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Prognostic relevance of angiopoietin-2, fibroblast growth factor-2 and endoglin mRNA expressions in chronic lymphocytic leukemia

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Elevated levels of circulating angiogenic cytokines and increased expression of genes encoding angiogenic factors have been reported in recent years in patients with chronic lymphocytic leukemia (CLL) but data regarding prognostic and predictive significance are still limited. Therefore, in the present study based upon our prior pilot results, we measured mRNA expressions of angiopoietin-2 (Ang-2), fibroblast growth factor-2 (FGF-2) and endoglin (CD105) by reverse transcription quantitative PCR in purified CD19+ cells from 70 untreated CLL patients (median age, 63 years; males, 64%; Rai III/IV stages, 29 %; unmutated IgVH genes, 60 %) and evaluated their possible association with established prognostic factors and clinical course of the disease. Higher expression of Ang-2 was significantly associated with unmutated IgVH genes ($n = 55$, $p = 0.003$). Higher CD105 expression was significantly associated with unmutated IgVH genes ($n = 55$, $p < 0.001$), high CD38 expression ($n = 66$, $p = 0.022$), high ZAP-70 expression ($n = 66$, $p = 0.010$), Rai stage I-IV ($n = 70$, $p < 0.001$), progressive clinical course of CLL ($n = 70$, $p = 0.001$) and shorter time to treatment ($n = 70$; $p < 0.001$). Expression of FGF-2 was not significantly associated with any of the prognostic markers. These results indicate that elevated expression of Ang-2 and in particular CD105 by CLL cells is associated with unfavorable prognostic features and clinical outcome; thus, both cytokines appear to play an important role in biology and progression of CLL and warrant further investigation.

Key words: chronic lymphocytic leukemia, angiogenesis, angiopoietin-2, fibroblast growth factor 2, endoglin, prognosis

Chronic lymphocytic leukemia (CLL) is the most common leukemic type of adults in Western hemisphere. It is characterized by clonal proliferation and accumulation of morphologically mature lymphocytes in bone marrow, peripheral blood and lymphatic tissues [1]. Clinical course of CLL is extremely heterogeneous with some patients succumbing to the disease within less than 3 years while others living for decades without need of therapy. The process of angiogenesis (new blood vessel formation from pre-existing vasculature) has been shown to be crucial for growth and metastasizing ability of solid tumors mainly by increasing the oxygen and nutrients supply to malignant cells [2]. Angiogenesis in "liquid" tumors was supposed to be less important, nevertheless numerous recent studies have shown enhanced angiogenesis in many hematological malignancies including chronic myeloid leukemia [3], acute myeloid leukemia [4], acute lymphocytic leukemia [5], and CLL [6-8]. It has been demonstrated that elevated markers of angiogenesis correlate with unfavorable prognosis of CLL [9-11].

Angiopoietin-2 (Ang-2), an antagonist of Ang-1 and its receptor Tie2, acts in a concerted action with VEGF and plays an important role in disruption of old blood vessels, thus facilitating their remodeling and subsequent sprouting of new vasculature [12]. Increased Ang-2 plasma concentrations were reported in patients with unmutated sequence for variable region of immunoglobulin heavy chain (IgVH), high expression

of ZAP-70 and CD38 as well as in patients with intermediate and high cytogenetic risk and at advanced Binet stages [10, 13]. In addition, elevated mRNA expression of Ang-2 in CLL cells was associated with unmutated IgVH genes and shorter progression free survival [14-16].

Endoglin (CD105), a member of transforming growth factor-beta receptor complex, is one of key angiogenic activators that regulates endothelial cell adhesion, migration, proliferation, apoptosis and permeability [17-20]. Elevated plasma levels of soluble endoglin (sCD105) have been reported in patients with CLL [21]; furthermore, increased levels of CD105 mRNA in CLL have been detected by ribonuclease protection assay in a very small pilot study [22]. Therefore, data regarding endoglin expression in CLL cells are still very scarce.

