Effect of heat shock protein-90 (HSP90) and vascular endothelial growth factor (VEGF) on survival in acute lymphoblastic leukemia: an immunohistochemical study

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Abstract Background The significance of vascular endothelial growth factor (VEGF) and heat shock protein-90 (HSP90) has received only limited attention especially in acute lymphoblastic leukemia (ALL). In this study, we assessed expressions of HSP90 and VEGF in bone marrow samples of patients with ALL and effect of these expression quantities on the mean overall survival. Patients and methods Using immunohistochemical methods, we assessed expression of HSP90 and VEGF in 22 cases of ALL. Results Expression of HSP90 was detected in 19/22 (86.4%) and 3/22 (13.6%) of patients with ALL, for strongly positive and moderate-weakly positive, respectively. Negative HSP90 expression was not detected in patients with ALL. Expression of HSP90 in patients with ALL and in control group were statistically significant (P < 0.001), however, did not reflect the mean overall survival (P = 0.910). Mean OS was evaluated 992 ± 181 and 724.8 ± 88.2 days for moderate-weak and high HSP90 expression, respectively. VEGF expressions were not significantly different between ALL and control groups (P < 0.087). We did not find any relationship between HSP90 and VEGF expressions in bone marrow specimens of patients with ALL. Conclusion This study demonstrated that HSP90 expression grades in patients with ALL were significantly higher than that in controls and presence of strong HSP90 expression was associated with worse overall survival. VEGF expression in patients with ALL was not different from that in control samples. Determination HSP90 with immunohistochemical method in bone marrow can provide information about prognosis.

Keywords Acute lymphoblastic leukemia · Heat shock protein 90 · Vascular endothelial growth factor · Immunohistochemical staining · Survival

Introduction

Heat shock proteins (HSPs) are molecular chaperones that were initially observed in cells exposed to elevated temperatures [1]. Molecular chaperones assist in the folding of nascent polypeptides into functional tertiary structures, and these molecular chaperones prevent misfolding and aggregation of polypeptides while they exit the ribosome [1]. Molecular chaperones play a crucial role in regulating the balance between protein synthesis and degradation [1]. Molecular chaperone HSP90 (90 kDa) is the new target for recent cancer therapy that encourages researchers. In normal cells, HSP90 expression is omnipresent in the cytoplasm where it interacts with client proteins [1–3]. The level of HSP90 is lower in normal cells than in neoplastic cells and is also present in latent form in normal cells. HSP90 does not form complexes with other chaperone proteins in normal cells [4]. In neoplastic cells, HSP90 is expressed two to ten times greater than in normal cells, which provides a survival advantage for neoplastic cells [4, 5]. This increased HSP expression has been consistent
with chemotherapeutic resistance and carcinogenesis. Increased activity of HSP has also been shown in aggressive and resistant tumors [6, 7]. HSPs play a major role in cytoprotection. Large quantities of HSP90 seem to play an important role in the survival of neoplastic cells; thus, HSP90 has become a fascinating target for inhibition. Apoptosis can be inhibited by overexpressed HSP27, HSP70 and HSP90 in several malignant cells. This inhibition effect can be emerged by direct physical interaction between HSPs and apoptotic molecules [8]. Increased levels of HSPs may be consistent with accumulation of hidden mutations in tumors and thus can make these malignant cells more aggressive and progressive [9]. These findings revealed that HSP90 inhibitors could be effective against many tumor types.

The angiogenic factor VEGF-A (commonly referred to as VEGF) has been well established as a key regulator in physiological endothelial cell growth, permeability and in the process of angiogenesis [10, 11]. In a study, researchers suggested that VEGF is required for stimulation of HSP90-associated oncogenes e.g. BCL-2 and Araf [12].

Many studies were published about increased microvessel density in bone marrow from patients with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). In those studies, the importance of angiogenesis in leukemias has been emphasized [13–19]. Although the mechanism of the angiogenesis is unclear and not explained completely, another study has showed a positive correlation between VEGF expression and increased microvessel density in adult patients with ALL and AML [14]. Although the role of angiogenesis and angiogenic factors is becoming clear in acute myeloid leukemia (AML), their role in B-lineage acute lymphoblastic leukemia (B-ALL) is less certain [13].

