A high definition picture of key genes and pathways mutated in pediatric follicular lymphoma

Mature B-cell lymphomas in childhood are a well-defined group of B-cell neoplasms, encompassing both aggressive and indolent tumors. Biologically, indolent pediatric B-cell lymphomas derive from either germinal center B cells [follicular lymphoma (FL)] or B cells outside the germinal center (marginal B-cell lymphoma).[1] Classic or adult-type FL is very rare in children and adolescents, whereas specific FL variants are more frequent than in adults and include pediatric-type nodal follicular lymphoma (PTNFL) and primary FL of the testis (PFLT).[1]

PTNFL has long been considered a localized variant of FL with high-grade morphology and a benign clinical course,[2] distinguished from typical adult FL by the absence of the t(14;18)(q32;q21) translocation and lack of BCL2 expression.[3] In the World Health Organization 2016 classification of lymphoid neoplasms, PTNFL was recognized as a definite clinico-pathological entity, not restricted to the pediatric age since it occurs, albeit rarely, also in adults.[1] Although the mutational landscape of adult FL has been extensively investigated,[4,5] only a few genetic alterations involved in the pathogenesis of PTNFL have been reported so far by three studies.[6-8] Among genes recurrently mutated in adult FL, only *TNFRSF14* and *IRF8* alterations were detected in PTNFL. Mutations in *IRF8* DNA binding domain[8] and mutations activating *MAP2K1*[6,9] have been proposed as potential drivers of the development of PTNFL. However, the biological mechanisms and signaling pathways involved in this malignancy remain to be fully elucidated and little is known about the molecular features of histological variants of FL in children.

In this study, the genetic landscape of a series of pediatric FL was investigated by whole exome sequencing (WES) analysis. Along with a few previously known mutations, new variants and mutated genes were identified and pathway-derived networks of functionally connected genes were outlined by leveraging system-based genetics approaches.

A total of 21 cases were collected, from December 2002 to May 2017, from *Associazione Italiana di Emato-Oncologia Pediatrica* (AIEOP) centers. All cases were originally diagnosed as PTNFL or PFLT and centrally reviewed by expert hematopathologists, according to the national AIEOP protocol for pediatric B-cell lymphomas (*Online Supplementary Table S1* and *Online Supplementary Figure S1*). Both primary tumor and paired peripheral blood samples of nine cases (7 PTNFL and 2 PFLT) were profiled by WES (Illumina HiSeq4000; 150 bp paired-end reads). The remaining 12 cases (11 PTNFL and 1 PFLT), for which peripheral blood was not available, were subjected to targeted screening of *TNFRSF14* exon 1 muta-

