Combined interphase fluorescence *in situ* hybridization elucidates the genetic heterogeneity of T-cell acute lymphoblastic leukemia in adults

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Supplementary Methods and Results

Patients

All patients provided informed consent to collection of samples. Biological analyses were done in accordance with the Declaration of Helsinki. Screening studies were approved by the Institutional Review Board of "La Sapienza" University, Rome, Italy.

Combined interphase fluorescence in situ *hybridization*

Quick-FISH probe preparation: after re-suspension in a formamide hybridization mixture with cot-1 DNA (Invitrogen, Milan, Italy), directly-labeled clones were ready. Slides were prepared using diagnostic cytogenetic pellets. At least six assays were spotted and the corresponding hybridization areas delimited by a round 12 mm coverslip stick with rubber cement to avoid cross-contamination. Slides and probes were co-denaturated on a plate at 75°C for 10 min, hybridized over-night at 37°C, and washed with nonidet P-40 at 75°C and at room temperature. One hundred and twenty to 200 nuclei and/or five abnormal metaphases were analyzed with a fluorescence microscope (Provis, Olympus, Milan, Italy) equipped with a CCD camera (Sensys, Photometrics, Tucson, AZ, USA) run from image analysis software (Vysis, Stuttgart, Germany).

Mutational analyses

PCR-based denaturing high performance liquid chromatography[1] (WAVE-MD™ System Transgenomic, Omaha, NE, USA) equipped with a DNASep Cartridge, was used to study *NOTCH1* mutations in exons 26, 27 (coding for HD-N/HD-C, a heterodimerization domain), and 34 (coding for TAD – a transactivation domain – and PEST – Proline, Glutamate, Serine, Threonine domain). Exon 26 was amplified using primers NOTCH1ex26FW1: 5'-AGGCCAGCATGCAGTTCTAA-3', and NOTCH1ex26FW1: 5'-TAGCAACTGGCACAAACAGC-3'. Exon 27 was amplified using primers NOTCH1ex27RE1: 5'-AGAGTACTGCTTGCCATGGC-3', and NOTCH1ex27REV1: 5'-TAGCAACTGGCACAAACAGC-3'. Exon 34 was amplified using primers NOTCH1ex34FW1: 5'-CAAACATCCAGCAGCAGCAAA-3', and NOTCH1ex34REV1: 5'-TACTTGAAGGCCTCCGGAAT-3'. The DNA sequence for *NOTCH1* refers to NT_02430.16.

Denaturing high performance liquid chromatography was also used to investigate *FBW7*, an ubiquitin ligase which targets NOTCH1 for ubiquitination and degradation. WD40 repeats III and IV (exons 8-9) were amplified in 35 cycles. Exon 8 was amplified using primers FBW7ex8FW1: 5'-GTGATGGGATCATTTTATACGG-3', and FBW7ex8REV1: 5'-GAAGTCCCAACCATGACAAG-3'. Exon 9 was amplified using primers FBW7ex9FW1: 5-'ACCTTGACTAAATCTACCATGT-3', and FBW7ex9REV1: 5'-TATGATTCATCAGGAGAGCATT-3'. The DNA sequence for *FBW7* refers to NT_016354. Annealed PCR fragments (25 µL) were injected into the DNASep HT cartridge. Products were eluted at a constant flow rate of 0.9 mL/min with a linear acetonitrile gradient determined by Navigator software (Transgenomic) according to the amplicon size and GC-content. The elution gradient for homo- and hetero-duplexes was produced by combining two buffers: buffer A (0.1 M triethylammonium acetate pH 7) and buffer B (0.1 M TEAA with 25% acetonitrile). Direct sequencing was performed on samples with abnormal chromatographs using normal samples as controls.

Comparative genomic hybridization

Comparative genomic hybridization was performed in patients n. 6, 14 and 17 using an Olympus microscope equipped with a CCD camera and a CytoVision digital image analysis system (Applied Imaging, Genetix Limited, New Milton, Hampshire, UK).[2] Gains and losses of genomic material were defined by comparing green-to-red ratio profiles with the 99% dynamic standard reference interval.[3] The comparative genomic hybridization profile showed: (i) in patient 6 a gain of the short arm of chromosome 6, of 6q22-q25, and of 7q31; (ii) in patient 14 a gain of 10q23.3-qter and a loss of 7q33-qter; and (iii) in patient 17 a gain of 1p33-pter and of the long arm of chromosome 9 and a loss of 6q11-q21 and of the short arm of chromosome 9.

