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Mapping the genetic features of T-ALL cases through simplified NGS approach

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ABSTRACT

Background: Despite the irruption of massive sequencing technologies in clinical routine, the genetic diagnosis of T-cell acute lymphoblastic leukemia (T-ALL) continues to be based on traditional techniques. The integration of old and new technologies with diagnostic and prognostic purposes represents a major challenge.
Methods: A High-Throughput Sequencing (HTS) approach was applied to analyze the genetic landscape of two patients diagnosed with T-ALL and one early T cell precursor acute leukemia. Orthogonal standard techniques were used to confirm the findings of NGS analysis.
Results: By using a single test, a complete genetic map including 2 previously unreported missense mutations in *BCL11B* gene are reported. Cooperating oncogenic lesions including *CDKN2A/B* deletions, *SIL-TAL1* rearrangement and *FLT3* amplification were also captured by using a single test.
Conclusions: HTS is a useful approach that allows simultaneously analyzing mutations, CNVs and the clonal repertoire in T-ALL patients. This approach may simplify the genetic assessment of ALL.

1. Introduction

The genetic diagnosis of ALL-T requires several techniques that must be used in a coordinated way. As an example, karyotype and FISH may identify relevant rearrangements, but are limited by their low resolution in the detection of smaller aberrations (especially karyotyping) and the inability to cover the whole genome and genetic regions (FISH). Therefore, these techniques should be complemented by SNP-arrays. Similarly, the analysis of the T cell repertoire applying Sanger-based sequencing of IG-TCR targets according to EuroClonality guidelines is used for the monitoring of minimal residual disease (MRD), but this procedure is laborious and time-consuming [1–4]. Finally, a comprehensive study of somatic and germline mutations in genes with prognostic significance can only be made with the use of NGS techniques. The increased expenses associated with the use of several laboratory techniques, and the need of coordinating them within the laboratory workflow, makes the diagnostic process complex, occasionally redundant, and inefficient.

To overcome these limitations, NGS approaches for simultaneous identification of Ig/TCR clonal marker and gene mutations have been previously proposed [5]. However, these methods were not initially designed to analyze copy number variations (CNVs) which may have prognostic implications [6,7]. Therefore, it is desirable to incorporate this analysis into the NGS approach.

In this work, we describe a customized NGS approach that enabled us to analyze large genomic regions in a single test with the aim of exploring gene mutations, rearrangements and CNVs, as well as to identify the TCR repertoire. In this way, we show the genetic profile of 3 patients diagnosed with T-ALL and early T precursor acute leukemia (ETP-ALL) and report 2 novel mutations in the *BCL11B* gene.

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2. Material and methods

Acute leukemia (AL) diagnosis was based on bone marrow (BM) aspirate and/or peripheral blood (PB) morphological examination showing ≥20% precursor blast cells. Lymphoid origin of precursor cells was confirmed by immunophenotyping with multi-parameter flow cytometry according to the immunological classification of acute leukemias [8]. Diagnostic categories were stablished according to the World Health Organization classification of tumors of hematopoietic and lymphoid tissues [9]. Patients were treated with intensive pediatric-type chemotherapy followed by allogeneic stem cell transplant in case of early ETP-ALL or high MRD after induction.

The study was approved by the Regional Ethics Committee for Clinical Investigation of the Principado de Asturias. Samples were obtained before chemotherapy was initiated and after informed consent, in accordance with the Declaration of Helsinki.

2.1. High-throughput sequencing (HTS)

DNA from BM and/or PB was extracted using QIAsymphony SP® (Qiagen). Samples were prepared for a customized library capture protocol. The library was designed using the SureSelect XT Reagent library preparation kit (Agilent) for Illumina paired-end multiplexed sequencing. Target regions were enriched with a customized Agilent probe kit including codifying regions ±50 pb from exons of 70 genes (ABL1, ABL2, ACD, ATM, BAX, BCL11B, BCR, BIRC3, BUB1, CDKN2A, CDKN2B, CHEK2, CRLF2, CRLF3, CTCF, DNMT3A, EP300, EPOR, ETV6, EZH2, FBXW7, FLT3, GFI1, GNB1, HLF, IDH1, IDH2, IKZF1, IL3, IL7R, JAK1, JAK2, JAK3, KMT2A, KRAS, LIG4, MGA, MLLT10, NBN, NF1, NOTCH1, NRAS, NUP214, NUP98, PAX5, PBX1, PDGFRB, PHF6, PIK3CD, PIK3R1, POT1, PTPRC, RUNX1, SF3B1, SH2B3, SRY, STAT1, STAT3, STAT5A, STAT5B, TAL1, TAL2, TCF3, TERC, TERT, TLX1, TLX3, TP53, TYK2, WT1) associated with ALL and 383 VDJ segments of TCR and BCR. Genes frequently rearranged with others with prognostic relevance were completely covered. Cluster preparation was carried out using a cBot (Illumina, San Diego, CA) device, and library sequencing was performed using the Illumina platform.

