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SUPPORTING INFORMATION

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Chromosome 6q deletion correlates with poor prognosis and low relative expression of FOXO3 in chronic lymphocytic leukemia patients

To the Editor:

Detection of genetic changes has improved the current risk stratification in chronic lymphocytic leukemia (CLL).^{1,2} Among the known recurrent chromosomal abnormalities,¹ 6q deletion is less frequent and controversy surrounding its prognostic significance still remains.^{3,4}

The study aimed at a genetic analysis of a group of CLL patients with chromosome 6q deletion for determination of its prognostic significance, extent, the minimal deleted region (MDR) using array comparative genomic hybridization (arrayCGH), and finally to determine the relative expression of candidate genes located therein.

TABLE 1 Characteristics of patients with 6q deletion (N = 70)

Characteristics	Patients with 6q deletion
Sex - males, N (%)	53 (75.7%)
Age, med (min - max)	63 (34–87)
Binet staging (A/B/C/UNK), N (%)	24 (34.3%)/23 (32.9%)/19 (27.1%)/4 (5.7%)
Mutation status IVGH (mutated/unmutated/both), N (%)	4 (5.7%)/65 (92.9%)/1 (1.4%)
Treated in time of examination, N (%)	28 (40.0%)
Deceased, N (%)	28 (40.0%)
Follow up of living patients, med (min - max)	4.7 (0.4–15.7)
Follow up of all patients, med (min - max)	4.8 (0.1–15.7)
FISH (changes/complex changes/solo del 6q), N (%)	29 (41.4%)/17 (24.3%)/24 (34.3%)
CG + FISH (changes/complex changes/solo del 6q), N (%)	21 (30.0%)/39 (55.7%)/10 (14.3%)
ATM deletion, N (%)	14 (20.0%)
p53 deletion, N (%)	17 (24.3%)
13q deletion, N (%)	30 (42.9%)
Trisomy 12, N (%)	2 (2.9%)

N-number, CG- conventional cytogenetics, UNK- unclassified, med-median, complex changes-finding of three or more changes.