Molecular studies

TAF_Iα/NUP214 *and* TAF_Iβ/NUP214 *fusion transcripts.*

To detect TAF_Iα and TAF_Iβ isoforms the NUP_2916R primer (exon 20) was used with the TAFa_283F (5'-GAAACCAAGACCACCTCCTG-3') and TAFb_38F (5'-AGCT-

Online Supplementary Table 1S. Clinical, hematologic, and Immunophenotypic features in 23 patients with T-ALL.

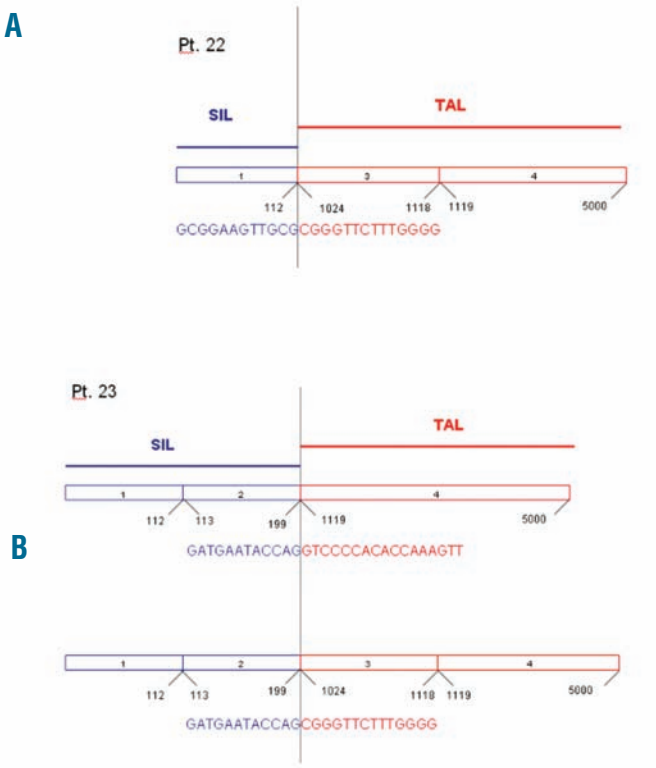
Patient	S/A	Immunophenotype (EGIL)*	Organomegaly and extramedullary sites	WBC ($x10^9$/L)	Follow-up (in months)
1	M/48	pre-T	spleen, lymph nodes	19.1	Resistant, died +1
2	M/47	pre-T	spleen, liver, lymph nodes	89.5	CR, alive +13
3	M/38	pre-T	mediastinum	24.0	CR; allogeneic BMT, alive +27
4	M/19	pre-T	lymph nodes	3.28	CR, auto-BMT, relapse; Cord blood transplant, died +23
5	F/30	cortical	mediastinum, kidney	16.3	CR; relapse, died +12
6	M/26	cortical	mediastinum, spleen, liver, lymph nodes	79.0	CR, alive +3
7	M/64	cortical	mediastinum, spleen, liver, lymph nodes	33.3	CR; 1st relapse; CR after second line therapy; allogeneic BMT; 2nd relapse, haplo-BMT, died +24
8	M/38	pre-T	spleen, liver, lymph nodes	82.6	CR, 1st relapse; CR after second line therapy; 2nd relapse, died +8
9	F/41	pre-T	spleen, liver, lymph nodes	214.0	Resistant, died +3
10	M/19	cortical	mediastinum, spleen, lymph nodes	125.7	CR, alive +48
11	M/20	cortical	mediastinum, lymph nodes	66.8	CR, alive +23
12	F/53	cortical	mediastinum, lymph nodes	51.2	Died +1
13	F/39	mature	mediastinum	35.1	CR, alive +25
14	M/41	cortical	mediastinum, spleen, liver, lymph nodes	65.4	CR, 1st relapse; CR after second line therapy; 2nd relapse, died +15
15	M/20	cortical	mediastinum, spleen, liver, lymph nodes	17.1	CR alive +5
16	M/28	cortical	lymph nodes	65.7	CR alive +29
17	F/26	pre-T	mediastinum, lymph nodes	56.7	Died +11
18	M/40	pre-T	spleen, lymph nodes	17.5	CR alive +17
19	M/17	cortical	spleen, liver, lymph nodes	14.1	Partial response; CR after second line therapy; 1st relapse, died +9
20	F/38	cortical	spleen, liver, lymph nodes	125.0	CR; 1st relapse, died +19
21	M/22	cortical	n.a.	70.6	CR, 1st relapse; second line therapy; 2nd CR, alive +16
22	F/25	unclassifiable	mediastinum, spleen, lymph nodes	62.7	CR, alive +9
23	M/37	pre-T	spleen, lymph nodes	48.3	CR, alive +8

*A, age; M, male; F, female; WBC: white blood cell count; n.a.: not available; CR: complete remission; BMT: bone marrow transplantation. *Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A et al. Proposal for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 1995; 9: 1783-6.*

Online Supplementary Table S2. Clinical, hematologic, and genetic findings in the seven *TAF_I-NUP214*-positive cases of T-ALL.