2.2. Bioinformatic analysis

An end-to-end in-house pipeline developed by HealthinCode (A Coruña) was applied for bioinformatic analysis of single indels and CNV variants. For SNP and indels detection, paired-end reads were first aligned with BWA. Duplicated reads were removed with Novosort. Realignment of indels and base recalibration were done according to GATK best-practices. Variant calling was performed on the final alignment file using the variant callers Samtools, BCFtools, HaplotypeCaller, UnifiedGenotiper and VarDict. The results were merged and a consensus quality calculated. The annotation process was carried out using a proprietary service that integrates information from various databases [10]. CNVs were analyzed using a comparative depth-of-coverage strategy [10,11].The method increases sensitivity and specificity by evaluating the base-to-base sequencing depth. An automatic selection of controls allows to decrease the noise and to evaluate each event of loss or gain by studying various factors to assign a score and establish a variety of filters. Regions with high level of homology such are *CDKN2A* and *CDKN2B* were submitted for multiple alignments. Details about the CNV methods are shown in Supplementary material.

Analysis of TCR and BCR repertoire was performed with Mixcr using external IMGT library for alignment [12,13].

2.3. CNV, clonality and gene rearrangement validation

CNVs detected by coverage analysis were confirmed by Multiplex ligation probe-dependent amplification (MLPA) using the available salsa-kits from MRC Holland. In addition, CNVs in *CDKN2A/B* genes and their dosages were confirmed with the kit *SALSA MLPA Probemix P419 CDKN2A/2B-CDK4*.

Multiplex PCR assay with fluorescent oligonucleotides was performed in two reactions (TCRGA and TCRGB tubes) to analyze TCRG V-D-J rearrangements [2]. Two different V and 2 common J primers, 6FAM and HEX fluorescently-labeled, were used in each tube. PCR products were run by capillary electrophoresis in a ABI3130xl instrument and analyzed by GeneMapper 4.0 software. The size of the amplified products is given in bp [1,2]. The interpretation of the results was carried out according to the recommendations of Langerak et al. [1].

Finally, gene rearrangements were confirmed by FISH and/or PCR.

2.4. Familial study

Familial study of candidate variants associated with the disease was carried out in family members by bidirectional Sanger sequencing using specific primers targeted to the fragment sequence of interest.

2.5. Statistical analysis

Analysis of TCR/BCR repertoire was performed with the tcR R package. The graphical representation of TCR repertoire was performed using the circlize R package. The diversity of the TCR repertoire was calculated applying the Shannon's Index [14]. Values of diversity between 2 and 3 were considered normal, whereas those with H score < 2 were classified as low diversity populations [15].

3. Results

Three patients with morphologic diagnosis of acute leukemia were included in the study. Lymphoid lineage and intrathymic differentiation stages of the leukemic cells were stablished by flow cytometry analysis. According to EGIL classification [8], the differentiation stage was T-II, early T and T-IV in patients 1, 2 and 3, respectively. A detailed description of demographics, physical examination and laboratory

Table 1
Demographics, physical and laboratory findings.

Patient	1	2	3
Sex	Male	Male	Female
Age	22	49	54
Physical findings	Enlarged lymphatic nodes, liver and spleen	Normal	Normal
CNS involvement	No	No	No
White Blood Cells $X10^9/L$	156	33	29
Percentage of bone marrow blasts	76	72	87
Immunophenotype of leukemic cells	T-II cyCD3, CD7, CD45d, nuTdT, CD99, CD5, CD2, CD13	Early-T cyCD3, CD7, nuTdT, CD99, CD123, CD13, CD34, HLADR	T-IV cyCD3, smCD3, CD45, CD38, CD7, nuTDT, CD99, CD2, CD4, TCRab, CD26, CD28,
Treatment	Intensive pediatric type chemotherapy	FLAG-ida followed by SCT	Intensive pediatric type chemotherapy
Response	Yes	Yes	Yes
Date	2/May/2018	15/July/2021	11/December/2019
Relapse	Yes	No	Yes
Date	16/April/2019		7/October/2020
Treatment for relapse	FLAG ida, SCT 22/July/2019		FLAG-ida
Final status	Dead 18/January/2020	Dead 22/December/2021	Dead 16/March/2021

CNS: central nervous system. FLAG-ida: fludarabine, cytarabine, idarubicin Granulocytic Colony Stimulating Factor. SCT: allogeneic stem cell transplant.

findings is shown in Table 1.

Once the diagnosis of T-ALL lymphoid linage was stablished, the genetic characterization of the disease was carried out with conventional techniques including chromosome banding, Fluorescence In situ Hybridation and PCR analysis for clonal TCR rearrangements and specific translocations, such STIL-TAL. In parallel, a next generation sequencing approach with a panel covering frequently reported genes with diagnostic and prognostic significance in ALL was carried out. The results obtained with NGS were compared to those obtained with standard techniques. The genetic findings of the 3 patients are summarized in Table 2.