Fibroblast growth factor 2 (FGF-2, formerly basic fibroblast growth factor [bFGF]) is another crucial cytokine involved in the process of angiogenesis [23]. Elevated circulating levels of FGF-2 in CLL have also been reported [24, 25], but their prognostic relevance remains controversial [25, 26] and knowledge regarding possible association of FGF-2 mRNA with prognosis of CLL is scanty [27, 28]. Moreover, FGF-2 expression may be post-transcriptionally regulated by antisense molecule (nucleoside diphosphate-linked moiety X motif 6 gene [NUDT6]) responsible for altered FGF-2 mRNA stability [29].

Based on these findings, the aims of our study were to clarify possible association of Ang-2, CD105 and FGF-2 mRNA expressions by malignant lymphocytes of CLL patients with traditional and modern prognostic factors and clinical course of this hematological malignancy.

Patients and methods

Patients. Gene expressions of Ang-2, FGF-2 and CD105 were analyzed in 70 untreated patients with CLL diagnosed according to NCI-WG criteria [30]. They were 45 males and 25 females with median age of 63 years (range, 37 – 84) (see Table 1 for details). The study was conducted according to the Declaration of Helsinki, was approved by local ethics committee and all study participants provided a written informed consent.

Table 1. Patient characteristics

	n
Total number of patients	70
Males	45 (64%)
Male : Female ratio	1.80
Median age (range), years	63 (37-84)
Median follow-up (range), months	18 (0-36)
Median time to treatment, months	12
Median overall survival, months	NR*

* Not reached; overall survival was 87% at 3 years.

Separation of CD19$^+$ Cells. CD19+ cells were separated from peripheral blood anticoagulated by K_2EDTA by gradient centrifugation technique using RosetteSep® Human B Cell Enrichment Cocktail (STEMCELL Technologies, Canada) according to manufacturer's instructions. Purity was ≥ 95% as verified by Epics XL flow cytometer (Beckman Coulter, USA) and anti-CD19 antibody (Beckman Coulter, USA).

RNA isolation. 3x10^7 CD19+ cells were homogenized in 1 ml of TRIzol Reagent (Life Technologies, USA) immediately after separation and frozen at -80°C until the time of RNA isolation. RNA isolation was performed according to manufacturer's instructions. RNA quantity and purity were assessed by UV-spectroscopy and aliquot of RNA was reversely transcribed to cDNA.

cDNA synthesis. cDNA was synthesized from 5 μg of total RNA by SuperScript III reverse transcriptase (Life Technologies, USA) according to manufacturer's instructions using provided oligo-dT primers.

Real-Time quantitative PCR. Two microliters of each cDNA were analyzed by quantitative PCR using TaqMan Gene Expression Master Mix and TaqMan Gene Expression assays (Life Technologies, USA) (ANG-2: Hs01048042_m1; ENG: Hs00923996_m1; FGF-2 Hs00266645_m1; HPRT1: Human HPRT1 (HGPRT) Endogenous Control; NUDT6: Hs00246601_m1) according to manufacturer's instructions in a total reaction volume of 20 μl. All PCR reactions were performed on RotorGene 6000 instrument (Corbett Life Science, Australia). PCR thermal profile was as follows: 50°C for 2 min (UDG incubation), 95°C for 10 min (enzyme activation), followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data analysis was performed using the ddCt method with HPRT1 used as the housekeeping gene. Ct (cycle threshold) values were obtained by '*Quantitation*' function of Rotor-Gene software (version 1.7.87) using manually set threshold.

CD38 and ZAP-70 expression and IgVH mutation status analysis. CD38 and ZAP-70 expression was determined using Epics XL flow cytometer (Beckman-Coulter, USA). CD38 expression was assessed on CD19+/5+ positive cells using CD38-PE antibody (Beckman Coulter, USA) and ZAP-70 was assessed in gated CD19+ cells using intracellular ZAP-70-PE antibody (Caltag Laboratories, USA). Positivity threshold was 20 % for ZAP-70 and 30 % for CD38 as universally accepted [31, 32]. IgVH mutation status was determined as described in detail elsewhere [33].

