FINAL REPORT

INVESTIGATION OF THE GENETIC BACKGROUND OF FAMILIAL MYELODYSPLASTIC SYNDROMES AND ACUTE MYELOID LEUKAEMIAS (OTKA-PD-108805)

Duration of the project: 2013/09-2015/09 (24 months)

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Content

1. Scientific background and main goals of the project

While the majority of myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) cases are sporadic, the rare familial cases provide a useful model system for the investigation of the molecular mechanisms of leukaemogenesis (Owen et al. 2008a). Germline mutations of 3 haematopoietic transcription factors: *CEBPA*, *RUNX1* and *GATA2* have been identified as causative or predisposing lesions to MDS/AML in these families (Song et al. 1999; Smith et al. 2004; Hahn et al. 2011). The explosion in the next generation sequencing technologies (NGS) and the heightened awareness by clinicians led to detection of an increased number of familial MDS and AML cases, suggesting that inherited susceptibility to these malignancies is likely to be more common than previously appreciated (Churpek et al. 2013). In addition, novel predisposing genes were also identified in the last few years including *SRP72*, *DDX41* and *ETV6* (reviewed by West et al. 2014).

However, despite the progress made so far, the genetic background of many familial cases remains obscure, suggesting that there might be other yet undiscovered genes predisposing to MDS/AML. These cancers may present in childhood or adulthood with a wide range in age of presentation (2-76 years) and variable penetrance with some carriers remaining asymptomatic until late adulthood (Liew and Owen 2011; Holme et al. 2012). The subtle initial clinical presentation or indeed the complete lack of antecedent haematologic abnormalities (warning features) before the development of the disease may mask the diagnosis. Identification of familial cases has significant clinical implications in management of younger patients, in particular for donor selection for allogeneic haematopoietic stem cell transplantation (HSCT) to prevent use of an unsuspected carrier sibling as donor.

In 2012, we identified the first Hungarian family with MDS/AML with 4 affected individuals (Figure 1A), which served as the basis for the present research project application. As part of our preliminary analyses we identified the predisposing/causative mutation in this family with 5 individuals harbouring the same inherited *RUNX1* p.R201* (c.601C>T) mutation (Figure 1B). Individuals II-1 and II-2 (identical twins) presented with MDS progressing to AML in 2002 within 2 months of each other at age 4 (with distinct karyotypes) and died within 1 year from the diagnosis. II-3 was diagnosed with MDS in 2010 at age 6. Her sister, II-4 was considered as donor for stem cell transplantation. However, she was also noted to carry the same *RUNX1* mutation, thus II-3 underwent an unrelated HSCT in 2012. Their mother, I-1 also harbours this mutation, although she is asymptomatic to date despite being 42 (Figure 1C).