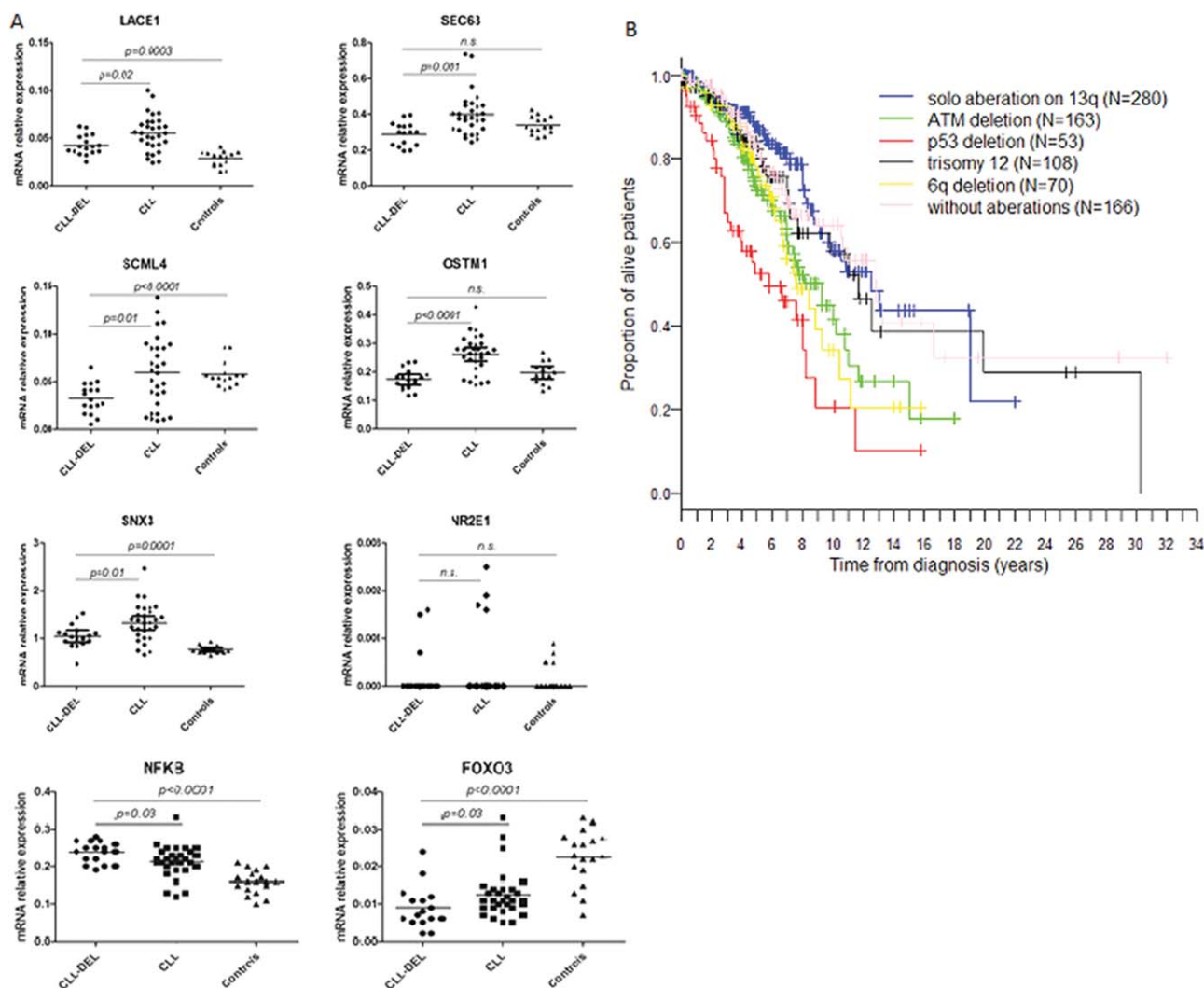


FIGURE 1A. Distribution of relative mRNA expression (ratio target gene/reference PGK1 gene) of *LACE1*, *SEC63*, *NR2E1*, *SCML4*, *OSTM1*, *SNX3*, *FOXO3* and *NFKB1* in peripheral blood of patients with CLL without 6q deletion (CLL), CLL with 6q deletion (CLL-DEL), and healthy controls (Controls). Group means are indicated by horizontal bars, error bars indicate 95CI. **B.** Overall survival according to chromosomal aberrations (N=791)

Peripheral blood and/or bone marrow samples from 1158 CLL patients diagnosed and treated in three Czech hematology centers (Brno, Olomouc and Pilsen) were examined in 2001–2016 using conventional cytogenetics (CG) and FISH. The patient characteristics are listed in Supporting Information Table S1. Chromosome 6q deletion was found by CG (53) and FISH (38) in a total of 91 (7.9%) patients. Complete data and material for further analysis were available for only 70 patients, including 42 patients examined at CLL diagnosis and 28 treated patients examined in the course of the disease (Table 1).

Deletion 6q was confirmed as a single aberration in 10 patients, together with one additional aberration in 21 and as part of a complex karyotype in 39 patients. Evaluation of the frequency of other changes using a FISH CLL panel¹ showed that 13q deletion was the most frequent change regardless of the number of changes, occurring in 30/70 (42.8%) patients. *TP53* deletion was present in 17 (24.3%) patients, with 13 of them having a complex karyotype. The results concerning the presence of additional changes identified by CG and FISH are shown in Supporting Information Figure S1.

The extent of 6q deletion was mapped using arrayCGH. As shown in Supporting Information Figure S2, the range of 6q deletion was very heterogeneous. Analysis showed a "large deletion" covering the entire arm of chromosome 6 (7 patients) and an "intermediate" deletion, a deletion always affecting the 6q telomeric region but not the 6q12 centromeric region (12 patients); deletions other than the two were classified as "interstitial" (51 patients). Given the group size, statistical analysis of the clinical significance of the extent of various deletion types was not conducted. ArrayCGH determined the MDR as a deletion in the region 107.7–108.7 Mb in the band 6q21 containing the genes *SCML4*, *SEC63*, *OSTM1*, *NR2E1*, *SNX3*, *LACE1*, and *FOXO3* (Supporting Information Figure S2). To determine the impact of the deletion on the expression of genes located in the MDR, their relative expression was analyzed using quantitative RT-PCR with the TaqMan assays (Supporting Information Methods). We analyzed 17 patients with 6q deletion, 30 CLL patients without 6q deletion and 19 healthy controls. Low relative mRNA expression of *FOXO3*, *LACE1*, *SNX3*, and *SCML4* was observed in CLL patients with chromosome 6q deletion as compared to those without