In the current study, we have evaluated the prognostic significance of pro-angiogenic VEGF and HSP90 expression in bone marrow with immunohistochemical method in patients with ALL.

Materials and methods

Between 2006 and 2009, a total of 27 cases of ALL were collected from the files of the Department of Pathology, the University of Kocaeli, and the Medical Faculty of Kocaeli. Five of 27 patients were not included in this study because of technical reasons (e.g. insufficiency of the specimens). The present study was carried out on 22 patients with ALL (14 men and 8 women), and the mean age was 29 ± 17.6 (only three patients were under 17 years old). Age- and sex-matched 50 cases were selected for a control group among patients who were evaluated as having metastatic solid tumor, idiopathic thrombocytopenic purpura or lymphoma without evidence of bone marrow involvement. The bone marrow specimen of one of the patients, a 74-year-old woman, was evaluated for HSP and VEGF; however, she was not considered for survival analysis as she refused the treatment. Therefore, we followed up with a total of 21 patients for survival analysis. The patients’ bone marrow was evaluated after hematoxylin–eosin and specific immunohistochemical staining by hematopathologist. According to the immunophenotypical subtypes, the patients were subgrouped as precursor B-ALL (n: 11 cases), common ALL (n: 6 cases), mature B-ALL (n: 2 cases) and T-ALL (n: 3 cases).

For analysis, patients were divided into three groups according to their risk categorization (low risk, n: 5; intermediate risk, n: 4; and high risk, n: 12). Instead of two patients, all adult patients were treated according to CALGB protocol. Ph(+) two adult patients received imatinib together with this protocol. Child patients (n: 3) were treated according to the modified BFM 90 protocol. All treatment protocol included CNS prophylaxis. Three patients were refractory to treatment (one of these patients was Ph positive). Complete remission (CR) was defined as the presence of not more than 5% blast cells in the bone marrow aspirates. The bone marrow samples were obtained at the time of diagnosis. The biopsy specimens were fixed in Holland’s solution and embedded in paraffin. The study protocol was approved by the Ethics Committee of the University of Kocaeli Medical Faculty.

Immunohistochemical staining and scoring

Paraffin wax–embedded bone marrow sections were deparaffinized and rehydrated in a graded ethanol series and distilled water. HSP90 and VEGF antibodies were purchased from Thermo Fisher Scientific (USA). HSP90 and VEGF rabbit polyclonal antibody were prepared according to the general and manufacturer’s instruction. Staining pattern was observed as nucleo-cytoplasmic and cytoplasmic for HSP90 and VEGF, respectively. The percentages of blasts that stained positive were evaluated at 400× and 1,000× magnifications. Semiquantitative grading of staining based on intensity was conducted. Blast cells stained positive less than 5% were evaluated as negative. According to the staining intensity and percentage, three grades were assigned: strongly positive (or high), moderate-weakly positive and negative. Stained samples can be seen in Figs. 1, 2, 3 and 4.

Statistical methods

Associations between patient characteristics and among levels of HSP90 and VEGF staining intensity and percentage (covariates) were assessed for continuous variables by Mann–Whitney and Kruskal–Wallis tests and for categoric variables by Fisher’s exact or χ² tests.
A *P*-value < 0.05 was considered significant. Overall survival (OS) was the primary outcome studied. The Kaplan–Meier method was used to compute survival, and log-rank tests were used to compare survival between groups. Survival was defined as the number of days from initiation of treatment until the date of death or until the date of last follow-up. Patients still alive at the date of last contact were censored.

**Results**

In this study, we investigated the expression of HSP90 and VEGF in patients with ALL by the immunohistochemical method. Expression of HSP90 strongly positive and moderate-weakly positive were detected in 19/22 (86.4%) and 3/22 (13.6%) of patients with ALL, respectively. Negative HSP90 expression was not observed in patients with ALL. When we assessed the VEGF expression in patients with ALL, we observed 8 (36.4%) and 14 (63.6%) of patients as stained strongly and moderate-weakly positive, respectively. We evaluated that VEGF expression in the control cases did not differ from that of our study group. High HSP90 expression was detected only in one control subject. Moderate-weakly HSP90 expression was detected in 45/50 (90%) of control cases, and 4/50(2.0%) of control cases had negative HSP90 expression.