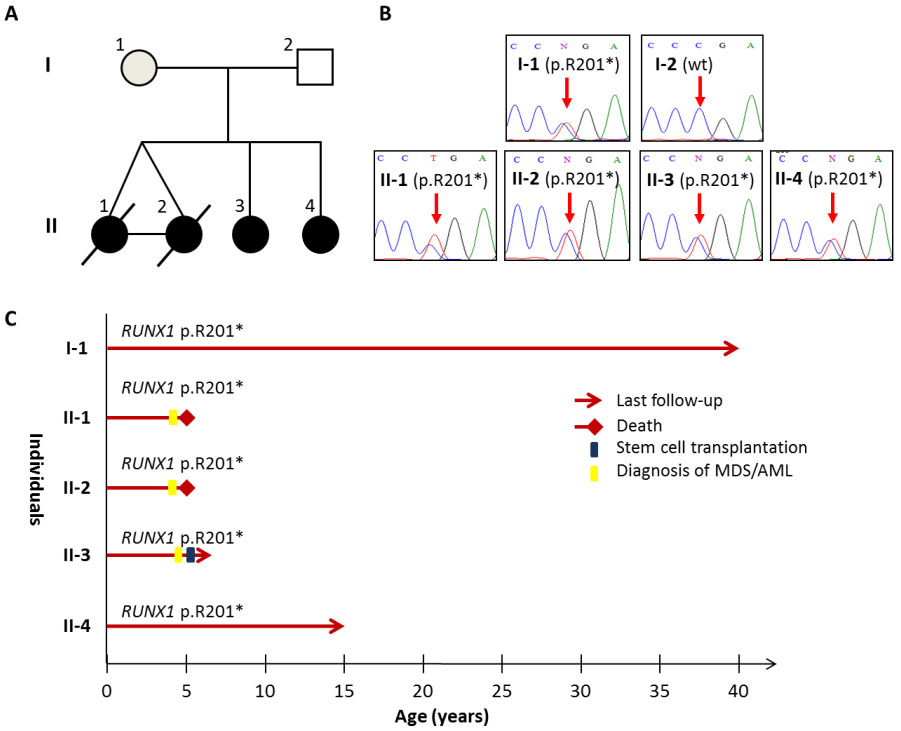

Figure 1. (A) Genogram for the *RUNX1*-mutated pedigree. The squares denote males and circles denote females. The black boxes indicate individuals with MDS/AML. The *RUNX1* p.R201* (c.601C>T) mutation was detected in I-1, II-1, II-2, II-3 and II-4. The germline origin of the mutation was confirmed from salivary DNA of II-3 and II-4. The grey boxes indicate individuals with *RUNX1* mutation without myeloid malignancy (I-1, II-4). I-2 showed wild type *RUNX1*. **(B)** Illustrated is the heterozygous non-sense *RUNX1* mutation leading to generation of a stop codon in individuals: I-1, II-1, II-2, II-3 and II-4. I-2 demonstrates a wild-type *RUNX1* sequence. **(C)** Schematic summary of the clinical history of the 4 individuals with *RUNX1* mutation. * Sister II-4 was also diagnosed with MDS in 2014 at age of 14 (as described later).

We hypothesized that the nature of secondary genetic events may explain the clinical heterogeneity observed within and between the pedigrees, such as our *RUNX1* family (*Figure 1*), as also suggested in our recently published family with concurrent *GATA2* and *ASXL1* mutations with the secondary *ASXL1* mutations representing an important trigger for the development of overt disease (Bodor et al. 2012). Delineating the molecular pathogenesis of these pedigrees results in better understanding of the genetics of familial leukaemias, but also provides important information regarding further management of the carriers within these families and indeed may lead to identification of novel therapeutic targets.

Based on these, the main aims of our project proposal were as follows:

- To perform whole exome sequencing (WES) in the 5 members of our “model” pedigree with *RUNX1* mutations (*Figure 1*) to unravel the dynamics of secondary genetic changes responsible for the observed clinical heterogeneity.
- To establish a nation-wide collection of familial MDS/AML cases in collaboration with the other onco-haematology centres in Hungary
- To develop standardised diagnostic assays for the currently known culprit genes predisposing to MDS/AML for routine testing where familial involvement is suspected.

2. Results achieved during the 24 months research period

2.1. Whole exome sequencing of the *RUNX1* mutated model pedigree

The main goal of the present research project was to perform whole exome sequencing (WES) on our *RUNX1*-mutated pedigree with a view to identify the cooperating and secondary genetic events and to dissect the clonal architecture and chronology of genetic events in this family. To do so, we performed whole exome sequencing from bone marrow samples of the 4 sisters. Peripheral blood samples from both parents were used as germline controls. The library preparation was performed using the SureSelect method with the next generation sequencing performed on a HiSeq instrument. Variant calling was performed using the standard algorithms and all relevant variants detected by whole exome sequencing were confirmed by bidirectional Sanger sequencing.

