FINAL REPORT

Liquid biopsy based identification and monitoring of novel therapeutic targets in B-cell lymphomas (KH17- 126718 project, 2017.12.01-2020.05.31.)

Summary

Genomic and transcriptomic analyses were performed in various B-cell lymphomas (follicular lymphoma: FL, chronic lymphocytic leukemia: CLL and primary central nervous system lymphoma (PCNSL) using cutting edge molecular technologies. Besides the conventional tissue samples, the clinical value of the liquid biopsies (cell-free circulating tumour DNA) in sensitive disease monitoring was demonstrated in B-cell lymphomas. We developed an assay for sensitive detection and monitoring of EZH2 mutations in FL capable of sensitive therapy response monitoring. A multigene panel was developed for detection of genomic and transcriptomic aberrations of PCNSL patients. For CLL patients treated with innovative drugs (ibrutinib or venetoclax), we developed ctDNA compatible molecular assays for sensitive detection and monitoring of genetic alterations in BTK, PLCG2 and BCL2 genes, capable of predicting therapy resistance before the overt clinical relapse. Finally, next-generation sequencing (NGS), droplet digital PCR (ddPCR) and Nanostring technologies became available for analysis of other oncohematological entities via the significant technology development accomplished during the project. The project led to development and introduction of routinely applicable, tissue and liquid biopsy compatible molecular diagnostic tests utilizing cutting edge molecular technologies and thereby capable of sensitive therapy response assessment as well as early prediction of therapy resistance and relapse in various B-cell lymphomas.

Major scientific achievements

We published a proof of principle paper in Haematologica (*Kiss et al. Haematologica 2019, D1 journal*) demonstrating spatial and temporal subclonal heterogeneity for the first time in a CLL patient treated with ibrutinib, and we demonstrated applicability of the liquid biopsy specimens in sensitive detection and spatial representation of mutations associated with ibrutinib resistance (Figure 1).

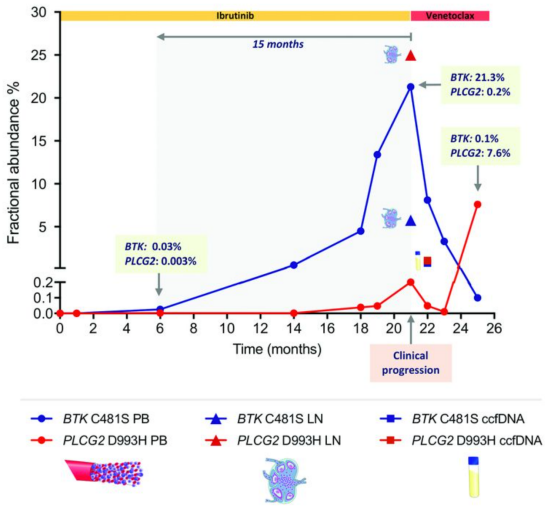

Figure 1: Spatiotemporal dynamics of *BTK* and *PLCG2* mutations (*Kiss et al. 2019*).

We performed an ultra-deep NGS analysis of all disease-relevant mutation targets in a cohort of 20 CLL patients treated with ibrutinib. The study of paired specimens provided novel insights into subclonal evolution and resistance mechanism emerging during ibrutinib therapy (Figure 2). In addition to the canonical

variants, we identified novel BTK and PLCG2 mutations associated with therapy resistance. Using ddPCR, we demonstrated that emergence of BTK resistance mutations predicted clinical relapse on average by 10.5 months (*Gángó et al. International Journal of Cancer 2020, Q1 journal*).

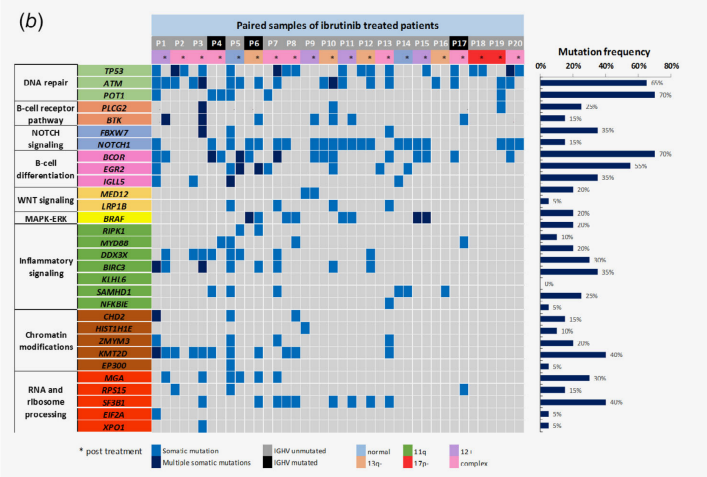

Figure 2: Heat ma displaying somatic variants detected in sequential samples of 20 patients treated with ibrutinib (*Gángó et al. 2020*).

