulin heavy chain genes (IgVH), cytogenetic aberrations, and both intracellular ZAP-70 and surface CD38 expression are recognized as parameters with established prognostic value. Molecules regulating the process of angiogenesis are also considered as promising markers. The purpose of this review is to summarize in detail the specific role of these prognostic factors in chronic lymphocytic leukemia.

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent leukemic disorder of adult Euro-American population. It is characterized by the clonal proliferation and accumulation of morphologically mature B-cells in bone marrow, peripheral blood, and secondary lymphoid organs. Malignant cells typically coexpress combination of surface markers CD19, CD5 and CD23. CD79b molecule is either absent or weakly expressed. Expression of CD20 is lower than in other B-cell malignancies. Surface expression of μ and δ haevy chains and clonaly expression of either κ or λ light chains of immunoglobulins is only faint in contrast to the other mature B-cell neoplasia such as non-Hodgkin lymphomas on which their expression is strong. CD22 and FCM7 are usually absent (40, 43). Majority of malignant CLL cells are arrested in G0 phase of cell cycle and are unresponsive to usual mitogenic stimuli (2). Leukemic cells are resistant to apoptotic death as potent antiapoptotic molecules such as Bcl-2, Mcl-1 or XIAP are overexpressed in B-CLL (45).

The natural course of CLL is highly variable. Whereas some patients suffer from aggressive disease with early need for treatment and significantly shorter overall survival, others have indolent disease without any need of treatment at all and identical survival with their CLL free counterparts of the same age (6). Care of patients with CLL represents significant burden for health care resources as it is accompanied by serious adverse effects associated with poor quality of life as well as shortened survival, caused by infections and secondary malignancies (10). It has been a long-term challenge for hematologists to distinguish between CLL patients with either good or poor prognosis. Many markers, both clinical and laboratory have already been tested for their putative prognostic value. Among them, classical and readily available parameters include Rai or Binet stage (based on the number of organs infiltrated by CLL clone, hemoglobin concentration, and number of platelets), parametrs of cell division (serum level of lactate dehydrogenase, thymidine-kinase and lymphocyte doubling time) and beta 2-microglobulin (41). Although Rai and Binet staging systems are able to stratify patients according to the extent of the disease, their prognostic relevance is limited in early stages as some of these patients will never progress, thus never require treatment and will die for reasons unrelated to CLL while others will need early therapeutic interventions and are likely to die of CLL. Many new prognostic parameters based on molecular biological and phenotypical analyses of B-CLL cells have recently emerged.

Cytogenetic aberrations in CLL

The concept that clonal evolution can ultimately lead to development of more dangerous clonal variants is well

established in oncology. It is manifested by the clonal evolution of unfavourable aberration over time in CLL. Only approximately 50 % of patients with CLL display cytogenetic abnormalities using conventional karyotype banding technique due to very low responsiveness of CLL cells to mitogenic stimuli in vitro; however, interphase fluorescent in situ hybidization (FISH) is able to detect aberrations in more than 80 % of patients. The most frequent abnormality is deletion 13q14 associated with favourable prognosis. Trisomy of chromosome 12 confers intermediate prognosis. Unfavourable aberrations include deletion 11q22.3 which is associated with bulky lymphadenopathy and especially deletion17p13 resulting in disruption of the p53 signalling pathway and development of resistance to conventional chemotherapy and ultimately most unfavourable clinical course. Several groups have reported multivariate analysis of various prognostic factors where both deletion 17p and deletion 11q retained statistically significant value with del 17p being the strongest predictor of poor outcome (12, 18, 57). Unsatisfactory results in 17p-deleted patients were confirmed in large prospective trials using fludarabine-based chemo/ or chemoimmunotherapy (5, 19) or frontline anti-CD52 monoclonal antibody alemtuzumab (23). Deletion of 17p is clearly and definitely established as the most powerful negative prognostic factor in CLL. Therefore, FISH using the probes for the four most frequent aberrations (deletion 13q, trisomy 12, deletion 11q, deletion 17p) is the only modern biological test which is recommended for use in routine practice by updated NCI-WG guidelines as it can direct the choice of treatment (20). FISH should not only be performed at the time of CLL diagnosis but also before every line of treatment as deletion 11q or deletion 17p may newly occur only during the course of the disease as a result of clonal evolution (51, 56). Deletion 17p is so unfavourable that CLL patients carrying this abnormality who require treatment should be seriously considered potential candidates for allogeneic stem cell transplantation which can overcome its negative prognostic impact (13).

