Original Articles

Aberrant Expression of *c*-Met and HGF/*c*-Met Pathway Provides Survival Advantage in B-Chronic Lymphocytic Leukemia

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Background: B-chronic lymphocytic leukemia (B-CLL) is characterized by accumulation of $CD5^+$ B lymphocytes. Decreased VLA-4 (Cd49d/CD29) and CD11a expression and defective adhesion in B-CLL have been previously shown, although there was no substantial data about its importance in immunobiology of B-CLL. The hepatocyte growth factor (HGF) receptor, *c*-met, plays a role in adhesion by acting on VLA-4. *c*-met and VLA-4 share crucial signaling molecules in cell survival. In this study, relationship between expressions of *c*-met and CD49d, CD11a, and additional common signaling molecules in B-CLL was investigated.

Methods: White blood cells from 24 patients with CLL were studied by flow cytometry and/or western blotting prior to and after culturing with recombinant HGF. HGF level from sera was measured with a bead-based flow cytometric assay.

Results: *c*-metα and *c*-metβ were expressed on B-CLL cells, while no expression was observed on normal donor CD19+ cells. This increase was inversely correlated with decreased expression of adhesion molecules. Serum level of HGF in B-CLL was found to be increased. In vitro experiments showed that HGF supported survival in B-CLL cells supporting the possible function of HGF/*c*-met pathway in B-CLL. Furthermore, expressions of critical signaling molecules shared by both VLA-4 and HGF/*c*-met systems including Bcl-XL, Akt, PI3K, and phospho-bad_{136} following HGF stimulations of B-CLL cells have been found to be increased.

Conclusion: Increased expression of *c*-met and HGF may bypass the importance of expression of critical adhesion molecules and support survival of B-CLL cells. *c*-met, being one of the surface tyrosine kinases, may serve as a target for future therapies in B-CLL meriting more attention.

Key terms: B-CLL; CD49d; *c*-metα; *c*-metβ; HGF; flow cytometry; bead-based cytometric assays

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The tyrosine kinase *c*-met is the high-affinity receptor for hepatocyte growth factor (HGF)/scatter factor, a multifunctional cytokine with pleiotropic effects. After HGF binding, *c*-met signaling has been shown to affect a wide range of biological activities, including cell motility, growth, proliferation, and protection from apoptosis. HGF/*c*-met pathway is necessary for the normal growth and development of various cell types, including hematopoietic progenitors in embryonic life and in adults (1,2). There is ample evidence indicating that pathways of HGF/*c*-met system and integrin family of adhesion molecules are linked and can crossmodulate their func-

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tions (3). For example, HGF/*c*-met system prevents cell death even when adhesion is blocked (4). This system exerts its effects primarily on VLA-4 and VLA-5 integrins, which are members of the β1 integrin adhesion molecule family (5). VLA-4 (6), ligand for VCAM-1 and fibronectin, plays pivotal role in B-cell lymphopoiesis and survival (7,8). B cells cannot proliferate and differentiate without this adhesion molecule (9).

B-chronic lymphocytic leukemia (B-CLL) is phenotypically characterized by accumulation of $CD5^+$ B lymphocytes (10). In addition to increased proliferation, $CD5^+$ B cells of CLL have a prolonged lifespan in comparison to their normal counterparts, although no paracrine or autocrine factor, which may provide them a survival advantage, has been described so far. Our group previously reported a decreased VLA-4 (CD49d/CD29) expression and decreased fibronectin adhesion in early stages of B-CLL as confirmed recently (6,11). It was an interesting point how B-CLL cells could live long without having critical adhesion molecules. Therefore, we investigated the relationship between CD49d and HGF receptor, *c*-met, and the expressions of Bcl-XL, phospho-bad_{136}, Akt, and phosphatidylinositol 3 kinase (PI3K) in response to HGF stimulation in B-CLL cells.