3.1. Analysis of germline and somatic variants

An average depth of 650×, 455× and 434× was reached in patients 1, 2 and 3, respectively. >99% of targeted regions reached an average coverage ≥200× in the 3 patients.

A mutation in *BCL11B* (NM_138576.3) was found in each patient: c.1742G > A: p.Gly581Asp in patient 1; c.376C > T: p.His126Tyr in patient 2 and c.1349C > T: p.Thr450Met in patient 3. All these variants were registered at frequencies below 0,01% in the human genotyping databases of general population. Number of heterozygotes registered In the GnomAD database were 3 for p.Gly581Asp, 9 for p.His126Tyr and 1 for p.Thr450Met [16] suggesting very low prevalence of these variants in general population. The alternative allele frequency of the variants was 53%, 50% and 46%, respectively.

The variant p.Gly581Asp is codified by exon 4 and located in a Gly-rich region placed between C2H2 type 3 and 4 domains. The variant p.His126Tyr is placed near the C2H2- type 1 domain and codified by exon 2. Up to date, no deleterious missense mutations in this region have been described. The variant, p.Thr450Met, is located in the C2H2-type 3 domain of the protein. It has been previously reported in 3 patients with T-ALL [17–19].

Variant p.Gly581Asp of patient 1 was also detected in his parent by Sanger sequencing, confirming its germline origin. Sanger sequencing was not possible in patients 2 and 3 since their parents were not alive.

Patient 2 harbored an internal tandem duplication (ITD) of the exon 14 and 15 in the *FLT3* gene, with an alternative allele frequency of 16%, suggesting a somatic origin. This mutation, firstly reported in acute myeloid leukemia (AML) [20], involves a fragment of 164 nucleotides in the c.1837_18137 + 1 of the cDNA and affects the juxta-membrane region of the receptor. The variant was confirmed by PCR amplification and capillary electrophoresis of PCR products, as previously described. The results are shown in Fig. 1.

3.2. CNV analysis

In patient 1, CNVs analysis showed decreased coverage in the whole *CDKN2A* gene and in 1 exon of the *CDKN2B* gene. Compared with controls, the dosage of the deletion was lower than 0.5 (deviation range 0–1), suggesting a mosaicism in the lymphoid population. Similarly, a whole deletion of both genes was present in patient 3, with a dosage near 0, indicating a complete deficiency of *CDKN2A*/B, which is compatible with homozygous deletion. These results are shown in Fig. 2. Confirmation of both deletions by MLPA is shown in Fig. 3.

3.3. Rearrangement analysis

A breakpoint in *TAL1* gene was identified in patient 1. The BLAT search from the sequence containing the misalignment revealed a gene rearrangement involving *STIL* and *TAL1* genes (1p33) leading to a deletion of the fragment chr1:47697571–47,779,404. This deletion was confirmed by FISH and PCR, as shown in Fig. 4.

3.4. TCR/BCR repertoire analysis

Patient 1 showed 586 clones grouped in 34 rearrangements, with overrepresentation of 1 clone in TRG, TRB and TRA. The most frequent V-D-J clonotype of TRG in this patient was *TRGV4-TRGJ1* (46%), followed by *TRAV41-TRAJ45* (20%), *TRAV41-TRAJ21* (16%) and *TRBV27-TRBJ2–3* (10%). The frequency of the remaining repertoire was lower than 1%.

No over-represented clone was observed in patient 2.

Finally, patient 3 showed 524 clones grouped in 25 different rearrangements, with overrepresentation of 1 particular clone in each TRG, TRB and TRA. The most frequent V-D-J clonotype of TRG was TRBV4–2-TRBJ2–7, with a frequency of 29%, followed by *TRGV5-TRGJ1* with a frequency of 25%.

Cord chart representation of clonal repertoire of V-D-J TCR is shown in Fig. 5. Summarize data of the whole VDJ-TCR repertoire and clonal diversity of the 3 patients are shown in Table 3. Detailed data of the whole VDJ-TCR repertoire of the 3 patients are summarized in the *supplementary material.*

Clonality analysis of TCRG repertoire by standardized fragment amplification PCR revealed 2 clonal peaks of 210 and 225 bp with VfI-J1.3/2.2 rearrangement in patient 1. Oligoclonal amplification was observed in patient 2, with a major peak of 220 bp with V9-J1.1/2.1 rearrangement. Two clonal peaks, 215 bp with VfI- J1.3/2.2 and 180pb with V9- J1.3/2.2 rearrangement were detected in patient 3. These results are shown in Fig. 6.

4. Discussion

In this study, we developed a capture-customized library based in HTS technology to analyze the genetic landscape of 3 patients diagnosed with T-ALL. By using this technique, we show 3 mutations in *BCL11B* gene. Two of these mutations have not been reported and one of them is of germline origin. Using a single test, we were also able to analyze the

Table 2
Summary of genetic findings detected in the 3 patients.