Intriguingly, WES revealed molecular addiction to the JAK-STAT signalling in 3 out of 4 siblings (Figure 2). The canonical V617F mutation in the *JAK2* gene was detected in one of the twins and the youngest sister. Although the other twin was wild type for *JAK2*, she harboured a loss of function mutation in *SH2B3* also known as LNK a negative regulator of the JAK-STAT signalling. Intriguingly, WES also identified acquired uniparental disomy (aUPD) of regions of 9p and 12q leading to homozigosity of the *JAK2* and *SH2B3* mutations, respectively. Collectively, lesions in the JAK-STAT pathway were detected in all 3 patients with an aggressive phenotype.

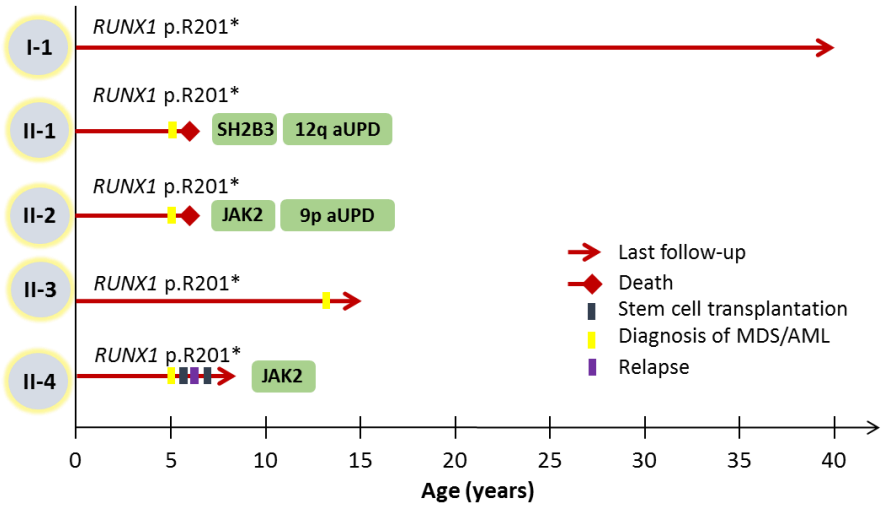

Figure 2. Lesions in the JAK-STAT pathway (*JAK2* and *SH2B3* mutations with corresponding acquired uniparental disomy of regions of 9p and 12q) were detected in all 3 patients with an aggressive phenotype.

In terms of the copy number alterations (CNA), gain of 21p (leading to apparent homozigosity of RUNX1) was detected in twin1 with monosomy 7 and loss of 9q detected in the other twin. The other 2 patients had a normal karyotype. Further, for the first time, mutations in the cell cycle checkpoint regulator *CDC27* were identified in 2 patients. The youngest sister carried a mutation in the *U2AF2* gene a member of the splicing machinery commonly mutated in MDS patients (Figure 3).

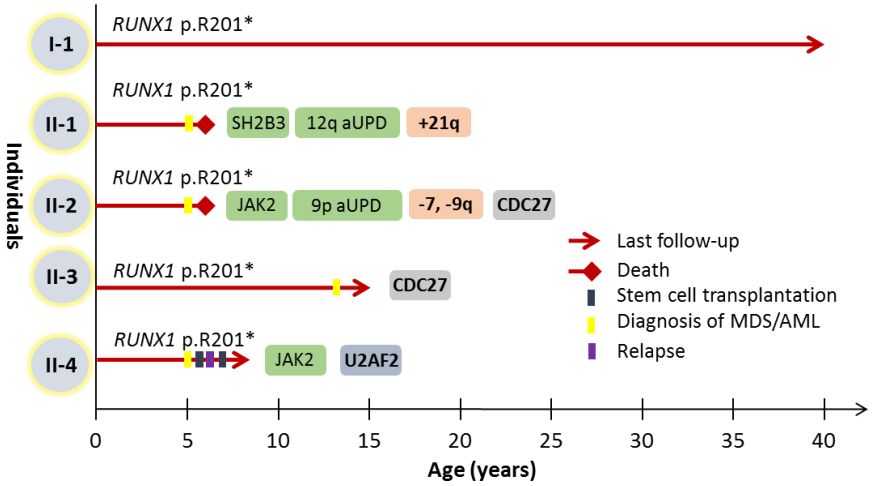

Figure 3. Cooperating somatic mutations and copy number alterations identified in the *Runx1*-mutated pedigree.