PATIENTS AND METHODS

Patients

We used a total of twenty-four patients with B-CLL [female/male: 6/18 with a mean age of 64 ± 10 (SD) years] where 77 ± 12% (mean ± SD) of the lymphocytes were both CD5- and CD19-positive. The median percentage of lymphocytes in peripheral blood was 80% (min–max: 43–96%), and the median of total lymphocyte count was 28.8×10^9/l (min–max: 7.8–243 $\times 10^9$/l). Bone marrow infiltration was between 75 and 95%. Twelve patients had early stage disease (RAI stages 0, I, and II), and 12 patients had late stage disease (RAI stages III and IV). Ten patients had no history of any previous treatment, and fourteen patients were on chemotherapy. The work had local research ethics committee approval, and written informed consent was obtained from all patients. Isolated B cells from 15 healthy donors and 10 umbilical cord blood (UCB) samples from full-term newborns were also studied.

Cell Isolation and Immunophenotyping

Mononuclear cells were obtained from heparinized blood samples by Ficoll-Hypaque (*d*: 1.077) density gradient centrifugation. B cells from healthy controls and UCB were isolated using a standard kit (Lympho-kwik, One Lambda, Canoga Park, CA). Purified monoclonal antibodies against *c*-metα and β were obtained from Upstate Biotechnology (Lake Placid, NY). Fluorochrome-conjugated antibodies CD5 (PE), CD11a (PE), CD19 [fluorescein isothiocyanate (FITC), PE, PerCP], CD49d (PE), and isotypic controls and FITC- or PE-conjugated antimouse IgG secondary antibodies were obtained from Becton Dickinson (San Jose, CA). Standard direct and indirect (for surface *c*-metα) staining procedures were applied. In indirect staining, cells were incubated with 1:100 dilution of *c*-metα mouse antihuman IgG antibody whose dilution was defined by previous serial dilution experiments. Following 30-min incubation in +4°C, cells were washed with phosphate-buffered saline (PBS, pH 7.3) and incubated with FITC- or PE-conjugated secondary antibody for 30 min. Stained cells were then washed, and second and/or third antibodies for CD19 and/or CD5 or CD49d that were directly conjugated were added. Fluorochrome-conjugated (FITC, PE, and PeRCP) mouse IgG1 and/or IgG2a isotypic controls were accordingly used in each setting (Becton Dickinson). Stained cells were examined by flow cytometry equipped with Cell Quest software (FACScan, Becton Dickinson). Daily calibration of Flow Cytometry by using CaliBrite beads (Becton Dickinson) was performed for quality assessment.

Western Blotting

Mononuclear cell lysates from lymphocytes of patients and isolated B cells from controls were prepared using a standard lysing solution. In experiments, lysates from patients with similar expression patterns for *c*-metα and CD49d were pooled and used as CLL mixture. BJAB (B lymphoblastic cell line), RAJI (Burkitt lymphoma B cell line), Bcl-1 (IgM-secreting B lymphoblastic cell line) B cell lines, and Jurkat (T cell line) were used as positive controls in the experiments. Twenty micrograms of protein was subjected to 5% SDS–polyacrylamide gel electrophoresis followed by transfer to PVDF membranes (Biorad, Hercules, CA). Membranes were probed with anti *c*-metβ, Akt, phospho-bad_{136}, PI3K (Cell Signaling Technology, Danvers, MA), and Bcl-XL (kindly gifted by JC Reed, The Burnham Institute, La Jolla, CA). Detection was performed by the enhanced chemiluminescence method (Roche, Basel, Switzerland).

Cell Culturing with HGF

To test the effect of HGF on the expression of signaling molecules in B-CLL cells, 3×10^6 B cells were cultured with 2 ng/ml recombinant HGF (rHGF; R&D Systems, Minneapolis, MN) in serum-free RPMI1640 medium for 24 h. Lysates were prepared as mentioned before.