the deletion and healthy controls (Figure 1A). Expression profiling of FOXO3, a negative regulator of cell cycle and/or apoptosis, revealed lower levels of FOXO3 transcripts in CLL patients with 6q deletion than in those without 6q deletion ($P = .03$) and healthy subjects ($P < .0001$). This was completed by examination of NF- κ B relative expression, a gene regulated by FOXO3, confirming its high expression (Figure 1A). We hypothesized that FOXO3 is the candidate tumor suppressor gene located at the 6q21 region since survival of normal and malignant B cells is largely dependent on signals from the PI3K-Akt-FOXO pathway. The PI3 kinase pathway contributes to cell survival in part through the activation of Akt and subsequent phosphorylation of the FOXO factors, which inhibits transcriptional functions of these pro-apoptotic genes.⁵ Additionally, pro-apoptotic FOXO3 competes for binding sites in DNA with the anti-apoptotic transcription factor FOXP1, associated with aggressiveness of CLL and B cell lymphoma.^{6,7} Therefore, we analyzed the prognostic role of FOXO3 mRNA levels in an independent cohort of 94 CLL patients (the 6q region status was not examined in them). We divided the cohort by median FOXO3 expression and performed a Kaplan-Meier survival analysis. Compared with cases having low-level expression of FOXO3, those with high-level expression had a significantly shorter median OS (13.1 vs 19.8 years, HR: 2.1 [CI 1.01–4.4]) (Supporting Information Graph S1). Furthermore, we evaluated some of the available clinical and laboratory parameters such as Binet stage, IGHV mutation status and treatment. As many as 92.9% patients with 6q deletion had unmutated IGHV status. We did not show any relationship with Binet stage or treatment at the time of examination (Supporting Information Table S2) or a statistically significant difference in the OS between patients with single 6q aberrations and those with 6q deletion and additional changes (Supporting Information Graph S2). Statistical analysis of the OS of patients with 6q deletions and all examined patients without 6q deletion showed a statistically significant difference, with the OS being shorter in patients with 6q deletions (Supporting Information Graph S3). The graph of the OS of 791 patients (with available FISH data) stratified according to the most common chromosomal changes such as deletions of 13q, 11q, 17p, and +12 shows poor prognosis of patients with 6q deletion (Figure 1B).

We confirmed that 6q deletion in CLL patients indicates poor prognosis and is related to lower expression of the FOXO3 gene which can become a candidate gene for targeted treatment. The FOXO3 gene belongs to a family of transcription factors characterized by a distinct forkhead domain. This gene likely functions as a trigger for apoptosis through the expression of genes necessary for cell death. Restoring the activity of FOXO3 promotes tumor cell death.^{8,9}

Overall, our data support the notion that homeostatic chemokines contribute to CLL resistance to cell death through inactivation of the transcription factor FOXO3, which may represent a novel therapeutic target in this hematopoietic malignancy.¹⁰

In conclusion, presented here are results of the so far largest group of CLL patients with chromosome 6q deletion, confirming that most frequently, the deletion is part of complex karyotypes and is associated with unfavorable prognosis. Low expression of the FOXO3 gene in the MDR is additional evidence of the tumor suppressive role of the gene in the 6q21 region and its role in CLL pathogenesis.

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CONFLICT OF INTERESTS

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

MJ designed the study, collected and analyzed the data, interpreted the results and wrote the paper. LK, AO, MH and KP performed FISH and arrayCGH analyses and contributed to the interpretation of the results. EK⁴ and RF performed expression analysis. VP, TP, MD, DL and KI treated the patients, collected their material and provided clinical information. EK⁵ performed statistical analysis. MM performed molecular analysis of FOXO3. All authors contributed to editing the manuscript.