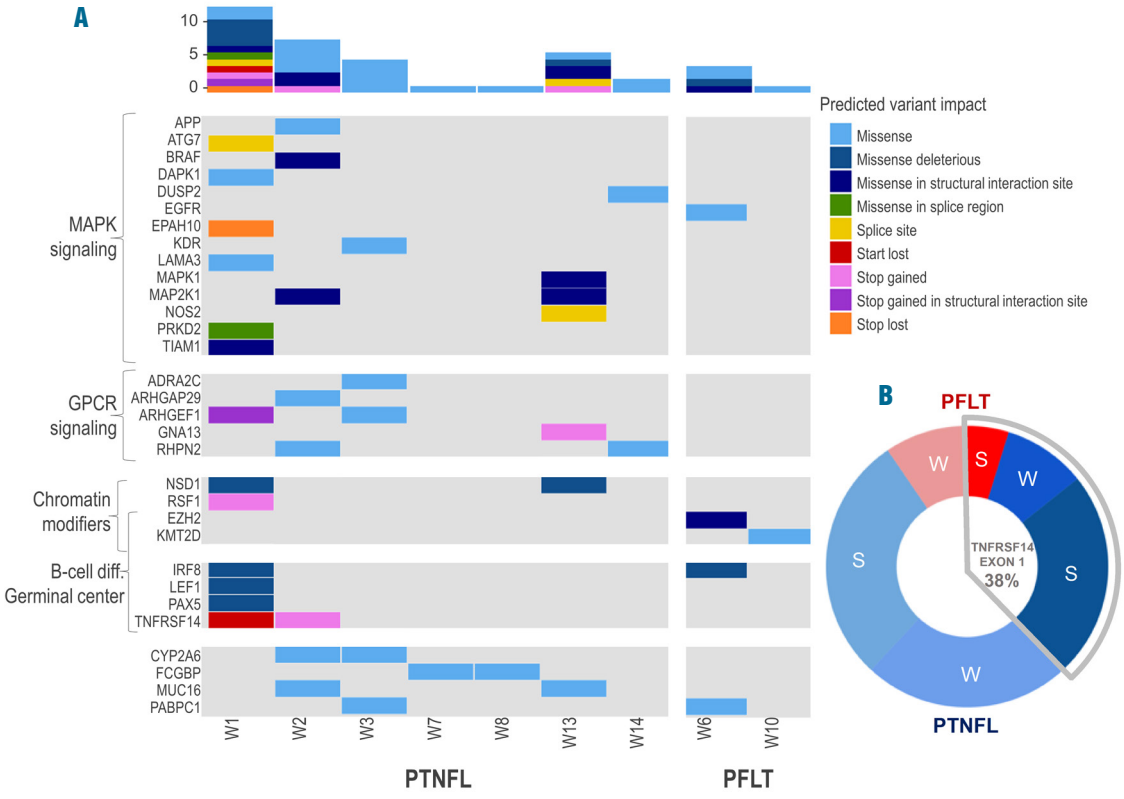

Figure 1. Main genes somatically mutated in childhood follicular lymphoma. (A) Mutation table representing patients grouped according to the primary site of the tumor and indicating the main genes carrying somatic mutations that were identified. The bar plot at the top shows the mutation load of each patient. Genes are clustered according to functional annotation. For each patient and gene, colors indicate the predicted impact of somatic variants according to transcript/protein annotation. Missence mutations were predicted as "deleterious" byMetaSVM and MetaLR; variants classified as "structural interaction site", according to SnpEff, have an impact on protein regions particularly important for folding. (B) Proportion of cases of childhood follicular lymphoma age profiled by whole exome sequencing (W) or screened by Sanger sequencing (S) that had mutations in *TNFRSF14* exon 1 (patients with nodal or testicular primary site disease are represented by different colors). PTNFL: pediatric-type nodal follicular lymphoma; PFLT: primary follicular lymphoma of the testis.

tions by Sanger sequencing.

A custom bioinformatics pipeline was used to call and prioritize somatic variants from WES data, using information from the following databases: dbSNP, COSMIC, Clinvar and ExAC (*Online Supplementary Figure S2*). After WES sequence read quality selection and alignment to the reference genome, average sequence coverage of 216x for tumor samples and 87x for paired peripheral blood samples was obtained (*Online Supplementary Figure S3*). Variant annotation, filtering and analysis identified 184 high confidence somatic variants in 169 genes, with "moderate" or "high" SnpEff predicted impact and a normalized allele frequency >0.1 (*Online Supplementary Figure S4 and Online Supplementary Table S2*).

Eleven "high" impact variants in eight genes, prioritized according to recurrence, functional criteria and/or previous reports in PTNFL or other lymphoid malignancies, were validated by Sanger sequencing (*Online Supplementary Table S3, Online Supplementary Figures S5* and *S6*): six variants in the recurrently mutated *ARHGEF1, MAP2K1* and *TNFRSF14* genes, and five in *ATG7, GNA13, RSF1, UBAP2*, and *ZNF608* (*Online Supplementary Figure S5*).

Ten genes were recurrently mutated in our cohort (Figure 1A), including *TNFRSF14, MAP2K1,* and *IRF8*, previously linked to PTNFL.[6-9]

In accordance with previous reports on *TNFRSF14* as a mutational hotspot in PTNFL,[6,7] two out of seven PTNFL cases profiled by WES carried *TNFRSF14* mutations. Screening by Sanger sequencing of *TNFRSF14* exon 1 in 12 additional patients disclosed mutations in six of them, including one PFLT case (Figure 1B and *Online Supplementary Table S1*). Overall 8/21 (38%) of the cases had *TNFRSF14* exon 1 mutations.