The median follow-up time was 493 days, and the mean remission time was 26.5 ± 6.65 months (interval:
13–33 months) in patients. Seven patients died because of relapse or treatment complications during their following time. The estimated mean overall survival (OS) time was detected 872.3 ± 118.5 days (interval: 639.9–1104.4) and 992 ± 181 days (interval: 637–1346.8) for all patients with ALL and the moderate-weakly positive HSP90 expression group, respectively. When we evaluated the mean overall survival (OS) time in strongly (high) positive expression group for HSP90, we observed the time to be 724.8 ± 88.2 days (interval: 551.8–897.7), which was shorter than the moderate-weakly positive group and all patients with ALL. However, these differences were not statistically significant (P = 0.910) (Fig. 5).

In regard to the mean OS, according to the HSP90 expression, we did not find any difference between risk groups in intergroup analyses. VEGF expressions were not significantly different between the control and ALL groups (P < 0.087 Pearson χ²). Finally, we did not find any relationship between HSP90 and VEGF expressions in bone marrow specimens of patients with ALL.

Discussion

Many studies showed that HSP90 can play a role in tumorigenesis and that inhibition of this protein can result in apoptosis of neoplastic cells [5, 20]. In a recent study, with an immunohistochemical method, HSP90 was moderate to strongly expressed in Burkitt’s lymphoma, follicular lymphomas and diffuse large B-cell lymphoma, 100, 61 and 59%, respectively [21]. In this study, none of grade 1 follicular lymphomas expressed HSP90 moderate to strong. The expression rates were observed in 31% of patients with T-ALL in the same study [21]. The enhanced expression of HSPs by leukemia cells may be associated with the active and indefinite proliferation of leukemia cells [22]. We determined the ratio of HSP90 expressions to be high and moderate-weakly, 86.4 and 13.6% respectively. Negative HSP90 expression was not detected in patients with ALL. According to these articles, HSP90 is most commonly expressed in high grade lymphomas. As previously mentioned, the enhanced expression of HSP90, particularly in high-grade tumors, could be a key for proliferation and survival. This effect may be experienced upon the interaction of HSP90 with several client proteins involved in cell proliferation and survival. These findings should prompt us to further investigate the tumorigenic role of HSP90.

Contrary to normal B cells, HSP27 overexpression can be seen in ALL. In another research, which was conducted on 18 patients with ALL, HSP27 was determined in 1–79% of blast cells (median 10%). HSP27 was exhibited in 20% or more the half of ALL cases in this research. HSP70 was expressed by 3–82% (median 39%). A total of 13 of 18 ALL cases (72%) exhibited 20% or more positive cells [23]. In patients who did not reach CR and in those with Philadelphia chromosome-positive leukemia, higher expression rates were observed. According to these studies, BCR-ABL has been reported to be an HSP90-associated protein, although we did not find high HSP90 expression in two BCR-ABL positive patients in our study.

According to Lauten et al.’s study, which assessed the relationship between HSP90 expression and glucocorticoid resistance, higher HSP90 was expressed in patients with childhood acute lymphoblastic leukemia than with healthy controls. However, they did not find a correlation between HSP90 expression and glucocorticoid resistance [24].

In an article, Biamonte evaluated the expression of the HSP90 alpha gene. According to this study, higher expression of HSP90 levels was associated with malign proliferation of leukemia cells [25].