Patient	1	2	3
Karyotype	46,XY, der(14)add [14](q32), −10 + 16/20?[cp3]/46,XY[9]	46,XY[20]	46,X-X?/46,XX, +mar[cp9]
FISH	Nuc sih(5SILx1), (TAL1x2), (5SIL con TAL1x1) [195/200]	Normal	Nuc ish(TCRA5̓, TCRA3)x2(TCRA5̓sep TCRA3)x1[166]
Variants	*BCL11B* c.1742G > A: p.Gly581Asp	*BCL11B* c.376C > T: p.His126Tyr *FLT3* c.1837_18137 + 1	*BCL11B* c.1349C > T: p.Thr450Met
Copy number variation	*CDKN2A/B* deletion		*CDKN2A/B* deletion
Gene rearrangements	*SIL/TAL1*	–	–
TCR repertoire	TRGV4-TRGJ1 TRAV41-TRAJ45 TRAV41-TRAJ21 TRBV27-TRBJ2–3		TRBV4–2-TRBJ2–7 TRGV5-TRGJ1

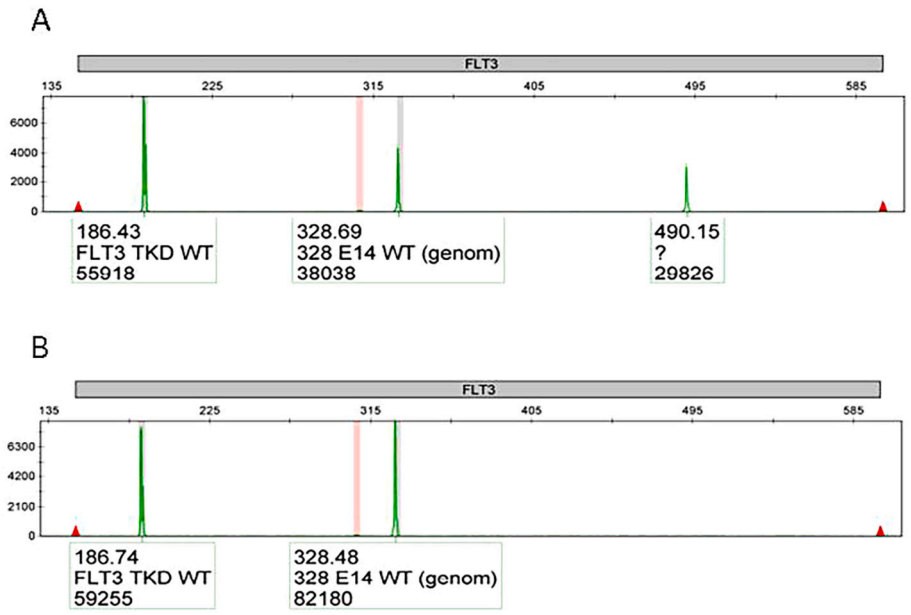

Fig. 1. PCR amplification of TKD and JM domains of *FLT3*. A) PCR amplification of patient 2. First and second peaks corresponds to wild type allele at TK and KM domain. Third peak corresponds to the presence of ITD. B) PCR amplification of negative control. The third peak of the ITD is not observed.