Statistical analysis. Statistical analysis was performed using software NCSS 2007 (NCSS, Kaysville, USA), Analyse It (Analyse It, Leeds, UK) and MedCalc (MedCalc, Mariakerke, Belgium). The association between expression of the analyzed genes and other prognostic factors was evaluated by Mann-Whitney test. As many patients had blood samples drawn significantly later than the date of diagnosis, overall survival and time to treatment was defined from the blood sampling to death / initiation of treatment. Overall survival and time to treatment curves were constructed by Kaplan-

Meier method and compared using log-rank test. As mRNA expression of analyzed cytokines in normal B cells is not known, expression of angiogenic factors > median was considered high. P-values lower than 0.05 were considered statistically significant.

Results

Prognostic markers. Low/intermediate/high-risk category according to modified Rai staging was present in 17/33/20 cases (24/47/29%). Clinical course of CLL at the time of blood sampling was stable in 38 and progressive in 32 patients. IgVH mutation status was assessed in 55 patients; IgVH genes were mutated in 22 cases (40%). Eleven out of 66 tested patients (17%) had high expression of CD38. Twenty-five out of 66 (40%) patients had high expression of ZAP-70. Favorable cytogenetics (negative result or 13q deletion as the sole aberration) were present in 22 out of 35 assessed patients (63%) (Table 2) [34].

Angiopoietin-2. Ang-2 expression was undetectable in 7 patients and the range of expression was more than 5 orders of magnitude. Patients with unmutated IgVH had significantly higher Ang-2 expression (n = 55, p = 0.003) (Figure 1A). There was no statistically significant association between expression of Ang-2 and high vs. low CD38 expression (n = 66, p = 0.724) (Figure 1B), high vs. low ZAP-70 expression (n = 66, p = 0.157) (Figure 1C), Rai stage 0 vs. I-IV (n = 70, p = 0.431) (Figure 1D) or stable vs. progressive clinical course of CLL (n = 70, p = 0.355) (Figure 1E). There was a trend towards shorter overall survival in the group of patients with high expression of Ang-2 (n = 70, p = 0.087) (Figure 1F) but no significant differences in time to treatment (n = 61, p = 0.403) (Figure 1G). There was no association between Ang-2 expression and favorable or unfavorable cytogenetics (n = 35, p = 0.712), leukocyte, lymphocyte or platelet count, hemoglobin or lactate dehydrogenase (LDH) (data not shown).

Endoglin (CD105). The range of endoglin expression was only 3.6 orders of magnitude. Patients with unmutated IgVH genes (n = 55, p < 0.001) (Figure 2A), high CD38 (n = 66, p = 0.022) (Figure 2B), high ZAP-70 expression (n = 66, p = 0.010) (Figure 2C), Rai stage I-IV (n = 70, p < 0.001) (Figure 2D) and progressive clinical course of CLL (n = 70, p = 0.001) (Figure 2E) had significantly higher CD105 expression. Patients with high expression of endoglin had a trend towards shorter overall survival (n = 70, p = 0.068) (Figure 2F) and significantly shorter time to treatment (n = 61, p < 0.001) (Figure 2G). There was no association between CD105 expression and favorable or unfavorable cytogenetics (n = 35, p=0.463), leukocyte, lymphocyte or platelet count, hemoglobin or LDH (data not shown).

Fibroblast growth factor 2. The range of expression of FGF-2 was more than 5 orders of magnitude. There was no significant association between FGF-2 expression and IgVH mutation status (n = 55, p = 0.323) (Figure 3A), high vs. low CD38 expression (n = 66, p = 0.253) (Figure 3B), high vs. low ZAP-70 expression (n = 66, p = 0.543) (Figure 3C), Rai stage 0 vs. I-IV (n = 70, p = 0.167) (Figure 3D), stable vs. progressive clinical course of CLL (n = 70, p = 0.187) (Figure 3E), overall survival (n = 70, p = 0.204) (Figure 3F) or time to treatment (n = 61, p = 0.192) (Figure 3G). Likewise, there was no association between FGF-2 expression and favorable or unfavorable cytogenetics (n = 35, p=0.282), leukocyte, lymphocyte or platelet count, hemoglobin or LDH (data not shown). In contrast to variable expression of FGF-2, it was very stable for NUDT6 (about 1 order of magnitude); thus, FGF-2 expression was not influenced by NUDT6 in our cohort of patients.

