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13TH SYMPOSIUM ON ADVANCES IN MOLECULAR HEMATOLOGY

GAB1 AS NOVEL THERAPEUTIC TARGET IN B LYMPHOID AND MYELOID MALIGNANCIES

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Introduction: Increased GAB1 levels have been described in several hematological malignancies and are associated with unfavorable prognosis (Mraz et al., Blood, 2014; Juric et al., JCO, 2007; Lenz et al., NEJM, 2008). GAB1 integrates and amplifies signals from cytokine and BCR receptors (van der Voort et al., JEM, 2000; Mraz et al., Blood, 2014), regulates cell migration/adhesion, and contributes to the adaptation of malignant B cells to BCR inhibitors (Seda et al., Blood, 2021). We tested if GAB1 targeting with novel inhibitors represents a potential therapeutic approach in hematological malignancies.

Methods: The role of GAB1 was tested using *GAB1* gene editing (Crispr/Cas9), siRNA or *GAB1* over-expression (pcDNA3.1), and two novel inhibitors (GAB1-001/4). GAB1 interaction partners were identified by co-IP and LC-mass-spectrometry.

Results: GAB1 downregulation by siRNA/knock-out impaired the migration of malignant B cells towards chemo-attractants such as SDF1, CXCL13, or conditioned media produced by bone marrow stromal cells (all $P < 0.05$). GAB1 downregulation/knock-out also impaired the activity of AKT and ERK in malignant myeloid cells and B cells. Moreover, GAB1 knock-out significantly impaired the proliferative capacity of MEC1 cells in the NSG mice ($P < 0.01$). The co-IP experiment revealed that GAB1 interacts with proteins involved in migration and cell signaling (GRB2, ACTR3, IP3R, GRAP2). GAB1 inhibitors (4 hours) impaired migration and BCR signaling in primary CLL cells, and at 48 hours induced primary CLL cells apoptosis (specific apoptosis: 25%; $N = 14$; $P = 0.003$). GAB1 inhibitors also induced significant apoptosis ($P < 0.05$, 48 hours) in 10 cell lines from various malignancies (% of induced apoptosis indicated in brackets): MCL (27%; JeKo), BL (20%, Ramos), DLBCL (30%, DOHH2), FL (17%, WSU-NHL), CLL (18%; MEC1), SLL (19%, OciLy5), AML (49%, 33%, 41% for ML2, OciAML3, MOLM13, respectively), and ALL (13%, Granta-452). The combination of GAB1 inhibitors and BTK-inhibitor ibrutinib exhibited a synergistic effect ($P < 0.05$) on cell apoptosis in primary CLL (induced apoptosis: 33% in combination vs. 25% for GAB1 inhibitor alone; $N = 14$), MEC1 (apoptosis: 26 vs. 18%), JeKo (apoptosis: 69 vs. 27%), ML2 (apoptosis: 63 vs. 49%), and OciLy5 (apoptosis: 32 vs. 19%) cell lines.

Conclusion: We have described the role of GAB1 in the proliferative fitness of various hematological malignancies. Novel GAB1 inhibitors impair cellular signaling, induce apoptosis and represent a potential therapeutic strategy in multiple hematological malignancies.

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CELL FREE DNA ANALYSIS – CURRENT INSIGHTS AND FIRST EXPERIENCES IN HODGKIN LYMPHOMA

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Circulating cell-free DNA (cfDNA) consists of small fragments of double stranded DNA released from cells undergoing apoptosis or necrosis into the peripheral blood. First it was mentioned in 1948, nowadays it is a widely used biomarker in prenatal diagnostics and in the last few years it has spread as a non-invasive technic called liquid biopsy also into the cancer diagnostics. A detectable amount of cell tumour DNA (ctDNA) released by apoptotic tumour cells mirrors the cancer genome. Screening of ctDNA fraction in cfDNA by next generation sequencing (NGS) can reveal clinically significant mutations and genetic variants associated with tumour. This provides an important advantage especially in lymphomas with problematic localisation and difficult availability of tumour material. The residual level of mutation detected in the time of diagnosis can be further followed by digital PCR (dPCR). dPCR is ultra-sensitive low-cost method which may improve monitoring and treatment adjustment in real time. Beside the MRD monitoring by dPCR, also the concentration and fragmentation of cfDNA itself seems to reflect the response to the chemotherapy. Nevertheless, despite big efforts to maximize the utilization of cfDNA as a biomarker for diagnosis, treatment and MRD monitoring, there are still significant limitations, e.g. low concentrations, short turnaround in plasma etc. We will present our first experiences with cfDNA-based approach in Hodgkin lymphomas including obtained NGS and dPCR data.