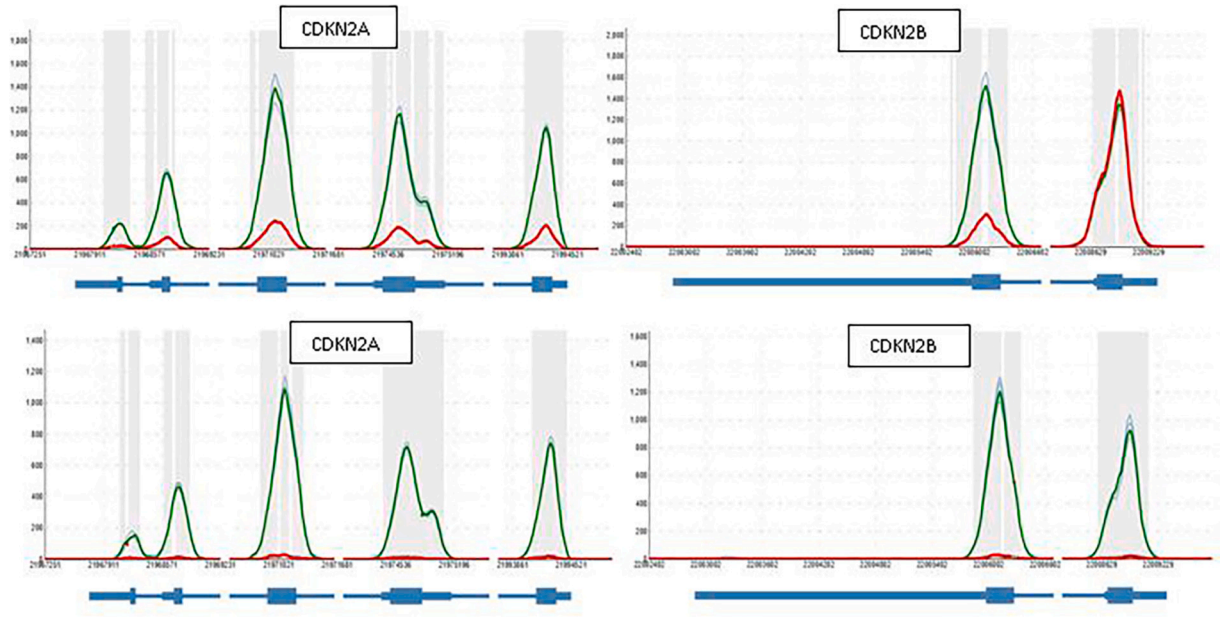

Fig. 2. Representation of the CNV involving *CDKN2A/CDKN2B* genes. Upper panel: patient 1. Lower panel: patient 3. Red lines represent the samples of the 2 proband cases. Green lines and grey bars represent the normalized coverage of controls. In patient 1, the deletion involves the whole CDKN2A and one exon of *CDNK2B* gene. In patient 3, the deletion involves both *CDKN2 A/B* genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TCR repertoire, showing clonal TRG restriction. Additionally, we demonstrated *CDKN2A/B* deletions in 2 patients, *SIL-TAL* rearrangement and *FLT3* amplification in 1 patient each. All the results were validated with standard techniques, providing evidence that NGS is a valid method in the clinical setting to capture the spectrum of genetic variation related to the development, diagnosis and prognosis of T-ALL.

T-cell differentiation is regulated by several genes among which *BCL11B* stands out. This transcriptional repressor promotes the differentiation of double positive and CD4+ T-lymphocytes through modification of gene expression [21]. Accordingly, *BCL11B* inactivation leads to maturation arrest [22,23]. In fact, loss-of function mutations and deletions of this gene have been described across the main molecular subtypes of ALL-T [24]. Its impairment leads to early arrest of T-cells at DN2-DN3 stage and decreased Tα/β rearrangements in favor of Tγ/δ ones. In line with the above, TRG of 2 patients with *BCL11B* mutations showed overrepresentation of TRG clones. Regarding prognosis, low expression of *BCL11B* has been associated with induction failure [24], and low survival [25]. In line with above, the patients in our study harboring these mutations died due to progressive disease.

The variant p.Gly581Ser in the *BCL11B* gene detected in one of the patients has been registered in ClinVar database without any condition associated. Therefore, according to the American College of Medical

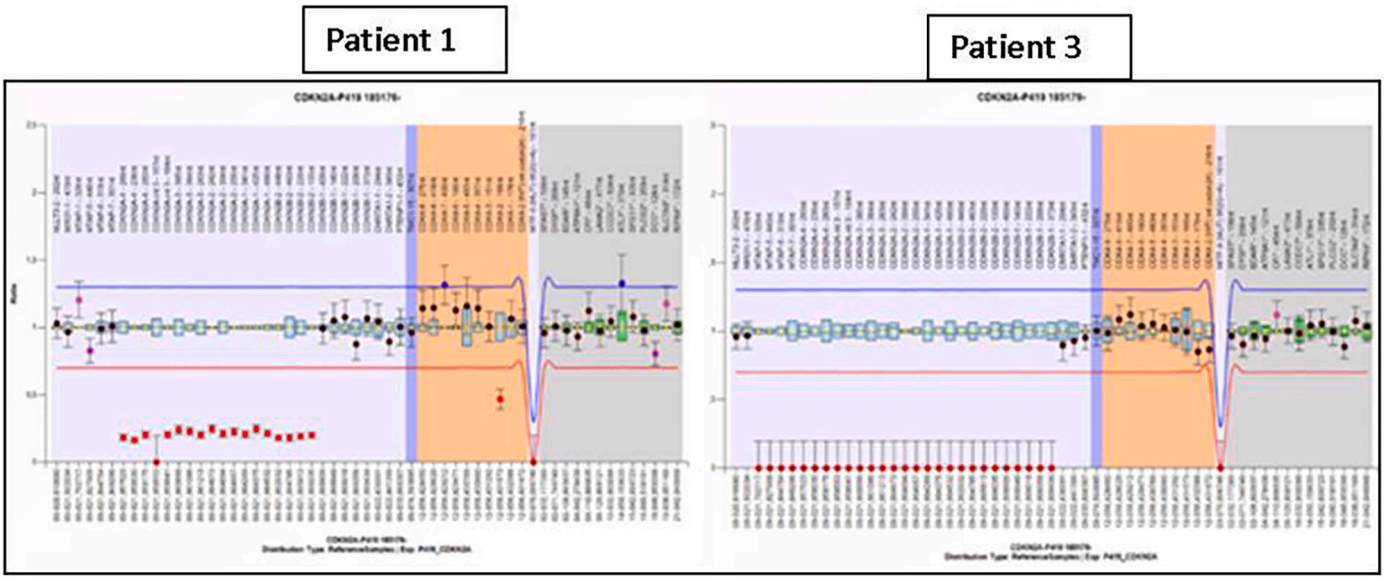

Fig. 3. MLPA analysis of patients 1 and 3. Red symbols indicate decrease of signal in the interrogated samples compared with controls in the target regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