Discussion

While the survival of many patients with CLL does not differ from their CLL-free counterparts, others die within 2 years from diagnosis. Currently used staging systems by Rai and Binet based on tumor burden are limited in prediction of clinical outcome, especially in patients diagnosed in early stages; therefore, new prognostic/predictive markers are needed.

Ang-2 plays a crucial role in disruption of blood vessels, thus facilitating their remodeling and angiogenesis [12]. Its expression is not relevant in solid tumors only but also in hematological malignancies. Increased Ang-2 plasma concentration was reported in patients with unmutated IgVH, high expression of ZAP-70 and CD38 as well as in patients with intermediate and high cytogenetic risk and at advanced Binet stages [10, 13]. Nevertheless Ang-2 source is not fully known and elevated Ang-2 mRNA concentrations in circulating leukemic cells were associated with unmutated IgVH and

Table 2. Prognostic parameters

	n
Rai modified risk	**70**
Low	17 (24%)
Intermediate	33 (47%)
High	20 (29%)
Cytogenetics	**35**
Favorable	22 (63%)
Unfavorable	13 (37%)
IgVH genes	**55**
Mutated	22 (40%)
Unmutated	33 (60%)
CD38	**66**
High	11 (17%)
Low	55 (83%)
ZAP-70	**66**
High	25 (40%)
Low	41 (60%)