The detection of different CNAs, regions of aUPDs provided us with the opportunity to estimate cancer cell fractions and decipher the possible sequence of the genetic alterations as well was to differentiate between clonal and subclonal variants. Based on these, the proposed order of genetic events detected in the twins is summarised in this figure with the germline *RUNX1* mutations representing the predisposing event. Mutations of *JAK2* and *SH2B3* appeared to be clonal followed by UPD of the respective regions leading to homozygous mutations. In the case of patient II.2 monosomy represents a dominant clonal event while the other CNAs and *CDC27* appear as subclonal cooperating events (Figure 4).

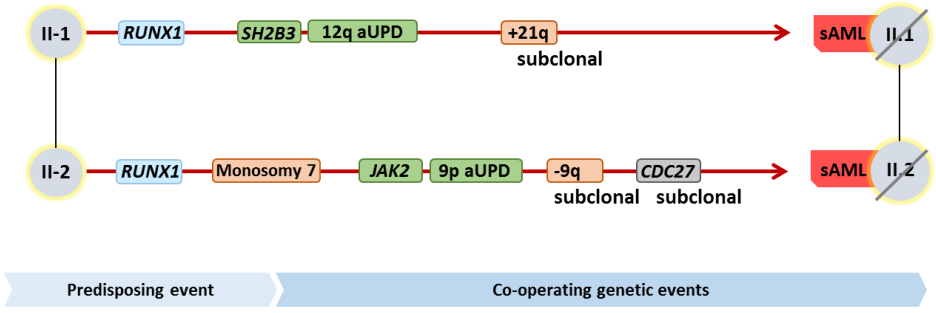

Figure 4. The proposed chronological order of the various genetic alterations in two members (twins) of the *RUNX1*-mutated pedigree.

To test whether these cooperating (*JAK2*, *SH2B3* and *CDC27*) variants identified in our pedigree are present in sporadic AML as well, we performed mutation analysis on a cohort of 110 sporadic AML patients, however only 2 patients with *JAK2* mutations were identified and all cases were wild-type for *SH2B3* and *CDC27*.

For patient II-4, who relapsed 15 months after her allogeneic stem cell transplantation, we compared the whole exome profiles from between diagnosis and relapse. We normalised the variant allele frequencies of the significant variants to the germline *RUNX1* mutation and thus we were able to directly assess the pattern of clonal evolution at relapse. Our data suggested that relapse in K.II.4 was triggered by expansion of the *JAK2* V617F mutated subclone, whilst the dominant T1 *U2AF2* mutation, now appeared to represent a subclonal variant.

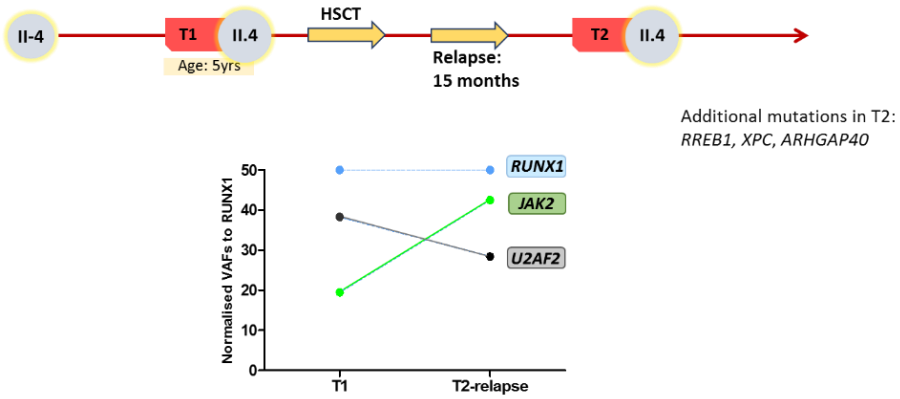

Figure 5. By comparing the diagnostic (T1) and relapse (T2) samples of patient II-4 we suggest that the relapse in this patient was triggered by the expansion of the *JAK2* V617F mutated subclone