Measurement of Cell Death and Survival

Cells from patients with B-CLL (1×10^6) were cultured with either 2 ng/ml rHGF or 10 μM genistein (Sigma, St Louis, MO) as apoptosis-inducing agent in RPMI1640 medium or only in medium as control. To provide stromal support, in some experiments, wells were coated with 5 μg/ml fibronectin (Sigma, St Louis, MA) for 2 h at room temperature, and cells were cultured with addition of rHGF. Cultures were harvested after 3 days. Cells were washed with PBS (pH 7.4), and cell death and/or cell survival was studied. Cells were either labeled with propidium iodide (20 ng/ml) or with Annexin V plus propidium iodide. In the latter, cells

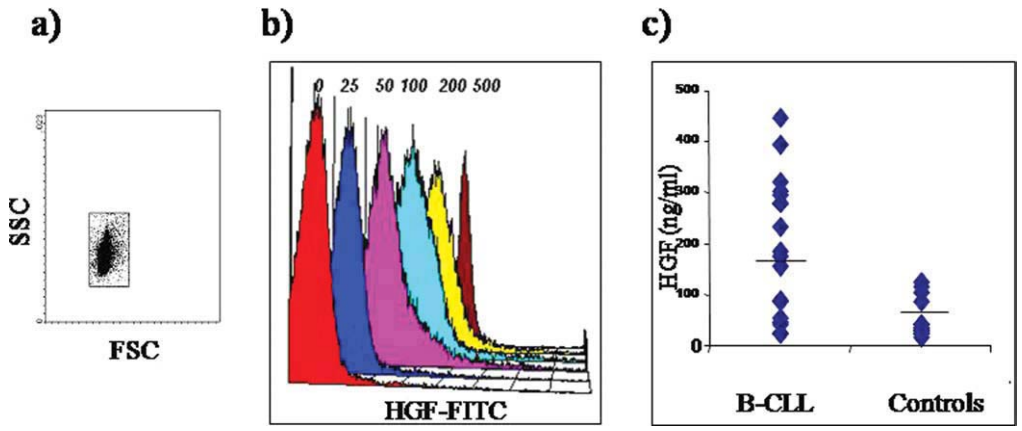

FIG. 1. Serum Levels of HGF. (**a**) Anti-human HGF-coupled 3.5-μm beads were used for semiquantitation of HGF levels of sera. (**b**) In control experiments, FITC-labeled HGF in various doses from 25 to 500 ng was incubated with anti-HGF-coupled beads. Results were evaluated by flow cytometry. Linear channel values of FL1 histograms were found to correlate very well with the doses of HGF. (**c**) Serum levels of HGF were significantly high in comparison to the sera of healthy controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were incubated with 1 μl of Annexin V (Annexin V Staining Kit, Biovision, Mountain View, CA) at dark for 15 min. Then propidium iodide (20 ng/ml) was added, and cells were acquired by flow cytometry.

Measurement of Serum Level of HGF

A bead-based semiquantitative flow cytometric assay was applied for measuring HGF levels. Sera from 20 patients with B-CLL and 15 healthy controls were examined. Antihuman HGF (Sigma, St Louis, MA) were coupled to 3.5-μm polystyrene microbeads (Bangs Laboratories, Fishers, IN). Recombinant human HGF (R&D Systems, Minneapolis, MN) was labeled with FITC (Sigma, St Louis, MA) and used as positive control. Anti-HGF-coupled microbeads were incubated with patients' sera followed by labeling with FITC-conjugated antihuman immunoglobulin (Sigma, St Louis, MA). For quantification, FITC-labeled rHGF was diluted serially and coupled to the same quantity of beads. Beads were acquired by flow cytometry. Levels of HGF were calculated by mean channel values in comparison to control beads that had a linear correlation with the amount of rHGF (Figs. 1a and 1b).

Statistical Analyses

Kruskal–Wallis test, Mann–Whitney *U* test, and Spearman correlation test were used in comparisons. A *P* value lower than 0.05 was regarded as statistically significant.

RESULTS

Expression of *c*-metα on B-CLL Cells

c-metα was found on B-CLL cells in contrast to normal peripheral B cells, and it was significantly higher in comparison to B cells from UCB ($P = 0.001$, Figs. 2a–2c). There was a significant inverse correlation between the expressions of CD49d or CD11a and the expression of *c*-met ($P < 0.01$ in comparison to controls, Figs. 2d and 2e). Inverse correlation was more prominent in early-stage patients in comparison to late-stage patients (cc: −0.4; $P = 0.03$). Therefore, it was demonstrated that *c*-metα and its signal-transducing part, *c*-metβ (Fig. 3a), were being expressed by B CLL cells in contrast to the decreased expression of critical adhesion molecules dissimilar to normal and UCB B cells.