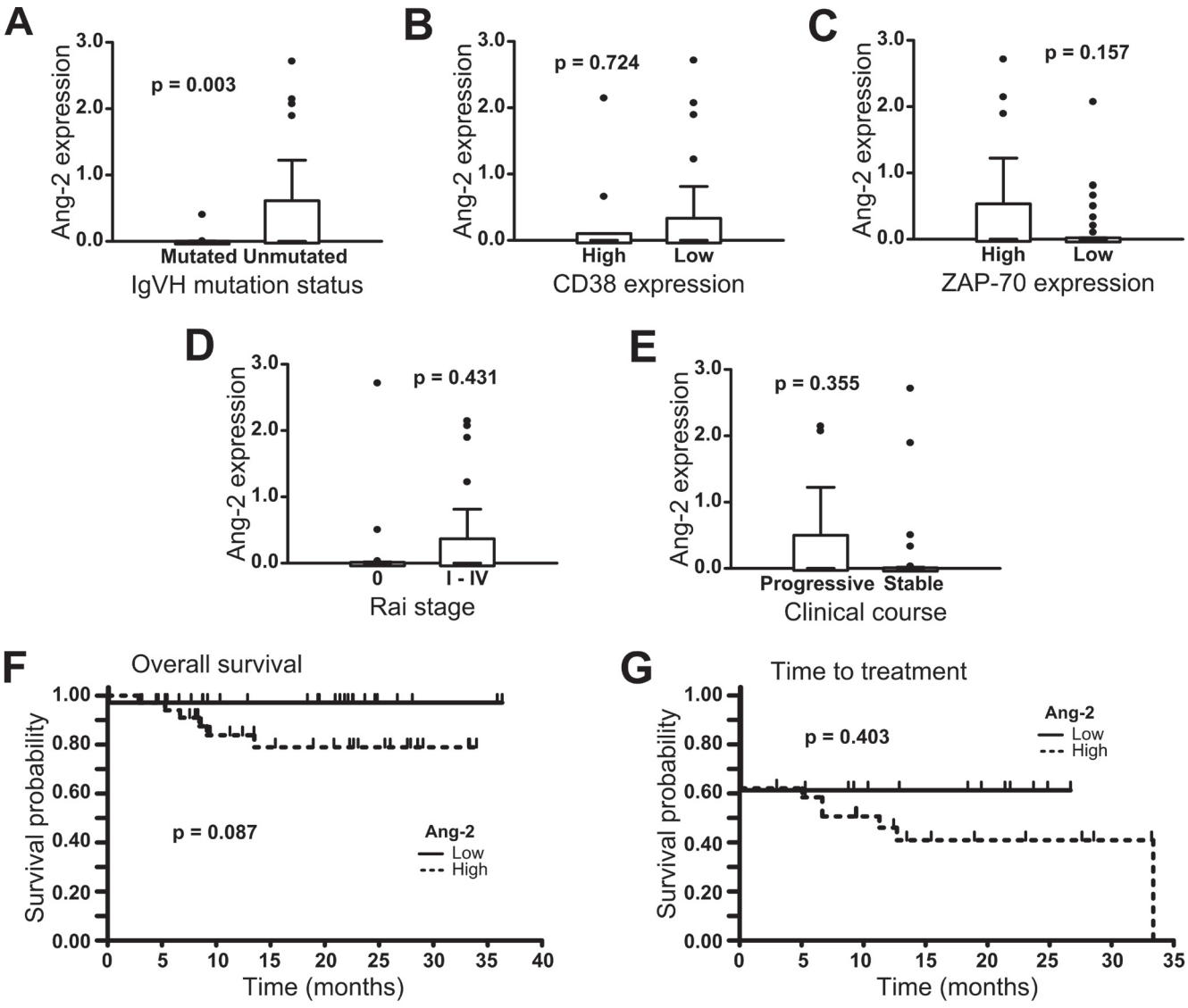

Figure 1. Distribution of Ang-2 expression according to IgVH mutation status (A), CD38 expression (B), ZAP70 expression (C), clinical stage according to Rai (D), course of the disease (E), Kaplan-Meier curves of overall survival (F) and time to treatment (G).

shorter PFS only [14-16], as was confirmed in our cohort of patients in this study where high Ang-2 expression was associated with unmutated IgVH and patients with high expression of Ang-2 had a trend towards shorter overall survival. Such a discrepancy could be explained by elevated expression of Ang-2 by other cells (such as endothelial cells) after autocrine or paracrine stimulation by other cytokines including FGF-2 [35].

Endoglin, a member of TGF-beta receptor complex, modulates cellular response to TGF-beta, especially cell proliferation and differentiation, but it is also a key player in vascular remodeling and angiogenesis. Increased soluble endoglin (sCD105) levels have been detected in many solid tumors [36] as well as in hematological malignancies [37] but the evidence about its role in CLL is still very limited. Elevated mRNA expression of CD105 was detected in malignant lymphocytes of a small cohort of CLL patients [22]. Higher sCD105 plasma levels were detected in CLL patients compared to healthy controls, in patients with progressive disease and advanced clinical stages; furthermore, these patients had shorter time to treatment. No association with IgVH mutation status or cytogenetic aberrations was found, however [21]. In our present study, we observed an association of CD105 mRNA elevated expression in CLL cells and unmutated IgVH, high expression of CD38 and ZAP-70, progressive disease and high Rai stage. Moreover, high expression of CD105 was associated with significantly shorter time to treatment and there was a trend towards