Neckers and Ivy have studied 22 patients with different types of acute leukemia and have found an association between the increased expression of the HSP90 alpha gene in leukemia cells and the active and indefinite proliferation of these cells. Their results also suggest that the high expression of the HSP27 gene may not be confined to a specific type of acute leukemia [26]. In our study, we observed that the expression of HSP90 did not significantly

![Fig. 5 Kaplan-Meier survival analysis for overall survival in weak-moderate staining group and strong staining group in ALL patients](Med Oncol)
correlate with risk groups, age and leukocyte counts. We found significantly strong HSP90 staining in patients with ALL when compared with controls; however, this evidence did not reflect OS (Fig. 5). Correlations have also been presented between expression of HSPs and that of drug-resistance and apoptosis proteins in patients with AML: MRP, MRK and BCL-2 [27]. In patients with AML, with lower expression levels of HSP27, HSP60, HSP70, HSP90 and HSP110, OS has been found longer than in the other patients demonstrating higher expression levels of the same HSP [27]. A study demonstrated that the coexpression of at least two proteins, including P glycoprotein, multidrug resistance–related protein, bcl-2 (flow cytometry), p53 (luminometric immunoassay) and heat shock protein 27 (Western blotting), was predictive for response to induction therapy in de novo AML comparing leukemic blasts of 20 responders with 20 nonresponders. The conclusion was determined that the overexpression of only one protein is possibly involved in the resistance and is insufficient to influence the prognosis for long-term survival in ALL, whereas the expression of more than one protein is predictive for reduced OS [28].

Angiogenesis plays a fundamental role in the neoplastic process and metastasis of solid tumors [29]. Although the angiogenic factor levels and the role of these factors in AML are becoming clearer, their role in ALL is not fully understood. In our study, patients with ALL did not show significantly increased VEGF expression in bone marrow when compared to the control group (P < 0.087). This finding was contrary to the results of Dias et al. [12]. Yetgin et al. analyzed serum levels of VEGF in 10 healthy controls and 31 children with ALL at the time of diagnosis and remission [30]. In the conclusion of this study, 26 of 31 patients had increased serum VEGF levels that suggest the clinical significance in ALL [30]. Faderl et al. and Kim et al. reported similar findings [31, 32]. With active angiogenesis, cell-associated VEGF may have an increased expression, which is observed in ALL cell lines [33]. As a result of incremented consumption of VEGF by increased angiogenesis, locally present VEGF may not be reflected in bone marrow. Perez-Atayde et al.’s study revealed higher microvessel density in the bone marrow of patients with ALL compared to the normal controls [16]. VEGF expression may also be minimal in the lymphoid system. Comparison of the VEGF protein levels in serum and the expression in bone marrow biopsies in adult ALL and AML cases indicated that patients with AML had slightly higher levels than patients with ALL [14, 34]. Additionally, compared with control groups, enhanced VEGF protein expression was determined in both patients with AML and patients with ALL [14, 34]. During the majority of childhood ALL, expression of VEGF in bone marrow or in peripheral blood can be detected [30, 35–38]. On the other hand, in another study, the VEGF levels were determined to be significantly higher in recurrent ALL compared with newly diagnosed ALL [36]. Interestingly, when we compare the data of adult and child patients with ALL, the latter seem to have lower expression levels of VEGF relative to the control groups [30, 37, 39, 40].

In conclusion, HSP90 is frequently expressed moderately or strongly in ALL. To determine prognostic predictivity and to benefit from HSP90 inhibitor therapy, immunohistochemical assessment for HSP90 also appears to be a convenient and relatively easy method in patients with ALL. The enhanced expression of HSP90 in leukemia cells may be associated with the active and infinite proliferation of leukemia cells. In the future, due to the aforementioned developments, HSP90 will be the important target for treatment of leukemia. In addition, the value of this immunohistochemical marker could be used as a potential discriminator of nonleukemic and leukemic cells.

Angiogenic factor VEGF has been established in the process of angiogenesis, but the prognostic significance of this molecule in adult ALL is unclear. Further investigation into the biology of angiogenesis in ALL is required. This study demonstrated that HSP90 expression grades in patients with ALL were significantly higher than that in controls and that the presence of strong HSP90 expression was associated with worse overall survival. VEGF expression in patients with ALL was not different from that in control samples. Determination HSP90 with immunohistochemical method in bone marrow can provide information about prognosis.

References