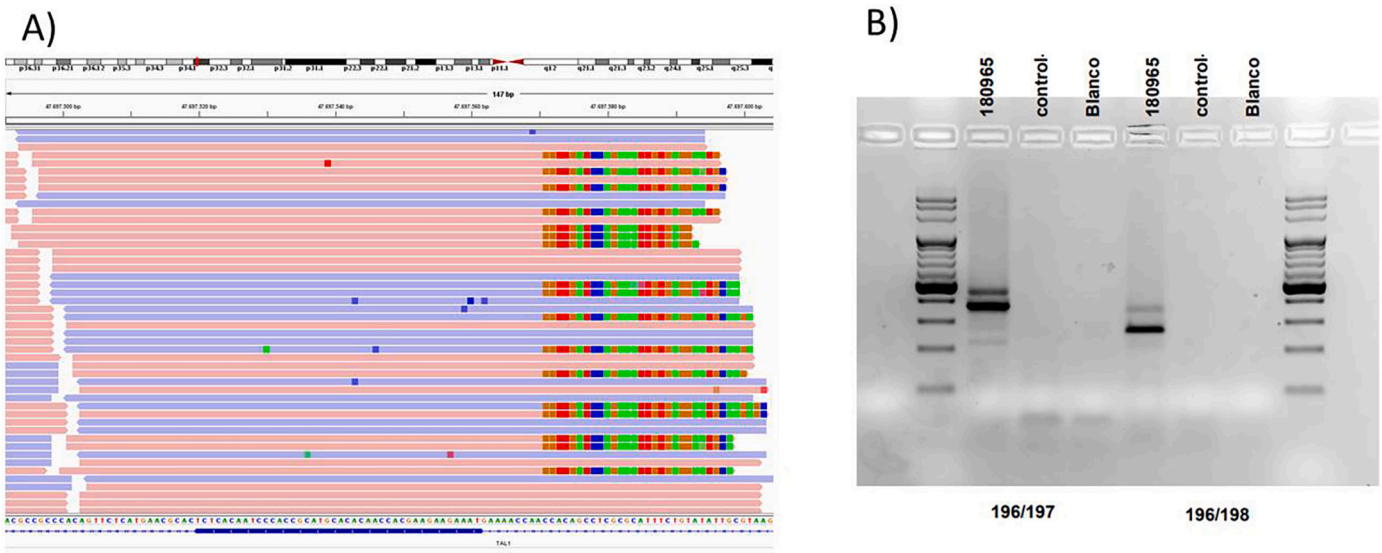

Fig. 4. A) IgIV alignments in region encompassing chr1:47697492–47,697,637 of TAL1. A breakpoint with ~50% of frequency of the total reads was observed. The blat of the misalignment sequence mapped with *STIL1* gene, suggesting the presence of a deletion in the chromosome 1p that leads to the fusion of *TAL1-STIL1*. B) PCR of the fragment involving STIL and TAL1 gene in the proband compared with the control without deleted sequence.

Genetics (ACMG) criteria, it should be classified as VUS [26]. However, this mutation is in exon 4, where most of the pathogenic mutations in T-ALL reported to date have been located [24]. Moreover, one mutation affecting a glycine residue (p.Gly596Ser) in the proximities of p. Gly581Ser has been previously reported in T-ALL [24]. Finally, the high clonality observed in this patient supports a pathogenic role. Ultimately, prediction of pathogenicity of these VUS-variants requires to perform functional assays.

Similarly to the change p.581Asp, the variant, p.Thr450Met, is also codified by exon 4 of *BCL11B* gene and located in the C2H2-type 3 domain. It has been previously reported in 3 patients with ALL [17–19] and the in silico predictors suggested a likely deleterious effect on the protein. Therefore, although this variant must be categorized as VUS, it should be further studied to confirm its pathogenicity.

The patient diagnosed with ETP-ALL harbored a missense mutation in *BCL11B* gene (p.His126Tyr). This particular variant has not been previously described in patients with T-ALL or ETP-ALL subtype, but the in silico prediction anticipated a likely tolerated effect. While *BCL11B* deregulation is the unifying event of a subgroup of ETP-ALL and lineage ambiguous leukemias, the leukemic transformation in these cases is driven by activation of the gene due to rearrangements or amplifications, not by loss-of-function mutations [27]. On the other hand, this patient showed different immunophenotypic features compared with case 1 and 3, suggesting other genetic background. In fact, no clonal TCR rearrangement was observed in this patient, which is expected considering the early stage of cell maturation characteristic of ETP-ALL. All in all, it may be concluded that *BCL11B* had not any pathogenic role in the leukemia development of this patient.