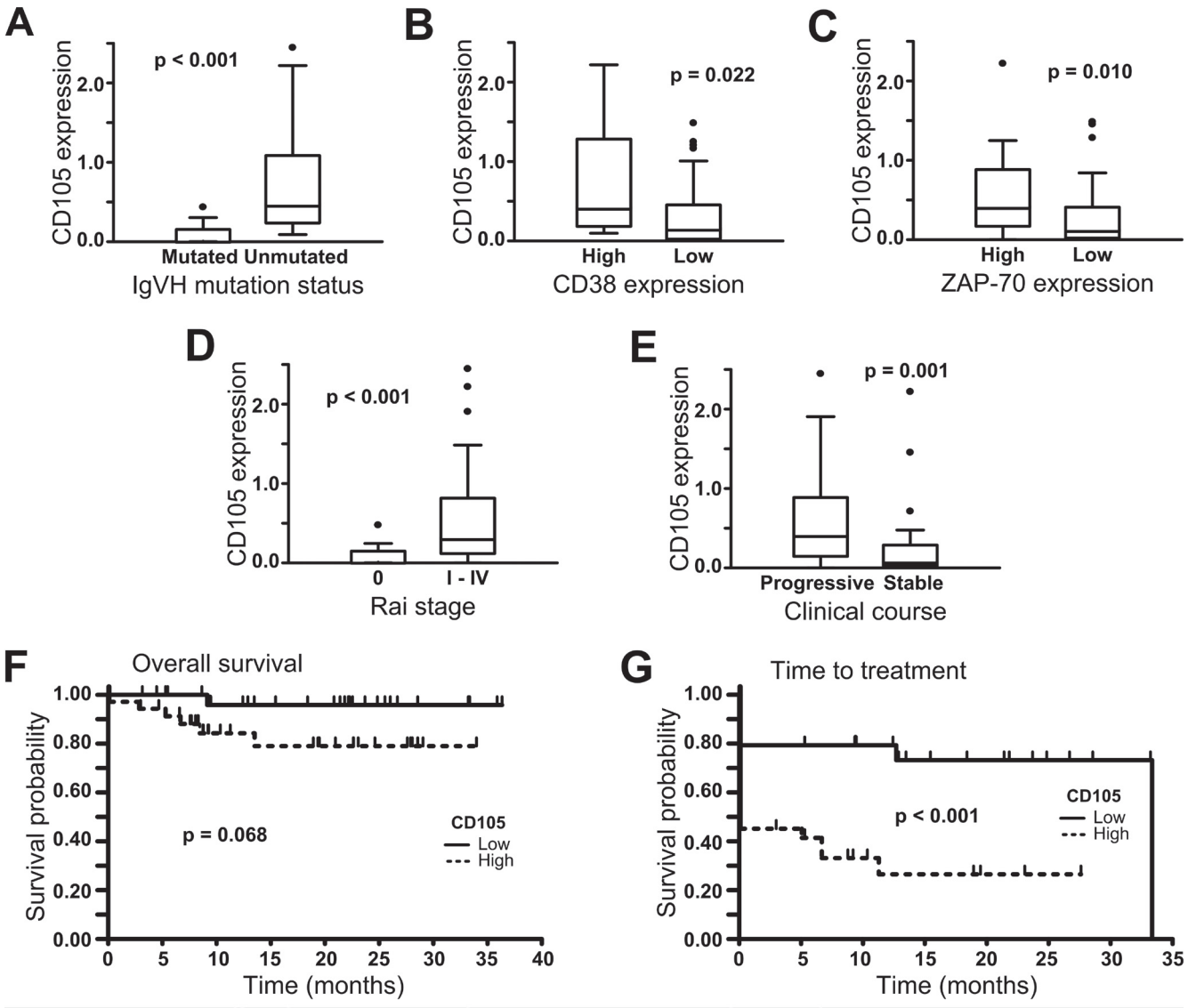

Figure 2. Distribution of CD105 expression according to IgVH mutation status (A), CD38 expression (B), ZAP70 expression (C), clinical stage according to Rai (D), course of the disease (E), Kaplan-Meier curves of overall survival (F) and time to treatment (G).

shorter overall survival in cohort of patients with high CD105 expression.

FGF-2 is a proangiogenic factor responsible for multiple processes including cell proliferation, new blood vessel formation or smooth muscle and tumor growth. It has been shown that FGF-2 plasma levels are increased in CLL [24] and decrease after fludarabine [38] or cladribine [39] treatment. Elevated circulating FGF-2 was associated with advanced clinical stage [40] and increased survival of CLL cells [27] and one of our prior pilot studies showed an association of unmutated IgVH genes with elevated plasma FGF-2 [25]. However, relationship of FGF-2 expression by CLL cells and prognosis remains unclear. Our current results show high inter-patient variability of FGF-2 expression by CLL cells but no association of FGF-2 expression and prognostic markers, supporting the hypothesis that FGF-2 in peripheral blood of CLL patients is not produced by malignant cells only and other cells (as in case of Ang-2) may contribute to the increase of circulating FGF-2 and paracrine stimulation of CLL cells [41]. Importantly, the transcript of FGF-2 antisense gene NUDT6 was shown to influence FGF-2 expression post-transcriptionally [29] but the expression of NUDT6 was very similar in all samples and could not be therefore responsible for the different FGF-2 mRNA levels.