HGF Levels in Patients with B-CLL

The mean serum level of HGF was significantly higher in patients with B-CLL in comparison to healthy controls (Fig. 1c, 195 ± 131 vs 53 ± 38 ng/ml, $P < 0.01$).

Expression of Survival-Related Signaling Molecules

It has been considered that the possibility of the interaction of *c*-met with its ligand HGF might lead to the activation of survival pathways, and this may in turn decrease the necessity of integrin-mediated signals for cell survival. To test this hypothesis, expression of survival-related signaling molecules shared by HGF/*c*-met and VLA-4 pathways were further investigated in B-CLL cells. Bcl-XL was found to be expressed in B-CLL cells. Stimulation of B-CLL cells with rHGF revealed a moderate increase in the expression of Bcl-XL (Fig. 3b). rHGF induced PI3K expression in B-CLL cells (Fig. 3d). Akt, a survival factor acting as downstream of PI3K, was found to be expressed spontaneously by B-CLL cells, and HGF increased its expression (Fig. 3c). Phosphorylation of bad by Akt is an important event that prevents binding of pro-apoptotic members to anti-apoptotic Bcl-2 and Bcl-XL and thus helps cells to survive (12). In our experiment, we demonstrated that HGF induced bad_{136} phosphorylation in B-CLL cells (Fig. 3e). Therefore, the interaction of *c*-met with its ligand HGF led to the activation of crucial survival pathways.

Survival Effect of HGF on B-CLL Cells

The ratio of cell viability measured with propidium iodide staining was 73.8 ± 8% in unstimulated B-CLL cells and 56.3 ± 13% in cells cultured with apoptosis-