BCL11B is also a tumor suppressor that cooperates with a variety of genetic defects to promote leukemogenesis. In line with this, the 2 patients with *BCL11B* mutations harbored *CDKN2A/B* deletions and one a *SIL-TAL1* rearrangement. Although these abnormalities are known for

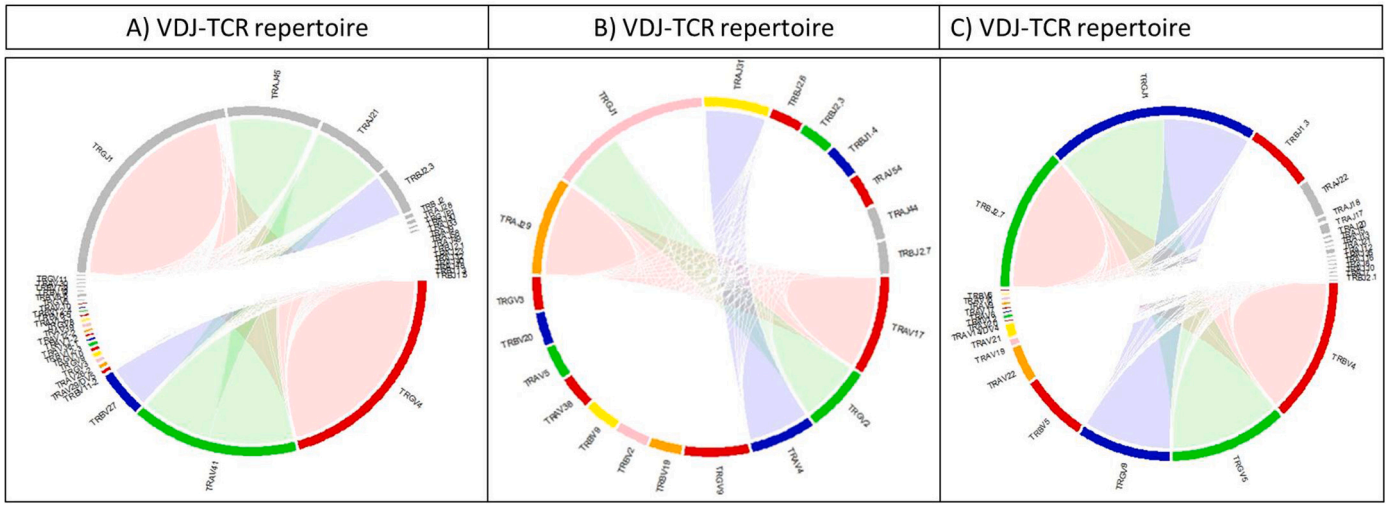

Fig. 5. Cord chart representation of clonal repertoire of V-D-J TCR. Upper panel. A) Patient 1: overrepresentation of TRGV4-TRGJ1, TRAV41-TRAJ45/TRAJ21 and TRBV27-TRBJ2–3. B) Patient 2: homogeneous clone distribution of the V-D-J TCR. C) Patient 3: overrepresentation of TRGV5-TRGJ1/TRGV9-TRGJ1, TRBV4–2-TRBJ2–7 and TRAV22-TRAJ22.

Table 3
Clone diversity of TCR according to Shannon index. Case 1 and 3 showed clonal expansion and non-diverse populations whereas case 2 did not.

Patient	ALL Phenotype	Sample origin	Average Coverage	No. Clones	No Different clones	Diversity (H Shannon)
Patient 1	T-ALL	BM	650	583	34	1,67
Patient one's mother	Healthy		409	57	44	3,66
Patient 2	ETP-ALL	PB	455	17	12	2,4
Patient 3	T-ALL	BM	434	524	25	1,81

T-ALL: acute Lymphoblastic Leukemia of T origin. ETP-AL: Early T precursor acute Leukemia. BM: Bone marrow; PB: peripheral blood.