Summary

By whole exome sequencing analysis of this *RUNX1* mutated pedigree we have

- Identified molecular addiction to the JAK-STAT pathway in all individuals characterized by aggressive disease, representing an important second hit which could be an indicator of leukemic transformation.
- Revealed the clonal architecture and dynamics of acquired mutations in this pedigree.
- Identified *CDC27* mutations as cooperating lesions in the germline *RUNX1* mutated setting.

We believe that the cases of familial leukemia and dysplasia represent important models for stepwise leukemogenesis and provide insights into molecular cooperating mechanisms. Better understanding and characterization of these cases has important implications for asymptomatic carriers with respect to bone marrow donor selections. Further insights into molecular cooperating mechanisms may also highlight novel directions for targeted therapies.

In terms of the publication, inclusion and whole exome sequencing analysis of the relapse sample (T2, figure 5) delayed the submission of this manuscript as we wanted to complete the data with this important piece of information. However, the manuscript is now ready for submission with the data and figures presented above. Considering the high number of affected individuals in this pedigree, the molecular findings and the interest in connection with this work at international meetings (see bellow), we are submitting the manuscript to the leading journal of the field (Blood).

2.2. Development of diagnostic assays

As part of the aims of the research project, diagnostic assays for routine screening of all relevant predisposing variants described in genes found to be associated with familial MDS/AML were developed and optimized. These include mutation screening in the following genes: ***CEBPA, RUNX1, GATA2, ETV6, ANKRD26, SRP72*** and ***TERC/TERT***. These diagnostic assays are now available for the Hungarian Hematology community. In addition to these Sanger sequencing based assays, we have also developed a next generation sequencing (NGS) based gene panel (using the AmpliSeq approach) incorporating all relevant predisposing genes allowing simultaneous analysis of these variants in the clinical diagnostic setting.

2.3. Additional families identified during the research period

During the 24 months research period we tried to heighten the awareness of the clinicians in connection with the occurrence of these familial MDS/AML cases and we indeed did identify a number of additional Hungarian pedigrees with suspected familial component. These are presented in figures 6-8. The two pedigrees with 2 and 3 affected siblings were tested for all known germline mutations of all knows predisposing genes (*CEBPA, RUNX1, GATA2, ETV6, ANKRD26, SRP72* and *TERC/TERT*) and showed wild type genotypes. Therefore, these pedigrees most likely belong the 30% of cases where the germline predisposing gene variant is unknown yet. Samples from these families will be analysed using whole exome sequencing to identify novel germline predisposing genetic events in these pedigrees. Additionally, we have recently identified another pedigree with familial thrombocytopenia with seven affected members. The family members are currently being tested for the known germline mutations in genes listed above. Identification of novel additional pedigrees was somewhat slower as we expected, however the continuous and repeated communication lead to heightened awareness and identification of novel affected families, especially in the second year of the project. These will be continuously tested *i,* for the known predisposing genes, or *ii,* for the presence of novel predisposing variants using whole exome sequencing.

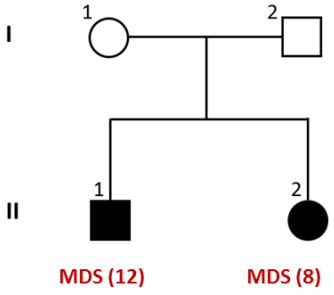

Figure 6. A novel pedigree with 2 siblings presenting with MDS. The numbers in parenthesis denote the age at diagnosis.

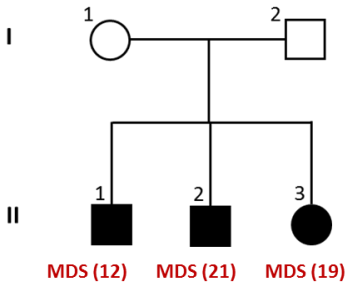

Figure 7. A novel pedigree with 3 siblings presenting with MDS. The numbers in parenthesis denote the age at diagnosis.

