PROJECT TITLE: ASSESSING THE COMBINED EFFECT OF MULTIPLE POLYMORPHISMS IN ORDER TO DEFINE THE GENETIC PREDISPOSITION TO MYELOPROLIFERATIVE NEOPLASMS

PROJECT CODE: PN-III-P1-1.1-PD-2016-1414

STAGE 2019: THE DEVELOPMENT AND IMPLEMENTATION OF REAL-TIME PCR TECHIQUES IN GENOTYPING A 5 SNPs PANEL IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA AND CONTROLS.

The aim of this stage (2019) included the following activity:

GENOTYPING A 5 SNPs PANEL IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA AND CONTROLS

Chronic myeloid leukemia (CML) in a classic myeloproliferative neoplasm characterized by the proliferation that originates from myeloid progenitors or precursors. CML was is the first cancer for which a genetic alteration has been described. The Philadelphia chromosome in patients with CML has been described for the first by Peter Nowell and David Hungerford in 1960. The two scientists observed that patients with CML had a shorter chromosome; initially they believed it was the chromosome 21 not 22 and they thought it was a deletion involved not a translocation. This is no wonder given the karyotyping techniques back then. Besides this, although a translocation is involved the amount of transferred genetic material is not equivalent: chromosome 9 gets a substantial amount whereas chromosome 22 receives only a small fragment, leaving it considerable shorter than normal. Only after another 13 years had the true nature of the Philadelphia chromosome been uncovered. In 1973 Rowley reported 9 patients suffering from CML, that had an addition to the chromosome 9 along side the deletion on chromosome 22. This is how the scientist

proves that a recurrent translocation is in fact involved, between chromosomes 9 and 22 (Rowley, 1973). Due to more modern karyotyping techniques, scientists could find out the exact breaking points of the two chromosomes, describing it as follows: t(9;22)(q34.1;q11.2).

Another 10 years had passed, so that the involvement of this translocation was understood; we are talking about a fusion of 2 genes: *ABL* on chromosome 9 and *BCR* on chromosome 22, the first time such an event was described in a human cancer. This BCR-ABL fusion brings a part of the ABL gene under the control of the promoter gene BCR, following an activation of the ABL tyrosine kinase which leads to a continuous signaling in the signaling pathways of myeloid precursors. The majority of patients are diagnosed during the chronic phase. Back in the days the disease would inevitably progress to an accelerated, blastic phase with high mortality rate and an overall survival of 2-3 years. Treatment was very limited. The appearance of Imatinib, the first tyrosine kinase inhibitor (TKI) marked a breakthrough in CML treatment and medicine. Imatinib competes with ATP while merging with the BCR-ABL hybrid protein, inhibiting it. This causes cellular death of the cells which carry the BCR-ABL mutation. TKIs are nowadays standard treatment in CML, being able even to cure the disease, notion named as TFR (treatment free remission). Further on, next generation TKIs appeared, like dasatinib, nilotinib or bosutinib changing the course of a once lethal disease.

A higher incidence of CML has been observed in relatives of patients, although the genetic basis of this phenomenon has not yet been as clearly understood as in case of other myelopriferative neoplasms like polycythemia vera (PV), essential thrombocythemia (TE) and primary myelofibrosis.

In the case of these last mentioned diseases three independent research groups have reported that the JAK2 46/1 haplotype is a major constitutional driver of MPNs, especially for JAK2 V617F positive patients (Kilpivaara et al, 2009; Jones et al, 2009; Olcaydu et al, 2009). 2010 has been the year when my research team and I, have replicated these results on a 149 Romanian patient group, by genotyping rs10974944 C>G SNPs, which mark the JAK2 46/1 haplotype through an own PCR-RFLP technique (Trifa et al, 2010a,b). This SNP was part of the Open Array panel applied in this project for testing 1000 patients and 400 controls.

Later, in 2014, the rs2736100 A>C TERT (telomerase) polymorphism, has been also described as a major driver of MPNs by a scientific research group from

deCODE Iceland (Oddsson et al, 2014; Jager et al, 2014). To mention is that the same polymorphism has initially been described as having a major role in lung cancer predisposition and later also in other solid cancers, especially gliomas. It seems that this particular polymorphism comes with an increased telomerase activity, respectively longer telomeres. In the general population, the presence of this polymorphism is associated with higher blood count of myeloid cells (erythrocytes, granulocytes, platelets). This polymorphism was also included in the Open Array panel applied in this project for testing 1000 patients and 400 controls.

Other studies from 2015 and 2016 show new polymorphisms in the context of genetic predisposition of BCR-ALB negative MPNs, in the following genes: MECOM, HBS1L/MYB, SH2B3, TET, ATM, CHEK2 (Tapper et al, 2015; Hinds et al, 2016).

Regarding CML, a study published in 2015 analyzed 16.561 polymorphisms in 1916 genes on 437 patients and 1.144 controls. The study identified 5 SNPs (rs14178, rs6651394, rs6668196, rs3777744, rs3768641) in the following genes: PSMB10, TNFRSF10D, PSMB2, PPARD, CYP26B1.

Results of 2019 stage: THE DEVELOPMENT AND IMPLEMENTATION OF REAL-TIME PCR TECHIQUES IN GENOTYPING A 5 SNPs PANEL IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA AND CONTROLS.

This stage contained many deliverables, as followed:

1. The contribution of the 16 analyzed SNPs in the development of BCR-ALB negative MPNs – a mathematic model regarding genetic predisposition in these diseases.

The contribution of the 16 SNPs genotyped in the last stage,

- JAK2 rs10974944
- TERT rs2736100
- MECOM rs2201862
- MECOM rs3851379
- HBS/MYB1L1 rs9376092

- THRB/RARB rs4858647
- SH2B3 rs3184504
- TET2 rs1548483
- CHEK2 rs555607708
- CHEK2 rs17879961
- ATM rs1800056
- GFIB rs621940

was statistically analyzed.