IgVH mutational status

It was proposed that the CLL cells are derived from naive B-cells of follicular marginal zone as these malignant elements express both CD5 which is seen on normal cells of fetal spleen and surface IgD which is characteristic for B cells without previous contact with antigens present in the germinal centre (4). Precise phase of B-cells differentiation is determined from the mutational status of genes encoding for variable parts of immunoglobulin chains. Rearrangement of V, D and J gene segments encoding for variable part of heavy chains of immunoglobulins is evident during an early step of B-cells differentiation in bone marrow forming B-cell receptor (BCR). An additional variability of BCRs is achieved by extensive mutations in V genes (somatic mutations) of mature B-cells after their antigenic challenges. B-cells with a high affinity BCRs to a particular

antigen are selected during this process. This affinity maturation is seen in a germinal center of secondary lymphoid follicles (4, 24). It is generally assumed that the origin of B cell clone in particular patient is derived form naive B cells if heavy chains V genes are more than 98 % homologous to the germline B cells despite the fact that current experimental data are challenging this oppinion. Schroeder et al found that 36 patients among 75 B-CLL patients revealed less than 98 % homology compared to the germline lineage. The presence of somatic mutations in B-CLL cells suggests their origin in memory B cells rather than in naive ones (52). Overall survival of patients with CLL cells bearing mutated IgVH genes is significantly prolonged compared to patients with unmutated genes malignant cells as proved by landmark studies by Hamblin and Damle (8, 22) et al. Hamblin's study also noted association of atypical morphology of malignant cells with more aggressive course of disease in patients with unmutated V heavy chains genes (22). These findings were further supported by Lin et al who also found that malignant cells of patients with unmutated VH genes express CD38 molecule on more than 20 % of malignant clone. The expression of CD38 on more than 20 % malignant cells was identified in 18 cases from 19 patients with unmutated malignant B cells. Vice versa, only 40 % of patients with unmutated IgVH genes displayed more than 20 % expression of CD38 molecule. Similar results were obtained regarding mutation or loss of p53 transcription factor. All patients with dysfunctional p53 gene harbored also unmutated malignant cells but only 42 % with unmutated IgVH genes were concomitantly mutated in p53 gene (33). Higher frequency of high risk genetic aberrations such as 17p and 11q deletions was detected in patients with unmutated IgVH genes by Kröber at al. The same investigators were able to prove the presence of prognostically favourable deletion 13q in patients with mutated IgVH (30).

The determination of the IgVH mutational status is recognized now as a significant prognostic marker which is independent to the classical staging of disease. As the costs of this test are fairly high and the availability of this molecular testing is still limited to the large research laboratories, there is a sustained long-term effort to identify the prognostically relevant parameters without these limitations. Surface and/or intracellular molecules which can be easily measured by flow cytometry were investigated. Among these, CD38 and ZAP-70 expression have emerged as potential surrogate markers for IgVH mutational status.

CD38 expression in CLL

CD38 molecule is a transmembrane glycoprotein with ectoenzymatic activity catalyzes the synthesis of cyclic ADP-ribose (cADPR), a metabolite of nicotinamide adenine dinucleotide (NAD+) with calcium-mobilizing (25). CD38 is broadly expressed on the surface of many cells of both haematopoietic and nonhaematopoietic origin depen-

dent on their either differentiation or activation (35). Binding of the agonistic anti CD38 monoclonal antibody to mature B-cells is followed by the upregulation of Bcl-2 antiapoptotic molecule thereby protecting malignant cells from apoptosis (65). CD38 is expressed in various leukemias and may be of prognostic significance. Expression of CD38 in CLL was first reported by Damle et al. in a pilot group of 37 patients. Patients whose CLL cells expressed CD38 on more than 30 % of malignant clone had mosstly unmutated VH genes and aggressive course of disease and vice versa, patients with low CD38 expression had predominantly mutated VH genes and long overall survival (8). The prognostic value of CD38 expression in CLL was further confirmed by Düring et al in a group of 133 patients. The patients with the expression of CD38 on more than 20 % of malignant cell had more advanced disease, poor response to therapy and shortened survival compared to patients with low CD38 expression (15).