To depict the functional interactions between genes somatically mutated in FL of childhood, a mutation network derived from Reactome and KEGG pathways was constructed. Pathways including at least one mutated gene were converted into gene networks and then the non-redundant union of pathway-derived networks was obtained (see *Online Supplementary Methods* and Esposito *et al.*[10] for methodological details). This meta-network (Figure 2 and *Online Supplementary Figure S7*) showed direct interactions and functional relationships between 66 genes somatically mutated in pediatric FL, mostly carrying previously non-described variants. Six connected components of multiple mutated genes were identified in the net: a large group of 51 genes ("main component"), a group of six genes, and four smaller groups. Notably, the net included nine genes recurrently mutated in the cohort. Almost half of the somatic mutations observed in the main component targeted genes belonging to two highly interlaced signaling pathways: "negative regulation of MAPK" and "G-protein coupled receptor" (GPCR) (Figures 1A and 2). *MAP2K1* carried known "high" impact missense variants (p.Lys57Gly in patient W2, previously reported in PTNFL;[6,7] p.Val60Gly in patient W13), hitting residues in protein-protein interaction domains, and *BRAF* showed the p.Lys601Asn mutation (patient W2), previously reported as oncogenic.[11] New variants in 12 additional genes of the mitogen-activated protein kinase (MAPK) pathway were uncovered and, overall, six of nine patients carried one or more mutations in genes directly involved in MAPK signaling (Figures 1A and 2). Specific mutations are particularly worthy of note: *MAPK1* p.Tyr131His falls in the catalytic domain, and ATG7 truncation due to a stop gain at amino acid 228 eliminates the C-terminal region of the protein, essential for dimerization and key molecular interactions. DUSP2 p.Ser249Asn falls in the tyrosine-protein phosphatase domain and the detected *PRKD2* mutation is predicted to both produce p.Phe81Ile substitution and impair the

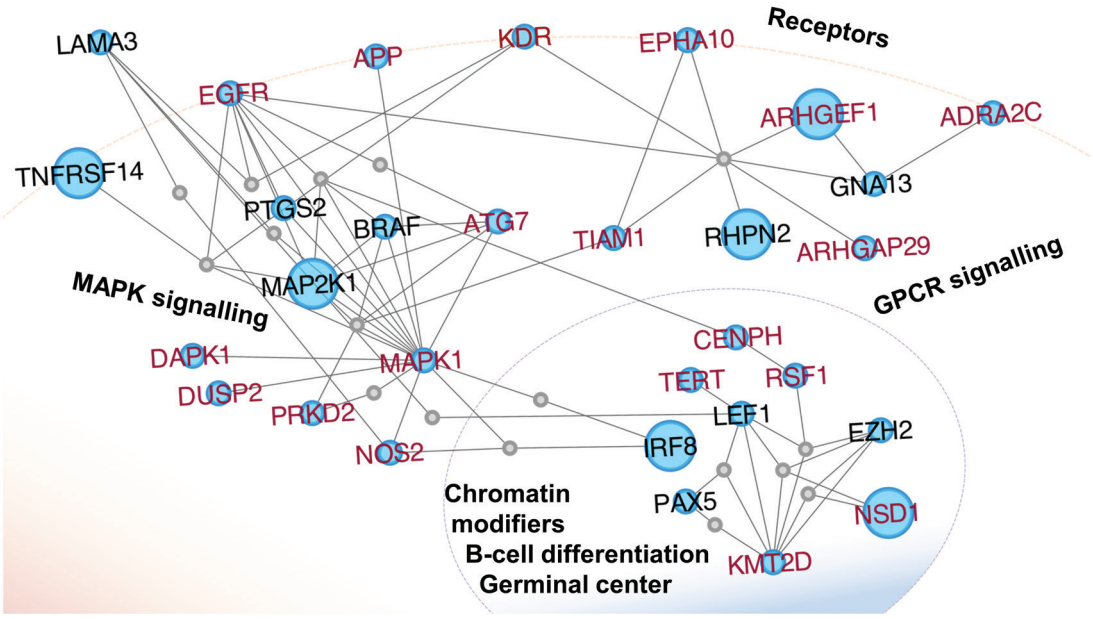

Figure 2. Main pathways hit by somatic mutations in childhood follicular lymphoma. The genes somatically mutated in the cohort are shown as an annotated Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome-derived meta-network. Several mutated genes are receptors, most genes are involved in mitogen-activated protein kinase (MAPK) or in G-protein coupled receptor (GPCR) signaling; chromatin-modifying enzymes and/or genes involved in B-cell differentiation and germinal center biology were also identified (see text and *Online Supplementary Figure* S7 for additional details). Node size is proportional to gene mutation recurrence in the cohort analyzed by whole exome sequencing; direct functional links between mutated genes are depicted, and small gray nodes indicate pairs of mutated genes connected through a single non-mutated gene in the network; red node labels indicate the genes for which new mutations were identified.