The JAK2 rs10974944 polymorphism, marker of the JAK2 46/1 haplotype, is the most important figure in genetic predisposition in PV, TE and PMF. Its weight lies in the risk score of genetic predisposition for developing these entities and its contribution is maximal in the development of JAK2 V617F positive diseases.

According to the results obtained in this study, the TERT rs2736100 polymorphism comes in second regarding genetic predisposition in PV, TE and PMF. In contrast to the JAK2 rs10974944 polymorphism, the TERT rs2736100 has an equal contribution in each of the three diseases, independent of their molecular marker, JAK2 V617F or CALR.

The two polymorphisms would explain over 70% of the attributable fraction of genetic variation in the development of chronic myeloproliferations. The MECOM rs2201862 polymorphism was associated with all MPN phenotypes and both molecular subtypes (JAK2 V617F and CALR positive), with a greater effect on the CALR positive patients. This polymorphism came in third in the mathematic model for prediction of the development of chronic myeloproliferations. SH2B3 rs3184504 and TET2 rs1548483 polymorphisms have been associated with MPNs, regardless of their phenotypic or molecular background, occupying the following positions after JAK2 rs10974944, TERT rs2736100 and MECOM rs2201862, in the mathematic model of MPN prediction. The ATM rs1800056 polymorphism is more frequent in patients with MPNs, regardless of their phenotypic or molecular background, with a low frequency in the population.

ATM rs1800056 polymorphism was seen more frequently in MPN patients, regardless of the phenotype or molecular subtype. However, it has a low frequency in the population.

The HBS1L-MYB rs9376092 polymorphism was positively associated only with ET (on behalf of JAK2 V617F and not CALR). This way we could only include

this polymorphism in the predictive mathematic model for ET, where it came in 4th, after JAK2 rs10974944, TERT rs2736100 and MECOM rs2201862.

The THRB-RARB rs4858647 polymorphism had only a weak correlation with PMF.

CHEK2 polymorphisms had a low frequency in the population analyzed in this study.

2. Oral presentations and posters at national and international conferences.

I was an invited speaker at the MPN&MPNr-EuroNet Fourteenth Meeting, held in Belgrade from the 8th to the 10th of May of this year. My presentation included my research activity regarding genetic predisposition of BCR-ABL negative myeloproliferative neoplasms and partial results obtained during this project.

I have participated at The 12th International Congress on Myeloproliferative Neoplasms, held in New York, 24-25th of October, at the European Hematology Conference in Amsterdam in June 2019 and at the Focus on MPN&MDS meeting in Belgrade in September 2019.

3. Elaborating a manuscript in order to be published in a famous hematology paper.

We have elaborated a manuscript based on the contribution of SH2B3 rs3184504 and TET2 rs1548483 polymorphisms in the development of MPN.

4. Genotyping protocols for the 5 SNPs panels for patients with CML and controls.

We elaborated individual protocols for genotyping each of the following SNPs: rs14178, rs6651394, rs6668196, rs3777744, rs3768641, in the next mentioned genes: PSMB10, TNFRSF10D, PSMB2, PPARD, CYP26B1. We used real-time PCR and Taqman probes for the genotyping of each SNP.

Genotyping these polymorphisms is based on the TaqMan chemistry, using specific hydrolysis probes for each SNP. Each SNP assay contains a mix of two probes, one for each specific allele and another for the extern primers used in the amplification of the SNPs containing DNA fragments, basically forming multiplex real-time PCR type reactions.

Each of the two probes is marked with a distinct fluorochrome. The fluorochromes are masked by a quencher and can only be freed if the probe hybridizes with the targeted DNA. The wild-type allele is marked with the FAM fluorochrome, while the variant allele is marked with the VIC fluorochrome in all SNPs of the Open Array panel. The genotyping mix uses ROX as a passive reference stain.

The hybridization process leads to the hydrolysis of the probe, the fluorochrome thus being freed and excited. The emitted light signal will be quantified after each amplification cycle. The quantity of the emitted fluorescence is proportional with the number of targeted DNA sequences matching each specific probe.

The fluorescent signal is converted into a graphic signal in shape of amplification curves (one for each allele). In this sense, homozygotes have one amplification curve, specific to the wild-type or variant allele, whereas homozygotes have both amplification curves. In the end the results appear as an amplification plot, grouped in three clusters for each SNP: wild-type homozygotes, heterozygotes, variant homozygotes. The genotyping soft has an auto-call option which generates automatically the genotype of each sample, if all steps have been followed optimally.

5. Genotype distribution and allele frequencies for the 5 analyzed SNPs in patients with CML and controls

We have genotyped the mentioned 5 SNPs in 250 CML patients and 200 controls.

In conclusion:

- we have fulfilled 100% the aim of the activity of the 2019 stage: **GENOTYPING A 5 SNPs PANEL IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA AND CONTROLS**

Also, we have accomplished all 100% deliverables proposed in the work plan for the 2018 stage, as following:

1. The contribution of the 16 analyzed SNPs in the development of BCR-ALB negative MPNs – a mathematic model regarding genetic predisposition in these diseases.

2. Oral presentations and posters at national and international conferences.
3. Elaborating a manuscript in order to be published in a famous hematology paper.
4. Protocols for genotyping the 5 SNPs panels for patients with CML and controls
5. Genotype distribution and allele frequencies for the 5 analyzed SNPs in patients with CML and controls

Following, the results obtained after genotyping the specific 5 SNPs in patients with CML, will be analyzed in order to build a mathematical model for the genetic predisposition of CML.

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