Interestingly, we also identified a family (from University of Debrecen) where identical twins were affected by acute lymphoblastic leukemia (ALL), with one of the sibling presenting with Philadelphia chromosome positive ALL, while the other sibling was diagnosed with Philadelphia chromosome negative

ALL ten years later (Figure 8). The samples from this family are currently being analysed by whole exome sequencing.

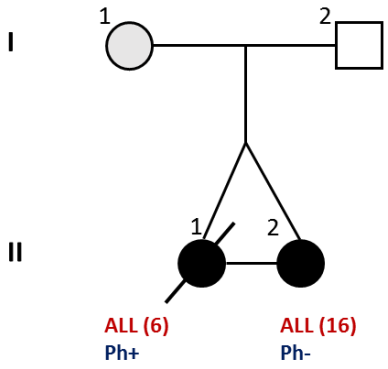

Figure 8. A novel pedigree with a twin couple where one the twins presented with a Philadelphia chromosome positive acute lymphocytic leukemia (ALL), while the other twin was diagnosed with a Philadelphia chromosome negative ALL ten years later.

2.4. International collaboration

Last year we became members of an International familial leukemia and myelodysplasia working group lead by Professor Fitzgibbon at the Barts Cancer Institute in London. This international group brings together experts of the field from the majority of the EU member countries and beyond providing a joint platform for collecting and characterizing these familial MDS/AML cases, generating international guidelines, ultimately contributing to better management of this special patient population (Figure 8). Any additional pedigrees identified in Hungary can be investigated within the frame of this working group.

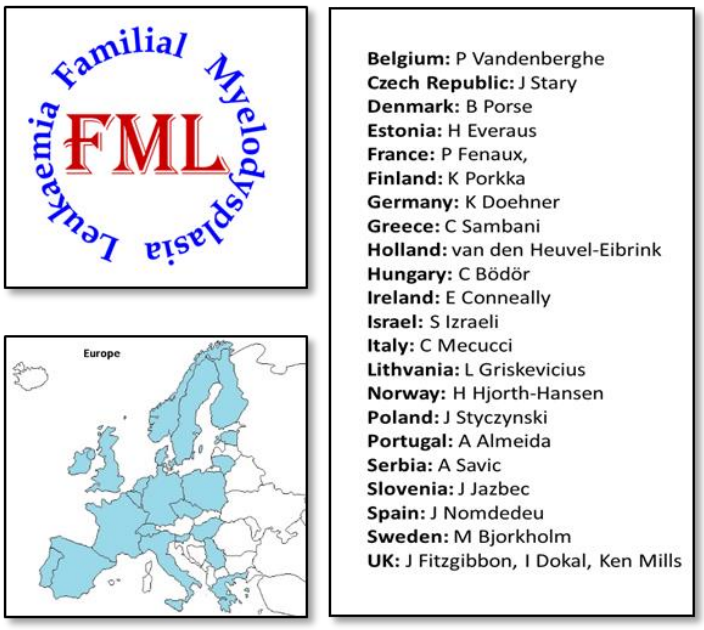

Figure n. The international *"familial leukemia and myelodysplasia-FML"* working group. Listed are the countries and representative experts from the individual countries.