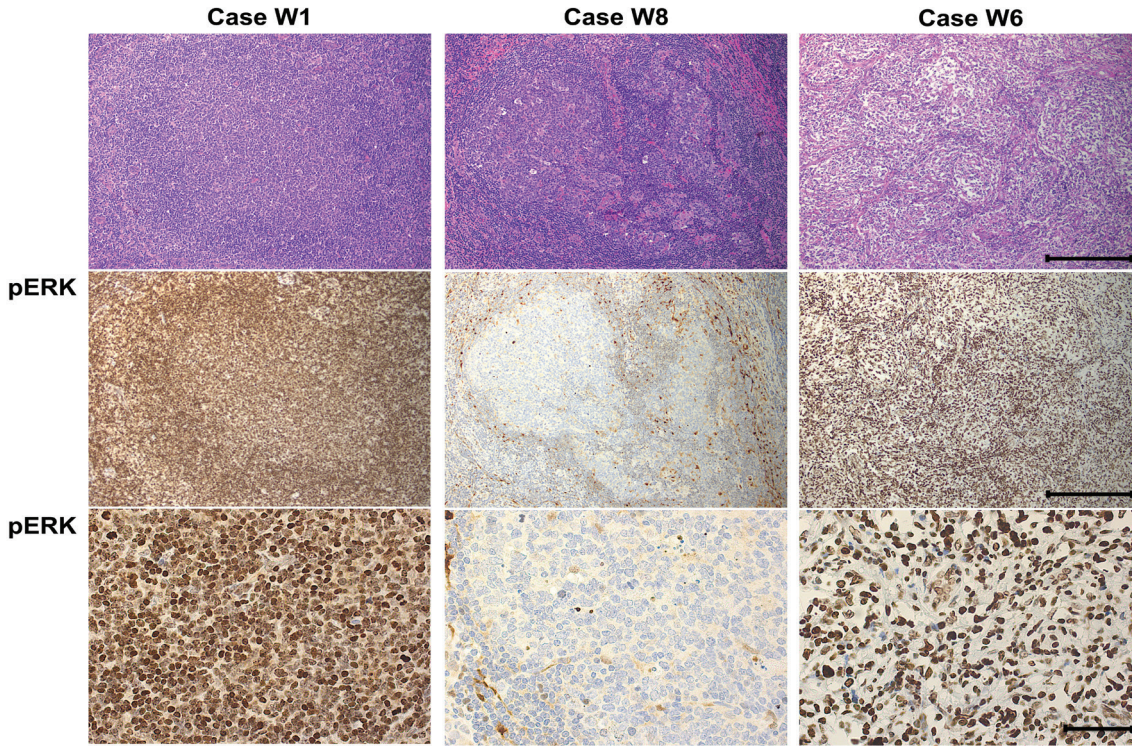

Figure 3. Immunohistochemical analysis of pERK in patients with mutated or non-mutated genes of the "negative regulation of MAPK" signaling pathway. In line with whole exome sequencing data and network analysis, patient W1 with pediatric-type nodal follicular lymphoma and patient W6 with primary follicular lymphoma of the testis, both carrying mutations in genes of the "negative regulation of MAPK" pathway, showed strong and diffuse immunohistochemical positivity for phosphorylated estracellular-regulated kinase (pERK). Case W8 without MAPK-related mutations was consistently pERK-negative (hematoxylin & eosin and peroxidase stains; original magnification, 10x and 40x). Bars in the first and second rows represent 250 μM, that in the third row represents 50 μM.

main transcript splicing, likely leading to a loss of function. To confirm the functional relevance of MAPK pathway mutations, the presence of pERK was immunohistochemically assessed in three available cases, negative for MAP2K1 lesions. In keeping with the molecular data, two cases with MAPK alterations, other than those previously reported in MAP2K1,[9] were strongly positive for pERK, while the MAPK unmutated case was consistently pERK-negative (Figure 3).