In conclusion, high expression of Ang-2 and especially endoglin was associated with unfavorable prognostic features and clinical outcome of CLL. Although larger patients cohorts with a long-time follow-up are needed for multivariate analysis and

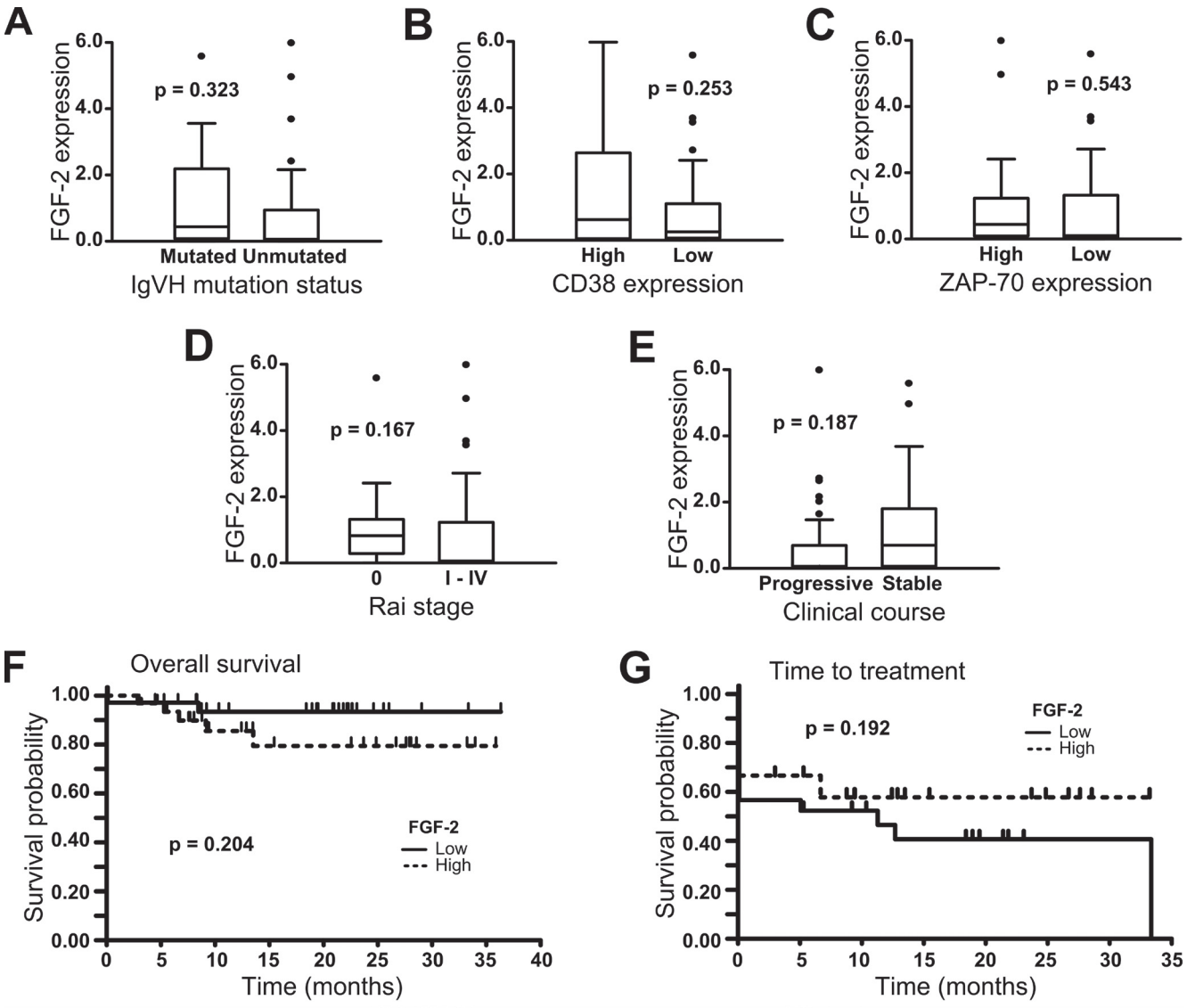

Figure 3. Distribution of FGF-2 expression according to IgVH mutation status (A), CD38 expression (B), ZAP70 expression (C), clinical stage according to Rai (D), course of the disease (E), Kaplan-Meier curves of overall survival (F) and time to treatment (G).

more precise definition of prognostic power of these cytokines, the data presented herein clearly show that angiogenesis plays an important role in biology and clinical course of CLL. Angiogenic factors represent promising molecular markers with significant prognostic value in CLL. Thus, further research in this field seems fully warranted and we plan to extend our study by analysis of expression at protein level by flow cytometry in upcoming experiments.

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