Pts*	S/A	Protocol	EGIL	Karyotype	*NOTCH1/FBW7*	Combined interphase CI-FISH	RT-PCR *TAF_I-NUP214*	Follow-up (months)
3	M/38	0904	pre-T	46,XY[15]	ex34 / no	del(9)(q34)/*ABL1*	n.d.	CR, allogeneic BMT alive +29
4	M/19	0904	pre-T	46,XY[15]	ex34 / n.d.	del(6)(q16)/*GRIK2* del(9)(q34)/*ABL1* del(12p)/*ETV6*	n.d.	CR, autologous BMT, Relapse Cord blood transplant died +23
X	M/47	n.d.	cortical	failed	no/ex9	del(9)(q34)/ABL1	n.d.	Refused treatment
Y	F/27	0496	pre-T	failed	ex27-ex34/no	del(9)(p21)/*CDKN2A-B* del(9)(q34)/*ABL1* del(11)(p13)/*LMO2* del(11)(q14)/*CALM*	n.d.	Resistant died +12
W	M/19	0904	pro-T	failed	nl/nl	del(9)(q34)/*ABL1* del(11)(p13)/*LMO2* del(11)(q14)/*CALM* del(12)(p13)/*ETV6*	d +22 positive d +50 positive	CR, in treatment alive +3
V	M/18	AIEOP	pre-T	failed	nl/nl	del(9)(q34)/*ABL1* del(5)(q35)/*TLX3*	n.d.	CR, +20 relapse died +24
Z	M/23	0496	pre-T	46,XY[12]	nl/nl	del(9)(q34)/*ABL1*	d +50 positive	CR, relapse, allogeneic BMT died +17

** Patients 3 and 4 are included in the 23 cases studied by combined interphase FISH study; patients X, Y, and W were investigated by multiplex-PCR (see also Figure 2); patients V and Z were discovered to have the* TAF_I-NUP214 *fusion by gene expression profiling; 0496 and 0904 are GIMEMA protocols for treatment of adult ALL; S, sex; A, age; M, male; F, female; n.d., not done; nl, normal; EGIL, European Group for the Immunological Characterization of Leukemias (Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A et al. Proposal for the immunological classification of acute leukemias. Leukemia 1995; 9: 1783-6); CR, complete remission; d +22, 22nd day after diagnosis; d +50, 50th day after diagnosis; BMT, bone marrow transplantation.*



Online Supplementary Figure S1. *SIL-TAL* fusion in patients n. 22 and 23. Panel A) In patient n. 22, *SIL* nucleotide 112 (exon 1) (left) was fused to *TAL1* nucleotide 1024 (exon 3) (right). Panel B) In patient no. 23 two alternative splicing isoforms were detected. In isoform 1 SIL nucleotide 199 (exon 2) (upper left) was fused to *TAL1* nucleotide 1119 (exon 4) (upper right); in isoform 2 *SIL* nucleotide 199 (exon 2) (lower left) was fused to *TAL1* nucleotide 1024 (exon 3) (lower right). Sequence numbers refer to NM_001048166.1 for *SIL* and NM_003189.1 for *TAL1*.

CAACTCCAACCACGAC-3') forward primers, respectively, in the first amplification round. The common reverse primer NUP_2601R (exon 18) and either TAFa_283F or TAFb_38F were used for the second amplification round. PCR products were cloned in pGEM-T easy vector (Promega) and sequenced.

Reverse transcriptase polymerase chain reaction study on the SIL-TAL1 *fusion.*

We designed new reverse primers in exon 4 of the *TAL1* gene to investigate patients n. 22 and 23 who were negative for the *SIL-TAL1* fusion according to multiplex-PCR but positive when assessed by combined interphase FISH. Briefly, 1 μg of total RNA was reverse transcribed using the Thermoscript kit (Invitrogen). Fusion transcripts were amplified using primers SIL-9F 5'-CGACCCCAACGTCCCAGAG-3') (exon 1)[5] + TAL_1927R (5'-GCTGACAACCCCAGGTCTTA-3') (exon 4) for the first amplification round and SIL_A67F (5'-TCCCGCTCCTACCCTGCAA-5') (exon 1)[6] + TAL_1925R (5'-TGACAACCCCAGGTCTTAGG-3') (exon 4) for nested PCR. PCR products were cloned in pGEM-T easy vector and sequenced.