3. Publications in connection with the research project

Peer reviewed articles *(with acknowledgement of the OTKA PD-108805 grant support)*

1. Disease evolution and outcomes in familial AML with germline *CEBPA* mutations. Tawana K, Wang J, Renneville A, **Bödör C**, Hills R, Loveday C, Savic A, Van Delft FW, Treleaven J, Georgiades P, Uglow E, Asou N, Uike N, Debeljak M, Jazbec J, Ancliff P, Gale R, Thomas X, Mialou V, Döhner K, Bullinger L, Mueller B, Pabst T, Stelljes M, Schlegelberger B, Wozniak E, Iqbal S, Okosun J, Araf S, Frank AK, Lauridsen FB, Porse B, Nerlov C, Owen C, Dokal I, Gribben J, Smith M, Preudhomme C, Chelala C, Cavenagh J, Fitzgibbon J. Blood. 2015, 126(10):1214-23. *IF: 10.452.*

2. The Molecular Pathogenesis of Acute Myeloid Leukemia. Tawana K, **Bödör C**, Cavenagh J, Jenner M and Fitzgibbon J. CML – Leukemia & Lymphoma. 2013, 21(3):67–77. http://www.remedicajournals.com/CML-Leukemia-and-Lymphoma/BrowseIssues/Volume-21-Issue-3/Article-The-Molecular-Pathogenesis-of-Acute-Myeloid-Leukemia

3. Clinical and genetic background of familial myelodysplasia and acute myeloid leukemia (Familiáris myelodiszpláziás szindróma és akut myeloid leukémia klinikai és genetikai háttere). **Kiraly PA**, Kallay K, Benyo G, Marosvári D, Csomor J, **Bödör C.** Submitted for publication to the Hungarian medical journal (“Orvosi hetilap”).

4. Convergence of somatic mutations within the JAK-STAT signalling pathway in a novel runx1-mutated pedigree. Manuscript in preparation with first authorship of **Kiraly P** and last authorship of **Bödör C**.

Conference presentations

We presented our data at different national and international conferences with an aim to heighten awareness in connection with familial leukemia.

International and national meetings:

1. 20th Congress of the European Hematology Association (EHA), Vienna, June 2015, Oral presentation: Convergence of somatic mutations within the JAK-STAT signalling pathway in a novel runx1-mutated pedigree. Presenting author: **Bödör C.**
   **The EHA congress is the main congress in the field of hematology in Europe with only 7% of the abstracts selected for oral presentation. Our abstract also received a travel award from EHA.*

2. European School of Hematology (EHS) meeting: International Conference on AML "Molecular and translational" - Advances in the Biology and Treatment, Budapest, September 2015, Poster

presentation: Convergence of somatic mutations within the jak-stat signalling pathway in a novel *RUNX1*-mutated pedigree. Presenting author: **Bödör C**.

3. Semmelweis Symposium on molecular oncology, Budapest, September 2015, Poster presentation: Familial acute myeloid leukemia and myelodysplastic syndrome. Presenting author: **Kiraly P.** (PhD student working on the project)

4. Annual Conference of the Hungarian Society of Hematology and Transfusion (MHTT), Budapest, May 2015, Oral presentation: "A JAK-STAT jelpálya komponenseit érintő mutációk konvergens evolúciója egy új, öröklődő *RUNX1* mutációt hordozó családban-Hungarian". Presenting author: **Kiraly P.** (PhD student working on the project)

5. PhD student conference at Semmelweis University, Budapest, April 2015, Oral presentation, Presenting author: **Kiraly P.** (PhD student working on the project) (Best oral presentation award received)

Book chapters

The following two book chapters were published in a book on targeted diagnostics and targeted therapies in oncology in 2013. Their updated versions will contain now a part on familial MDS/AML as well with the acknowledgement of the present OTKA-PD grant. The new edition (4th) is in print now and expected to be published in 2016.

1. Bödör C, Gyermekkori daganatok: Hematológiai daganatok, In: Kopper L, Tímár J, Becságh P, Nagy Z (editors). Célzott diagnosztika és célzott terápia az onkológiában 3, Budapest: Semmelweis Kiadó, 2013. pp. 174-176. (ISBN: 978-963-331-300-8). [Hungarian].

2. Bödör C, Hematológiai daganatok, In: Kopper L, Tímár J, Becságh P, Nagy Z (editors.). Célzott diagnosztika és célzott terápia az onkológiában 3. Budapest: Semmelweis Kiadó, 2013. pp. 181-188. (ISBN: 978-963-331-300-8). [Hungarian].

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