There are many doubts regarding the threshold value of CD38 positivity. Threshold of 7 % was proposed by Thoronton and Weisner (58, 63), 20 % by Ibrahim and Düring (15, 27) and 30 % by Damle and Hamblin, respectively (8, 22). There are some claims that there are no significant differences in a predictive value between 20 % and 30 % positivity threshold (26). In contradiction to the results of During and Thornton who reported stable expression of CD38 on CLL cells both in the course of disease and after the initiation of the chemotherapy, Hamblin and Ibrahim reported increased expression of CD38 molecule on malignant cells after the initiation of therapy in majority of patients. As mentioned above, increased CD38 expression was proved on malignant cells with unmutated IgVH genes in association with worsening of the disease (15, 22, 27, 58). However, it is claimed that the CD38 expression could be also changed by concomitant diseases such as infectious and immunopathological disorders via interferons and other cytokines release. There are no differences in the expression of CD38 molecule between peripheral blood and bone marrow cells (22, 48). Correlation between mutational status and CD38 expression is far from being 100 %, partly because CD38 expression can fluctuate in time irrespective to the disease progression. Furthemore, the consensus for positivity cut-off in CD38 expression has not been reached yet.

ZAP-70 expression in CLL

ZAP-70 (zeta-associated protein of 70 kilodaltons) is a tyrosine protein kinase belonging into the Src protein kinases family. It serves as an enzyme phosphorylating zeta chain of CD3-TcR complex of T cells. ZAP-70 is not expressed in normal B-cells as they utilize another enzyme Syk (spleen tyrosine kinase) kinase for phosphorylation of intracellular substrates during cellular activation. SH2 domains of both ZAP-70 and Syk kinases are specifically recognizing activation ITAM (Immunoreceptor Tyrosine-based Activation Motifs) amino acid sequences of either TcR of BCR recognizing complexes. The targets for SH2 domains of these kinases are phosphorylated tyrosine residues only. CD3 is a multicomponent molecular complex non-covalently associated with TcR heterodimers. CD3 complex consists of five chains, gamma, delta, epsilon, and either homodimer zeta-zeta or heterodimer zeta-eta, respectively. There is a single ITAM activation motif in each CD3 chains with the exception of both zeta and eta chains in which three ITAM motifs are found. ITAM motifs are essential for the downstream transduction of intracellular activation signals. Their activity is coordinated with either CD4 HLA II class or CD8 HLA I class costimulatory signals to T cells recognizing antigen via TcR. Lck kinase is activated immediately after successful recognition of peptidic fragment by TcR alfa beta or gamma delta heterodimers. Activated Lck kinase subsequently phosphorylates ITAM motifs of CD3 complex subunits which are capturing effectively ZAP-70 kinases from cytosol as ZAP-70 kinase is highly specific for already phosphorylated ITAM motifs of zeta chains. The activation of ZAP-70 kinase is mediated either by autophosphorylation or by Src/Lck kinases phosphorylation. The principal substrate for activated ZAP-70 kinase is an adaptor LAT (Linker of Activated T cells) protein. Phosphorylated adaptor proteins are assembling other cytoplasmic signaling proteins characterized by the presence of SH domains. Very complex multimeric signaling units called "signalosomes" are formed by this process leading ultimately to the activation of downstream signaling cascades (42). It has been suggested by previous studies that ZAP-70 kinase can replace BCR signaling in patients with Syk-1 kinase deficiency (29).