Gene expression profile

Total RNA (2-8 μg) was converted into double-stranded cDNA using a cDNA Synthesis System kit (Roche Applied Science, Mannheim, Germany) and the Poly-A control transcripts (Affymetrix, Santa Clara, CA, USA). cDNA was used for an *in vitro* transcription reaction to synthesize biotinylated complementary RNA (cRNA) using the Microarray RNA target synthesis kit (Roche Applied Science) and the labeling nucleotide mix (Affymetrix). For each sample, 11 μg RNA were fragmented and hybridized to HGU133 Plus 2.0 gene chips (Affymetrix).

Quantitative real-time polymerase chain reaction

One microgram of total RNA was retro-transcribed using the Advantage RT-for-PCR Kit (Clontech, CA, USA). Real-time quantitative-PCR analysis was performed using an ABI PRISM 7700 sequence detection system and the SYBR green dye (PE Biosystems, Foster City, CA, USA) method, as previously described.[6] Real-time PCR conditions were as follow: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 1 cycle at 95 °C for 15 s, and 1 cycle at 60 °C for 1 min, for a total of 40 cycles. For each sample, C_T values for *GAPDH* were determined for normalization; ΔC_T between *GAPDH* and target genes were calculated. Primers were designed using the Primer Express 1.0 software (PE Biosystems).

TAF_I was quantified using TAF_I-ex8_F (5'-TGACGAAGAAGGGGATGAGGAT-3') and TAF_I-ex8_R (5'-TCAAGACAGGGCGACTGAGCACAA-3') primers. Two pairs of primers were used to quantify *NUP214*: NUP214-ex15_F (5'-GACATCAGTGGAAAGATTCAGATCCT- 3') with NUP214-ex16_R (5'-AAGGTATGCAAGTCATCTGATTCTGT-3') and NUP214-ex36_F (5'-AGGCTGTTCAGACCGACGTT-3') with NUP214-ex36_R (5'-AGTAATCATGCGCCTTGTGAGTT-3'). Finally, the following primers were used for *GAPDH* quantification: GAPDH: forward- 5'-CCACCCATGGCAAATTCC-3' with reverse- 5'-GATGGGATTTCCATTGATGACA-3'.

Results

SIL-TAL1 *fusion*

In patient n. 22, the *SIL* nucleotide 112 (exon 1) was fused to *TAL1* nucleotide 1024 (exon 3). In patient n. 23 two alternative splicing isoforms were detected. In isoform 1 *SIL* nucleotide 199 (exon 2) was fused to TAL1 nucleotide 1119 (exon 4); in isoform 2 *SIL* nucleotide 199 (exon 2) was fused to *TAL1* nucleotide 1024 (exon 3) (*Online Supplementary Figure S1*). Sequence numbers refer to NM_001048166.1 for *SIL* and NM_003189.1 for *TAL1*.

References

1. Xiao W, Oefner PJ. Denaturing high-performance liquid chromatography: a review. Hum Mutat. 2001;17(6):439-74.
2. Matteucci C, La Starza R, Crescenzi B, Falzetti D, Romoli S, Emiliani C, et al. Interpretation of the complex karyotype and identification of a new 6p amplicon by integrated comparative genomic hybridization and fluorescence in situ hybridization on the U937-I cell line. Cancer Genet Cytogenet. 2002;135(1):28-34.
3. Kirchhoff M, Gerdes T, Rose H, Maahr J, Ottesen AM, Lundsteen C. Detection of chromosomal gains and losses in comparative genomic hybridization analysis based on standard reference intervals. Cytometry. 1998; 31(3):163-73.
4. Van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G,. et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia. 1999;13(12):1901-28.
5. Pallisgaard N, Hokland P, Riishøj DC, Pedersen B, Jørgensen P. Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. Blood. 1998;92(2):574-88.
6. Husson H, Carideo EG, Neuberg D, Schultze J, Munoz O, Marks PW, et al. Gene expression profiling of follicular lymphoma and normal germinal center B cells using cDNA arrays. Blood. 2002;99(1):282-9.