Detailed investigation and sensitive, time-resolved monitoring of the BTK C481 mutations was performed in 83 relapsed/refractory CLL patients during single-agent ibrutinib treatment. With a median follow-up time of 40 months, BTK^{C481S} was detected in 48% (40/83) of the patients, with 80% (32/40) of them showing disease progression during the examined period. Emergence of the BTK^{C481S} mutation preceded the symptoms of clinical relapse with a median of nine months. Subsequent Bcl-2 inhibition therapy applied in 28/32 patients harbouring BTK^{C481S} and progressing on ibrutinib conferred clinical and molecular remission across the patients (Figure 3). This study demonstrated the clinical value of sensitive BTK^{C481S} monitoring with the largest longitudinally analysed real-world patient cohort reported to date and validated the feasibility of an early prediction of relapse in the majority of ibrutinib-treated relapsed/refractory CLL patients experiencing disease progression (*Bödör et al. British Journal of Haematology 2021, Q1 journal*).

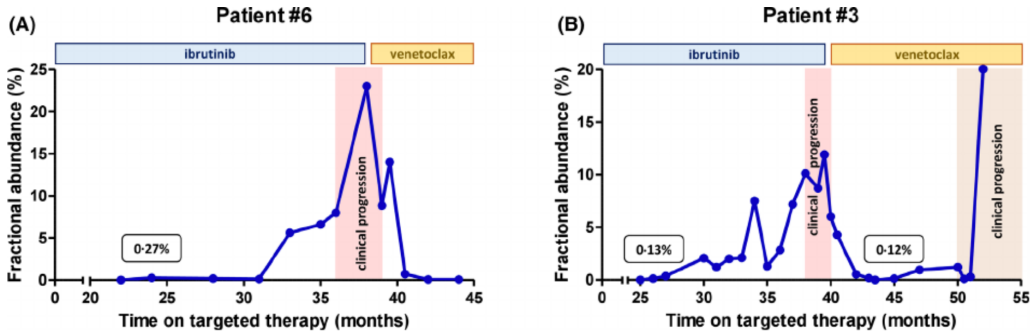

Figure 3: Temporal dynamics of the BTK C481S mutation in two patients with relapsed/treatment refractory (CLL) receiving ibrutinib and subsequent BCL2 inhibitor venetoclax therapies (*Bödör et al. 2021*).

Efforts to characterize molecular alterations and patterns of clonal evolution in patients treated with novel therapies including ibrutinib and venetoclax were also expanded into the field of Richter syndrome (RS) representing transformation of CLL into high grade B-cell lymphoma. A detailed immunophenotypic analysis of formalin-fixed, paraffin-embedded tissue specimens of RS phase was performed, followed by extensive molecular characterisation of CLL and RS samples, including the immunoglobulin heavy chain gene (IGH)

rearrangement, TP53 mutations, drug-induced resistance mutations in BTK and BCL2 genes and various copy number changes and point mutations detectable with multiplex ligation-dependent probe amplification (MLPA). Our study contributed to better understanding of RS pathogenesis in the era of targeted oral therapies. Rare phenotypic variants of RS occured under the treatment of ibrutinib or venetoclax, and genetic factors leading to RS were to those identified in patients treated with chemoimmunotherapy. We also reported the first *BCL2* G101V mutation in an RS patient treated with venetoclax (*Gángó et al. Pathology 2021, Q1 journal*).

In an international collaborative effort with Christoph Bock's group at CEMM in Vienna, we analyzed high-resolution time courses of ibrutinib treatment in patients with CLL, combining immune-phenotyping, single-cell transcriptome profiling, and chromatin mapping. We identified a consistent regulatory program starting with a sharp decrease of NF-κB binding in CLL cells, which is followed by reduced activity of lineage-defining transcription factors, erosion of CLL cell identity, and acquisition of a quiescence-like gene signature. This study also and it established a broadly applicable method for epigenome/transcriptome-based treatment monitoring (*Rendeiro et al. Nature Communications 2020, D1 journal*).

In follicular lymphoma (FL), we established a large nation-wide collection of plasma derived ctDNA samples (679 samples from 268 FL patients today). In our proof of principle paper, we demonstrated that circulating tumor DNA based *EZH2* mutation analysis performed by a highly sensitive droplet digital PCR method may be a valuable treatment monitoring approach in *EZH2* mutant follicular lymphoma. *EZH2* variant allele frequencies changed in parallel with the volume of metabolically active tumor sites observed on 18F-fluorodeoxyglucose positron emission tomography combined with computer tomography (PET-CT) scans (*Nagy et al. Genes 2020, Q2 journal*). Variant allele frequencies of *EZH2* mutations decreased or were eliminated rapidly upon successful treatment, with treatment failure being associated with elevated *EZH2* variant allele frequencies. We also demonstrated spatial heterogeneity in a patient with two different *EZH2* mutations in distinct anatomical sites, with both mutations simultaneously detected in the liquid biopsy specimen (Figure 4). This approach has been integrated in the molecular diagnostic algorithm of the home institution and is being currently tested on a large cohort of FL patients.