The panel of genes with predictive value for mutational status of IgVH genes segments in B-CLL patients was identified by DNA microarray technique by Rosenwald et al in their orginal study. Gene encoding for ZAP-70 tyrosin kinase was among such candidate genes (50). 93% concordance between IgVH mutational status and the presence of ZAP-70 kinase in malignant B cell was proved by quantitative PCR analysis as reported by a subsequent study of the same group (50). The expression of ZAP-70 was confirmed as a marker of mutational status of IgVH genes by Crespo et al. Their patients with the expression of ZAP-70 showed rapid progression of disease and shortened survival. Three methods, flow cytometry, immunobloting, and immunohistochemistry, respectively, were used to detect the presence of ZAP-70. The results of these three above noted techniques were equivocal in all patients with the only one exception. The discordance in this patient was very likely caused by an extraordinary high number of T-cells. The application of flow cytometry to detect ZAP-70 is extremely advantageous as this technique enables to identify the presence of ZAP-70 separately for CLL malignant clone, T cells and NK cells, respectively (7).

The pilot study by Crespo et al was followed by research done by Schroerse et al who confirmed previous findings

that the presence of more than 20 % of ZAP-70 positive malignant cells in CLL patients being significantly associated with more aggressive course of disease and shortened survival of these patients compared to patients with low ZAP-70. The results by Schroeder et al were extending the significant link of ZAP-70 positivity to other adverse prognostic factors such as lactate dehydrogenase, beta-2-microglobulin, thymidin kinase and CD38 expression, respectively. However, higher variability in the presence of ZAP-70 (8 %) compared to the expression of CD38 (2 %) was identified by these authors. Patients discordant for the presence of ZAP-70 and CD38 expression were reported by Schroers et al (47). Such results are resembling discrepancies between the presence of ZAP-70 and IgVH mutational status in some B-CLL patients as reported by Hamblin, Crespo, and Orchard (7, 21). Collectively, it seems apparent from these studies that the subgroup of patients with the unequivocal results of IgVH mutational status, and the positivity for CD38 and ZAP-70 really exists rather be caused by artifacts which are inseparable linked to the methods of detection. The prognostic value is enhanced by the simultaneous determination of both CD38 expression and ZAP-70 presence. Three groups of patients, the first one with favourable prognosis, the second one with intermediate prognosis and the third one with an adverse prognosis, respectively, could be stratified by the combination of CD38 expression and ZAP-70 positivity by this approach (53). Detection of ZAP-70 in CLL cells is substantially influenced by the method of detection. Confounding variables include: different clones of monoclonal antibody (2F3.2 vs. 1E7.2), direct or indirect immunofluorescence technique, methods of cell permeabilization, fluorochromes used (FITC, PE, ALEXA 488). Furthemore, results are influenced by variables in estimation of flow cytometer data, especially cut-off determination. Whereas some authors set the threshold of positivity based on the presence of ZAP-70 in T-cells and NK-cells, isotype control has been used by others (21, 31, 64). The most widely used method of ZAP-70 detection seems to be direct immunofluorescence method using 1E7.2 monoclonal antibody conjugated with ALEXA 488 fluorochrome. Despite international laboratory harmonization efforts, no definite consensus regarding ZAP-70 assessment by flow cytometry has been reached so far.

Angiogenesis in CLL

Angiogenesis is defined as a process of new blood vessels formation from already existing vasculature. Angiogenesis plays an essential role in solid tumors growth and their spreading by metastasis. For review see Ribatti et al 1999 (49). Angiogenesis is tightly regulated by the activities of both proangiogenic factors, such as hypoxia, and selected cytokines (VEGF, bFGF, PDGF, TGF-beta, IL-8), and inhibitors of angiogenesis such as thrombospondin-1, endostatin, and angiostatin. Under physiological conditions,