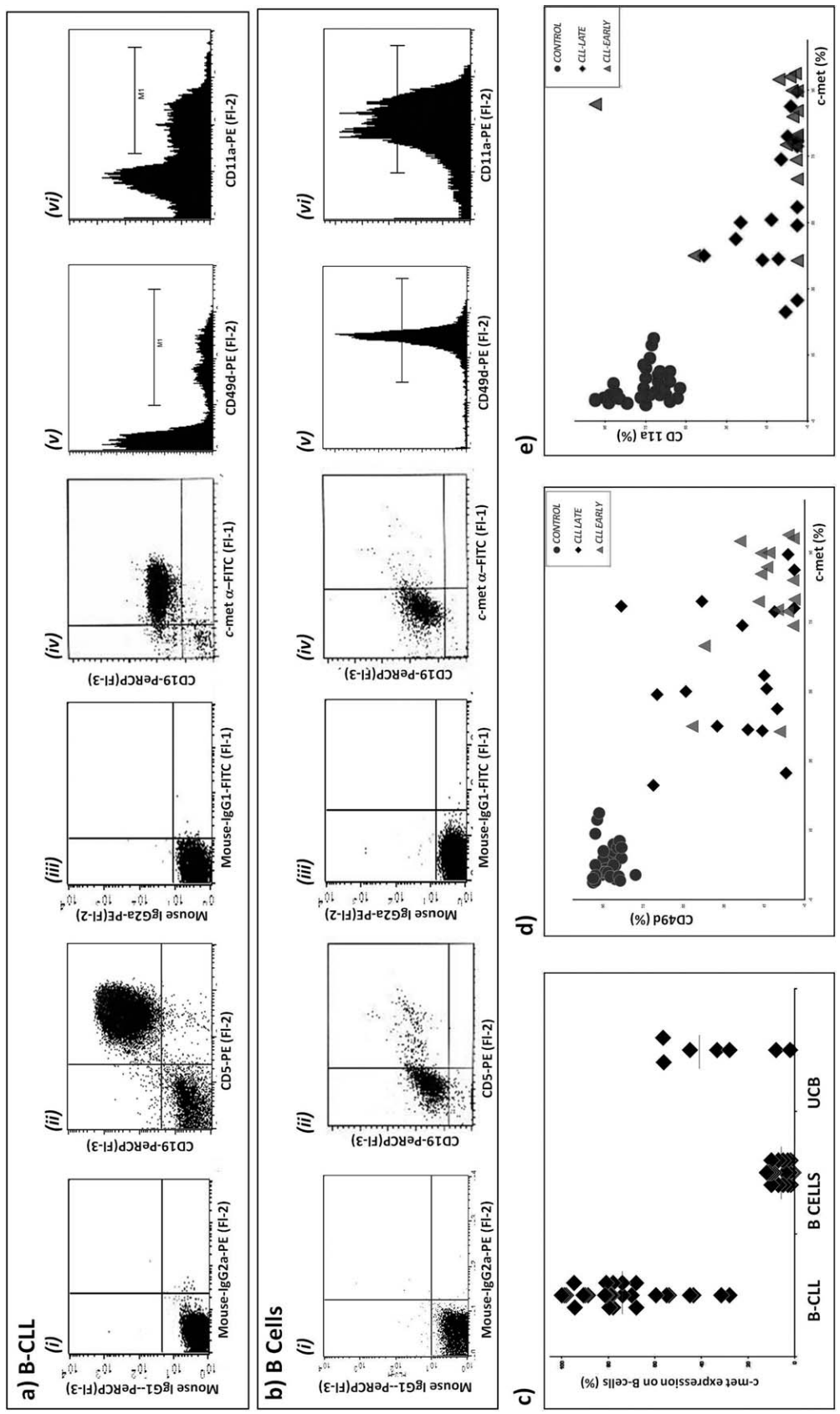

FIG. 2. Expression of *c*-met$_\alpha$ on B cells. A representative figure showing isotypic controls (***i*** and ***iii***), $CD19^+$ versus $CD5^+$ (***ii***), $CD19^+$ versus *c*-met$^+_\alpha$ (***iv***), CD49d (***v***), and CD11a (***vi***) on cells from a patient with B-CLL (**a**) and isolated B cells from a healthy donor (**b**). (**c**) Comparison of *c*-met$_\alpha$ between patients with B-CLL, isolated normal B cells, and mononuclear cells from UCB [mean values: 74 ± 20%, 6 ± 6%, and 37 ± 26%, respectively ($P = 0.001$)]. (**d**) Correlations between CD49d versus *c*-metα (cc: −0.6, $P < 0.01$) in controls and between early-stage and late-stage B-CLL cases (cc: −0.4, $P < 0.03$) and (**e**) CD11a versus *c*-met$_\alpha$ (cc: −0.7, $P < 0.01$). Kruskal–Wallis one-way ANOVA and Mann–Whitney *U* tests were used for comparisons. Pearson and Spearman tests were used for correlations.