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Targeted next generation sequencing identifies a novel β -spectrin gene mutation A2059P in two Omani children with hereditary pyropoikilocytosis

To the Editor:

Hereditary pyropoikilocytosis (HPP) is a red blood cell (RBC) membrane disorder characterized by RBC fragmentation, poikilocytosis and mean corpuscular volume of 50–60 fL. During the neonatal period and early childhood, patients often present with transfusion-dependent

hemolytic anemia.¹ HPP is a bi-allelic disorder due to homozygous mutations in spectrin gene (Sp^{HE}) or inheritance of one Sp^{HE} allele *in trans* to a low-expression α -spectrin $SPTA1$ allele ($Sp^{\alpha^{LELY}}$).² The mutated genes encode spectrin variants that possess trypsin resistant sites. Common α -spectrin variants account for a weakening of the integrity of the spectrin-based network when their defective domain(s) forms a part of or in the vicinity of the spectrin heterodimer self-association site. Both codon 28 of α -spectrin gene $SPTA1$ (the hotspot for mutations) and exon 30 of β spectrin gene $SPTB$ can give rise to Spectrin (Sp) $\alpha^{1/74}$, which is detected as a tryptic peptide with a molecular weight (M_r) of 74,000 in gel electrophoresis after limited trypsin digestion.³ Diagnosis of HPP can be challenging with coexistence of underlying hemoglobinopathy especially in infants.

Herein we describe the clinicopathological profile of two related Omani families with co-presence of HPP and hemoglobinopathies. Two probands (1-a and 2-a) presented with history of hemolytic anemia at 3 months of age requiring transfusion support. Both infants had history of neonatal jaundice requiring phototherapy, and so were the older siblings of child 1-a and two siblings of child 2-a (2-b and 2-e). The parents of proband 2-a are first degree cousins, and they are also first degree cousins of the father of child 1-a (Supporting Information Figure 1). Blood films of children 1-a, 2-a, 2-b, and 2-e showed polychromasia and poikilocytosis including spherocytes, elliptocytes, ovalocytosis, and tear drop cells. They also had evident signs of hemolysis (Table 1). Blood films of the parents showed numerous elliptocytes, in keeping with hereditary elliptocytosis (HE). All subjects, except father 2-d, had variable microcytosis most striking in child 2-b (Table 1).

The RBCs of both probands gave a broad fluorescence peak in the Eosin-5'-maleimide (EMA) Binding test (Supporting Information Figure 2A). Proband 1-a had more affected RBCs than proband 2-a because her EMA-labeled RBCs produced a lower fluorescence reading and the histogram showed a slight left shoulder to the known hereditary spherocytosis (HS) sample, indicating a subpopulation of more fragile RBCs.⁴ By contrast, the parents of both probands gave a single uniform peak with a fluorescence reading within the normal range, as expected for HE. In spectrin analysis, a significant increase of dimer content was found in both probands: child 1-a had a greater amount than child 2-a (Supporting Information Figure 2B). Although the dimer contents for both sets of parents were within the HE range, the RBCs of parents of proband 1-a had a greater dimer content than those for the parents of proband 2-a. In limited trypsin digestion of spectrin, a single intensely stained peptide with M_r of 74 000 was obtained for both probands (Supporting Information Figure 2C). Because there was no residual $\alpha^{1/80}$ peptide in their tryptic digests, both probands were considered homozygous for the $Sp^{\alpha^{1/74}}$ variant. The detection of $Sp^{\alpha^{1/74}}$ variant and the marked increase of dimer content suggested HPP. None of the probands and parents had reductions in α -spectrin and protein 4.1. In overall membrane protein quantitation after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of erythrocyte membrane proteins, elevated ankyrin:band-3 ratio was obtained for both probands: 0.277 for 1-a and 0.261 for 2-a (range for normal controls = 0.214–0.238). Although the detection of $Sp^{\alpha^{1/74}}$ variant and the marked increase of