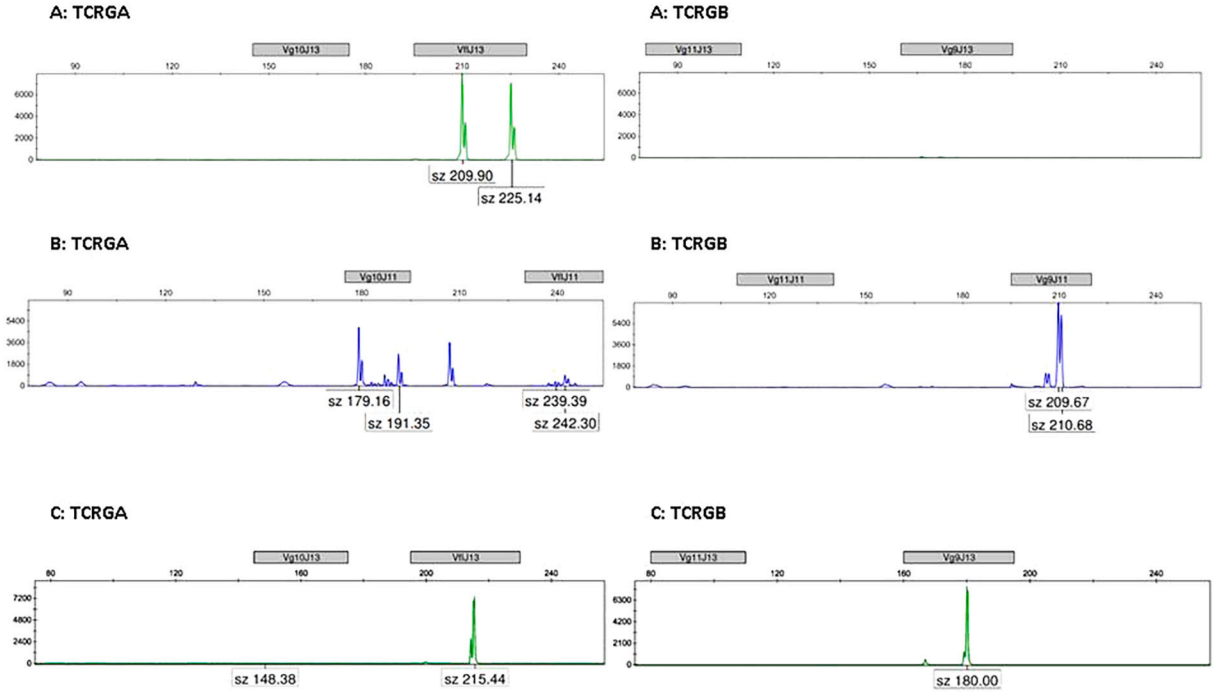

Fig. 6. Clonality analysis of TCRG repertoire by standardized fragment amplification. A) Patient 1: two clonal peaks (210 and 225 bp) are detected with VfI- J1.3/2.2 rearrangement. B) Patient 2: oligoclonal amplification is detected with a major peak (220 bp) with V9-J1.1/2.1 rearrangement. C) Patient 3: two clonal peaks, 215 bp with with VfI- J1.3/2.2 and 180pb with V9- J1.3/2.2 rearrangement are detected.

their pathogenetic role in T-ALL, their prognostic value is controversial [28–30]. However, some authors have pointed out the limited prognostic value of single genetic alterations instead of considering multiple genetic abnormalities [31].

Regarding the clonality assessment, we identified specific TCR rearrangements in 2 patients, providing a clonality marker that could be monitored later during the treatment of the disease, although this procedure is laborious and time-consuming [3].

Finally, *FLT3* duplication was the only genetic aberration detected in patient 2. The inability of our method to detect any other abnormality may be explained by the fact that ETP-ALL exhibits a high diversity of genetic alterations, among which mutations in signaling pathways are the most frequent. In fact, whereas *FLT3* mutations are extremely rare in *T-ALL*, their frequency in ETP-ALL raise up to 37% [32,33].

The main drawback of our work is the limited size of the library, which does not cover frequently deleted regions such are 5q, 6q or other genes such are *EED, SUZ12, SETD2, RELN*, among others, with stablished role in T-ALL and ETP-ALL. This reduced size of the library may explain the low number of CNVs detected in the patients. However, the main aim of the present work was not to develop a definitive panel but to show that it is possible to simplify the diagnostic workflow in a routine diagnostic laboratory. The current proposal does not replace standard techniques, but it does combine them, increasing the detection capacity and limiting costs. The confirmation of the NGS results with standard techniques support our approach. Future improvements in the design of library are warranted.

In line with above, the genetic information obtained from our panel in the ETP-ALL patient was very scarce. The diagnostic workflow in this entity begins with flow cytometry for lineage assignment. After this step, whether is more efficient to implement a large panel covering myeloid and lymphoid genes or to use separate panels for lymphoid and myeloid leukemia is a question not yet resolved.

5. Conclusions

In conclusion, we successfully applied an NGS-based method to analyze in one sample the genetic landscape of 3 patients with T and ETP-ALL and report 2 novel *BCL11B* gene mutations. The ability of studying SNV/indels, structural variants and Ig/ TCR simultaneously may simplify the genetic assessment of ALL in a routine clinical setting, but also increase the detection capacity and limit cost. Considering the importance of taking into consideration multiple genetic defects in the prognostic assessment of ALL, our approach may have practical implications in prognostication, treatment selection with targeted therapy and disease monitoring. Modifications in the library design may overcome the limitations of genetic diagnosis, especially in ETP-ALL.

Disclosure statement

This work was supported by Healthincode (Spain), Hospital Universitario Central de Asturias, Oviedo (Spain) and Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Oviedo, (Spain). J.M. G.A is employee at Healthincode. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval and consent to participate

This study was approved by the local ethics committee of Hospital Universitario central de Asturias, Oviedo, (Spain).

Data availability

Some data that have been used like code are partially confidential or they can be consulted in the supplementary materiak

Acknowledgments

We kindly thank the patients and their families for approving to be part of this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2022.109151.

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