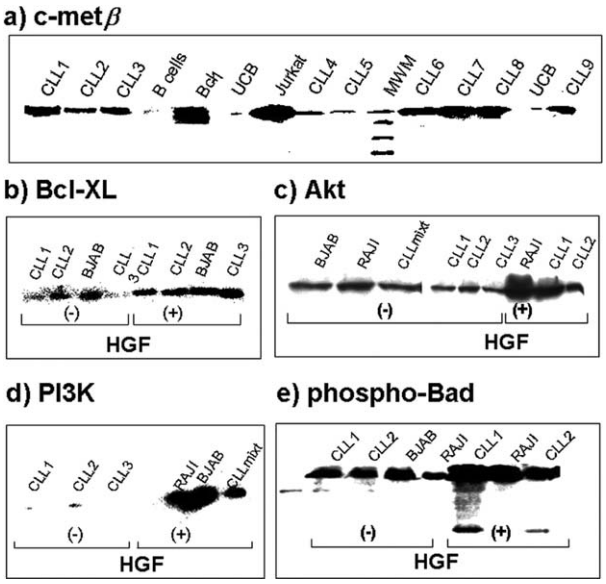

FIG. 3. Expressions of *c*-metβ, Bcl-XL, Akt, Phospho-bad$_{136}$, and PI3K in B-CLL before and following HGF stimulation. (**a**) *c*-metβ is expressed in patients with B-CLL (CLL1–9); isolated B cells, from UCB; normal controls; and cell lines (Bcl-1 and Jurkat). MWM: molecular weight marker. (**b**) Bcl-XL expression is increased in B-CLL (CLL1–3) after rHGF stimulation. BJAB cell line was used as Bcl-XL control. (**c**) Akt is expressed in B-CLL cells as well as in BJAB and RAJI cell lines. HGF increases its expression in both CLL cells and cell lines. (**d**) HGF induces expression of survival factor PI3K in B-CLL cells. BJAB and RAJI cell lines are positive controls. (**e**) Phospho-bad$_{136}$ is expressed in B-CLL cells following HGF stimulation.

inducing agent, genistein ($P < 0.01$), whereas this was 87.8 ± 6% in rHGF-stimulated cells ($P < 0.01$ for both, Fig. 4a). HGF has a survival-protective effect on B-CLL cells. No additional survival effect was observed in cells cultured with rHGF on fibronectin-coated wells (survival ratio: 86 ± 6%). Apoptosis inhibitory effect of HGF was also confirmed with Annexin V staining (Fig. 4b). The ratio of Annexin V was 24.8 ± 4% in control B-CLL samples. This was 10 ± 4.9% following HGF stimulation.

DISCUSSION

Adhesive interactions are critical for survival of all cells. Among several families of adhesion molecules, integrins are of a special interest for hematopoietic cells. For instance, adhesion with VLA-4 alone is sufficient for capturing peripheral blood hematopoietic progenitor cells within the bone marrow (13). Furthermore, VLA-4 (CD49d/CD29) provides essential interactions for B-lymphocyte development in addition to its necessity for lymphopoiesis in general with their features of activating several divergent pathways in cell survival (14–16). In that sense, an operational and highly expressed CD49d status has been expected in B lymphoproliferative disorders. However, our previous data confirmed recently demonstrated that VLA-4 has shown a decreased and variable pattern on B-CLL cells (6,11).

In normal physiologic conditions, cells cannot live without environmental signals. However, in B-CLL cases, it has been considered that the ability for proliferation and more importantly the capacity of longer survival of B-CLL cells may conquer their communicative defects encompassing adhesion molecules (17–19). Either an external stimulus that is independently capable of activating common signal transduction pathways (for example, an induction provided by a cytokine or growth factor) or an internal/individual activation of signal pathways bypassing and decreasing the importance of other signals (for example, presence of uncommon molecule on the "given" cell, which simultaneously uses protein tyrosine kinases on a common pathway) can be conceived as possibilities to explain underlying defects in malignant cell behaviors, in general. We focused on the latter with the context of defective expression of VLA-4 in B-CLL. In that sense, *c*-metα, which is one of the growth factor receptors, was a good subject as a second signal provider, since it has used the same signaling pathway as that of the integrin group of adhesion molecules (3,20–22). Both play roles in cell proliferation and survival (23). HGF/*c*-met pathway prevents cell death