optimal balance between proangiogenic and antiangiogenic factors is maintained (3). The classical marker of abnormal angiogenesis associated with an adverse prognosis is microvessel density (MVD) determined by microscopical evaluation of tumor slides immunohistochemically stained for the present of endothelial markers such as CD31, CD34 or von Willebrand factor. Prognostic value of these markers is established for many types of solid tumors such as lung carcinoma, renal cell carcinoma, melanoma, and various brain tumors (16, 17, 32). Recently, correlation between clinical development of different tumors and peripheral blood levels of selected proangiogenic cytokines (VEGF, bFGF) has been identified. It has already been fully appreciated that higher levels of VEGF are significantly correlated to the size of tumor in patients suffering from various solid tumors. Much less are known about the role of abnormal angiogenesis in malignancies of hematopoietic origin where this process has been underscored (11, 60). There is a substantial switch in this opinion now. Increased microvessels density in bone marrow of patients suffering from acute lymphoblastic leukemias, acute myelogenous leukemias, multiple myeloma, non-Hodgkin lymphoma and myelofibrosis, respectively, was equivocally found (1, 9, 44, 47, 61). There are more dense bone marrow microvessels in advanced active multiple myeloma compared to inactive multiple myeloma or monoclonal gammapathy of undetermined clinical significance (61). Results regarding the density of microvessels in CLL patients are rather confusing. Significantly higher density of blood microvessels in early CLL compared to controls was reported by Molica et al (39). Such results were supported by a study conducted by Kini et al (28). However, no such neovascularization in bone marrow of CLL patients has been identified by Aguayo et al (1). An apparent discordance in the results of various studies could have been caused by either different methodological approach (detection of vWF and CD34 determination) or by different evaluation of results, as well as the biological variables in patients cohorts studied. Smolej et al. recently reported that staining with anti-CD34 monoclonal antibody yielded significantly higher microvessel density counts compared to anti-vWF antibody staining (55).

It was reported by Mollica et al that an increased microvessel density in CLL patients is associated with worse prognosis (39). Moreover, Duensing et al showed that the CLL cells themselves are the source of angiogenic activator bFGF. In addition, an increased serum concentration of bFGF in CLL patients is significantly associated with more advanced disease and poor response to therapy with fludarabine (14, 37). Malignant CLL cells produce VEGF and concomitantly express receptors for this proangiogenic cytokine, thereby creating an autocrine proangiogenic signalling loop. Survival of B-CLL malignant cells is significantly prolonged by VEGF action which is responsible for increased expression of antiapoptic molecule Bcl2 (46). The level of VEGF in CD38 + CLL cells is 2 to 3 times higher

compared to CD38 – CLL cells. Both VEGF and bFGF levels were elevated in CLL patients compared to healthy controls as showed by Molica et al. However, only circulating VEGF was positively correlated to the ZAP-70, CD38 molecules expression, and IgVH mutational status of 23 patients with CLL (38). These results are in contrary to the results of our group. We found significantly increased level of VEGF in 27 untreated B-CLL patients negative for ZAP-70 (54). These results could be influenced by many variables including limited number of patients and differences in the determination of either ZAP-70 expression or VEGF levels.

Another proangiogenic factor which is closely linked with VEGF pathway is angiopoietin-2 (Ang-2). Its physiological role is the destruction of old blood vessels which is subsequently followed by neovascularization and blood vessel remodelling (59). Recently, Maffei et al have shown that circulating CLL cells are capable to produce an increased amount of Ang-2 (34). Results of our group (62) independently to results of others showed an increased expression of mRNA for angiopoietin in CLL patients with unmutated IgVH genes (36, 54). It could be suggested from these results that increased level of Ang-2 is associated with unfavorable prognosis of CLL patients. However, further studies exploiting extended spectrum of both pro- and antiangiogenic factors in larger patient cohorts are needed.

Conclusions

CLL is an extremely heterogeneous disease. Currently we have many modern prognostic factors at hand which can help us to assess the individual patient's prognosis more adequately. Deletion of 17p is by far the worst prognostic factor directly affecting response to treatment and overall survival. Other biological markers such as IgVH mutational status and expression of CD38 and ZAP-70 seem to be independent prognostic indicators which can add further value to prognostic stratification. However, the prognostic power of these markers has to be further validated in large prospective trials because some of them are likely to be treatment-specific and their impact may potentially be erased by effective treatment. Despite all the abovementioned prognostic factors, the decision whether or not to treat a CLL patient is currently always dependent upon the clinical activity of the disease only. Future efforts in this field will probably move from prognostic factors to predictors of therapeutic response and clinical outcome.

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