The "GPCR" signaling pathway showed recurrent mutations in five cases of PTNFL and was preserved in the examined PFLT cases (Figure 1A). A previously unreported ARHGEF1 p.Trp746* nonsense mutation was detected in patient W1 and the known ARHGEF1 pathogenic variant p.Arg566His (COSM6206921) was found in patient W3. Notably, *GNA13*, encoding Gα13, a direct interactor of ARHGEF1 (Figure 2), carried a truncating mutation (p.Glu311*) in patient W13, eliminating the C-terminal portion on the G-protein alpha domain. Two GNA13 missense mutations, both affecting Gly60 (p.Gly60Ser and p.Gly60Asp) in the nucleotide phosphate-binding region of the protein, had been found previously in two cases of PTNFL.[7] Moreover, disruption of the Gα13-dependent pathway by loss-of-function mutations has already been reported in more aggressive lymphomas, such as diffuse large B-cell and Burkitt lymphomas.[12]

In addition, the "chromatin modifying enzymes" pathway was hit in both PTNFL and PFLT: *RSF1* and *NSD1* genes, encoding for histone chaperone and methyltransferase, respectively, were mutated in PTNFL cases, while mutations in *EZH2* and *KMT2D* genes were found in PFLT (Figure 1A). The EZH2 p.Tyr646Phe mutation detected in our cohort has been found frequently in both FL and PTNFL of adult patients,[6] whereas to the best of our knowledge *NSD1* and *RSF1* variants have not been reported previously. Although one study described *KMT2D* as the most frequently mutated histone-modifying enzyme in PTNFL[7] and *KMT2D* disruption has been proven to alter germinal center B-cell development and promote lymphomagenesis,[13,14] mutations in epigenetic modifiers have so far been considered nearly exclusive to FL in adults.[6] Our data confirm that epigenetic mechanisms, hit in two of seven of the analyzed cases, can contribute to the pathogenesis of PTNFL. Interestingly, both the PFLT cases profiled by WES (patients W6 and W10) carried mutations of epigenetic modifiers. Further investigation in larger cohorts is needed to clarify the role of alterations in epigenetic modifiers in PFLT.

Besides *KMT2D* and *EZH2*, the above-mentioned *TNFRSF14* and *IRF8* genes are also connected with germinal center B-cell development (Figure 2). The *IRF8* mutations detected were p.Lys66Arg in patient W1 (PTNFL) and p.Tyr23His in patient W6 (PFLT). Moreover, LEF1 (Lymphoid enhancer binding factor 1), a transcription factor regulating B-cell development, carried a p.Ala150Val variant, reported in COSMIC as pathogenic. *PAX5* is also part of the transcriptional network under *IRF8* control. The PAX5 p.Gly183Ala mutation discovered in our cohort affects the same residue of the p.Gly183Ser variant previously shown to have an impact on B-lymphoid development by reducing the transcrip-

tion factor activity.[15] Overall, mutations in genes directly linked to B-cell differentiation and/or the germinal center reaction occurred in three patients in our cohort, two of whom also carried hits in the "negative regulation of MAPK" and "GPCR" pathways.

In conclusion, beyond confirming a few mutations previously reported in PTNFL, we identified several novel variants in genes involved in the MAPK pathway and provide evidence on the involvement of GPCR downstream signaling in the pathogenesis of PTNFL. We also detected mutations in genes encoding chromatin-modifying enzymes, some of which affected genes not previously associated with childhood FL. Our network analysis considerably extended previous data on the mutational landscape of FL of pediatric age, further indicating the signaling pathways of possible pathogenic relevance in these malignancies.

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Funding: the authors would like to thank the Fondazione Città della Speranza, Padova, Italy; Fondazione CA.RI.PA.RO, Padova, Italy (grant 17/03 to LM) and Camera di Commercio Venezia, Venezia, Italy. The study was also partially supported by AIRC (Investigator Grant – IG 2017 #20052 to SBo) and by the Department of Molecular Medicine of the University of Padova, Italy (PRID 2017 to SBo). AB is a PhD student of the Graduate School in Biosciences of the University of Padova, Italy.

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doi:10.3324/haematol.2018.211631

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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