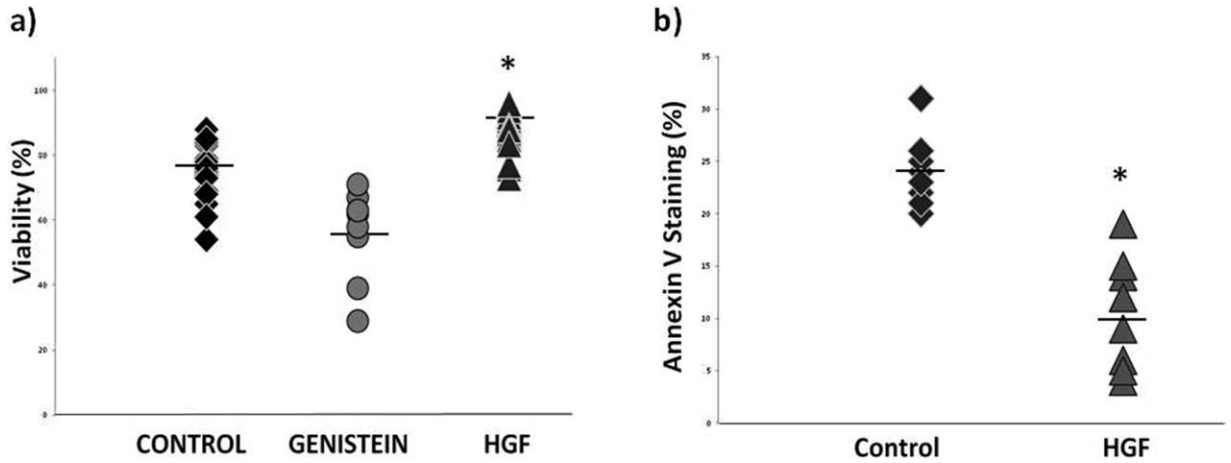

FIG. 4. Survival effect of HGF on B-CLL cells. (**a**) HGF stimulation provides survival-protective effects on B-CLL cells. Cell viabilities [calculated as 100 − cell death % measured with PI staining] were 73.8 ± 8% in unstimulated B-CLL cells, 56.3 ± 13% in cells cultured with genistein, and 87.8 ± 6% with HGF (*$P < 0.01$). (**b**) Annexin V staining in controls (24.8 ± 4%) and in HGF-stimulated samples (10 ± 4.9%, *$P = 0.01$)

even when the adhesion is blocked (4,24). Supporting our hypothesis, we have found that expressions of *c*-metα and β were aberrantly increased in B-CLL cells. This was inversely correlated with adhesion molecule expression, while no *c*-met expression has been observed on normal B cells. This inverse correlation was more prominent in early-stage B-CLL cases although we observed some variations between the cases. In accordance with our previous findings of increased expression of adhesion molecules in late-stage patients, increased but moderate levels of *c*-met and adhesion molecules together might fortify each other effect in late stages of B-CLL in addition to other unknown factors. Serum level of HGF, as a factor that activates signaling through c-met, has also been found elevated in B-CLL cases. In a previous study, serum level of HGF has not been concluded as high in comparison to that in other hematopoietic malignancies (25). However, it is difficult to estimate a threshold value of growth factors that is sufficient for activation of a given pathway without following its in vivo effects.

As a growth factor receptor, activated *c*-met has anti-apoptotic activity via PI3K. PI3K–Akt signaling plays a crucial role in cell survival (26). Sustained activation of PI3K/NF-kappaB pathway has been shown in B-CLL cells (27). In our experiment, we have observed weak expression of PI3K before rHGF stimulation. However, HGF seriously induced PI3K. Akt, which is downstream of PI3K, regulates NF-κB pathway, which promotes expression of anti-apoptotic genes, including Bcl-2 and Bcl-XL (28). Thus, activation of NF-κB by Akt decreases cell death. In a previous report, activated AKT has been demonstrated in freshly isolated B-CLL cells (29). Similarly, we have found constitutive expression of Akt in B-CLL cells. Expressions of Akt as well as Bcl-XL were increased following rHGF stimulation.

Another substrate for Akt;BAD, as a pro-apoptotic member of Bcl-2 family, has a capacity for binding to anti-apoptotic proteins, including Bcl-2 and Bcl-XL, and inhibits anti-apoptotic activity of these proteins (30–32). In our experiment, we have found a significant increase in expressions of both bad and phospho-bad$_{136}$.

This is the first study showing that both intracellular and extracellular parts of HGF receptor *c*-met are aberrantly expressed in B-CLL cells. It is conceivable that HGF/*c*-met pathway may play a role in survival of B-CLL cells, activating crucial survival-related molecules bypassing the necessity of having critical adhesion molecules. Furthermore, as being one of the surface receptor tyrosine kinase, *c*-met merits further investigations in immunobiology of B-CLL as a candidate for possible targeted therapies.

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