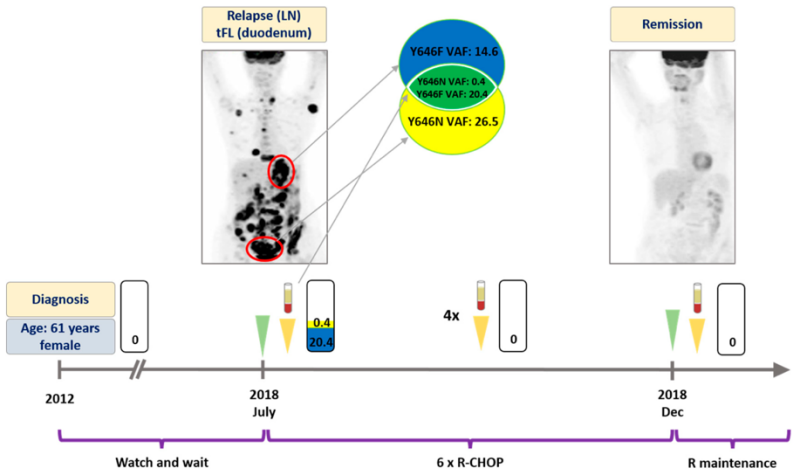

Figure 4: Detailed illustration of treatment monitoring in Patient#1. Spatial heterogeneity was captured in pretreatment plasma and rapid elimination of EZH2 Y646N and Y646F mutations was observed upon successful R-CHOP treatment. Since then the patient has been receiving rituximab maintenance therapy with good general health conditions. Numbers in the rectangles represent the VAF of the mutations, yellow color and blue colors indicate Y646N and Y646F variants, respectively. Green arrows indicate the time when the PET scans were performed, yellow arrows indicate the time when liquid biopsy specimens were collected. (*Nagy et al. 2020*).

In addition to the nodal FL cases, we defined the mutation patterns of TNFRS14 and EZH2 in cutaneous FL, a rare form of the disease (*Gango et al, Wirchows Archive 2018, Q1 journal*).

A multigenic NGS-based panel was developed for profiling of genomic alterations of patients with primary or secondary central nervous system lymphoma (PCNSL). As part of an international collaborative effort lead by our group, the molecular subtype of 77 PCNSL and 17 secondary CNS lymphoma patients was determined using the NanoString Lymphoma Subtyping Test (LST), a gene expression-based assay representing a more accurate technique of subtyping compared with standard immunohistochemical (IHC) algorithms. In addition, mutational landscapes of 14 target genes were determined using ultra-deep next-generation sequencing. Using the LST-assay, a significantly lower proportion (80% vs 95%) of PCNSL cases displayed ABC phenotype compared with the IHC-based characterization. The most frequently mutated genes included MYD88, PIM1, and KMT2D (Figure 5). In this study, we successfully applied the LST-assay for molecular classification of PCNSL, reporting higher proportion of cases with GC phenotype compared with IHC analyses, leading to a more precise patient stratification potentially applicable in the diagnostic algorithm of PCNSL (*Bödör et al. Journal of Neuropathology and Experimental Neurology 2020, Q1 journal*).

Figure 5: The NanoString LST readouts are illustrated in form of a gene expression heat map with the 15 target genes contributing to the model (*Bödör et al. 2020*).

Technologies established during the study period were used in different oncohematological entities as well and lead to publications in the field of paediatric acute lymphoblastic leukemia (*Kiss et al. Modern Pathology 2020, D1 journal*) and myeloid malignancies (*Bátai et al. Pediatric Blood & Cancer 2020, Q1 journal).*

Dissemination of the results

In total, we published **17 scientific papers** during the study period. **Three** papers were published in international **D1** journals (*Kiss et al. Haematologica 2019, Kiss et al. Modern Pathology 2020, Rendeiro et al. Nature Communication 2020*), **six** papers in international **Q1** journals (*Gángó et al. Pathology 2021, Bödör et al. British Journal of Haematology 2021, Gángó et al, International Journal of Cancer 2020, Bödör et al. Journal Of Neuropathology And Experimental Neurology 2020, Bátai et al. Pediatric Blood & Cancer 2021, Gángó et al. Wirchows Archive 2018)* and **one** research was published in international **Q2** journal (*Nagy et al. Genes 2020*). Altogether **7 papers** were published in the **Hungarian peer reviewed journals**. One original article (*Kotmayer et al. 2018*) and 6 review papers (*Illyés et al. 2021, Nagy et al. 2020, Kotmayer et al. 2020, Aczel et al. 2019, Fésüs et al. 2019, Bátai et al. 2018*) were published in *Hematology-Transfusiology*, the official journal of the Hungarian Society of Hematology and Transfusion with one review paper published in *Hungarian Oncology* (*Kotmayer et al. 2020*).

In addition to the scientific publications, we regularly presented our data at national in international scientific meetings including the annual conference of the Hungarian Society of Hematology and Transfusion and the annual meeting of the American Society of Hematology and European Hematology Association as well as various events of the European School of Hematology.

Our group also paid particular attention to outreach activities and communication of the scientific activities to the wider communities and patients advocacy groups. These activities included presentations at the Researchers' Night events in 2020, 2019 and 2018. Scientific workshops for patients with B-cell lymphomas were organised in collaboration with the Foundation of Hungarian Oncohematological Patients in 2018 and 2019.

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**All papers listed above have first and/or last authorship by the PI of this project or the members of this research group.*