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DETECTION OF NUMERICAL AND STRUCTURAL GENOMIC ABERRATIONS FROM FFPE TISSUES: CHALLENGES AND PITFALLS

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The analysis of numerical and structural genomic aberrations in genes that have predictive/prognostic value is essential for clinical decision-making, not only in haemato-oncology. However, genetic diagnostics from formalin-fixed paraffin-embedded (FFPE) tissue samples is challenging, depending on the input material, storage, DNA/RNA extraction method and sample post-processing. In this study, we compared different DNA isolation methods from FFPE samples, evaluated the suitability of obtained DNA for detection by next-generation sequencing (NGS) and digital PCR (dPCR). Data on mutations and deletions of the *TP53* gene, a predictive biomarker in many haematological malignancies, were compared with genetic analysis of DNA isolated from peripheral blood and bone marrow. In addition to an overview of preanalytics of fixed tissue samples, results of various DNA extraction and sample processing methods will be presented, including DNA quality metrics and results and quality metrics of NGS and dPCR analysis.

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METHYLATION PATTERN AS A BIOMARKER FOR HEMATO-ONCOLOGICAL MALIGNANCIES

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Along with the promoter methylation and CpG rich regions across the whole genome, the methylation status of distant enhancers has been shown to be a powerful determinant of cell-state and cancer. The lecture will summarise the recent availability of long reads that report on the methylation status of enhancer-promoter pairs on the same molecule, the joint effects of multiple enhancers per promoter and the detection of methylation pattern on cell free DNA and genomic DNA in cancer. Moreover, the perspectives of methylation analysis for screening and follow-up of patients with haematological malignancies will be discussed.

LIQUID BIOPSIES IN MULTIPLE MYELOMA

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Multiple myeloma (MM) is the second most common hematological malignancy. It is characterized by malignant plasma cell infiltration of the bone marrow and so-called CRAB symptoms (hypercalcemia, renal failure, anemia and bone lesions). The diagnostic gold standard is bone marrow biopsy. However, collection of bone marrow is invasive, painful and occasionally can lead to false-negative results due to single-site collection or complication of patient's condition. That is why liquid biopsies are becoming more interesting. They provide minimally invasive alternative to diagnosis, treatment monitoring and MRD monitoring from various body fluids. Moreover, as there are subclones of plasma cells found outside of the bone marrow, liquid biopsies are more and more important in diseases such as multiple myeloma.

In MM, circulating plasma cells (cPC) have been long known to be connected to worse prognosis of MM patients. However, there are other molecules, such as cell-free DNA, circulating microRNA and others found in peripheral blood or urine that may become new diagnostic and prognostic markers.

This study will summarize different approaches and studies of liquid biopsies in MM.

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NOVEL 5'UTR EPO MUTATION AUGMENTS INTERACTION WITH HIF2 AND CAUSES AUTOSOMAL DOMINANT ERYTHROCYTOSIS

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We studied 10 affected and 11 non affected relatives of a five generation kindred with autosomal dominant familial erythrocytosis. We have excluded other known inherited forms of erythrocytosis. i.e., mutations of *globin*, the 2,3 DPG generating *PBGM* gene causing increased Hb/O2 affinity (low p50), gain-of-function mutations of erythropoietin receptor (*EPOR*), germ-line *JAK2* mutations, and hypoxia inducible factor 2A (HIF2-A*(EPAS1)), PHD2(EGLN1),* and *VHL* mutations associated with augmented oxygen-sensing pathway. Those affected family members had moderately increased erythropoietin (*EPO*) levels, no splenomegaly, normal leukocyte and platelet numbers and normal p50 (presented at this mtg, *Blood*. 2003;102,162b). We sequenced whole exomes and adjacent portions of introns of two affected individuals and found a novel heterozygous 5`UTR *EPO* variant with change -136 nt upstream from the *ATG EPO*

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initiation site (NG_021471 –136 G>A). This variant segregated with the erythrocytosis phenotype in 15 relatives examined: the 7 affected subjects were heterozygous for this variant and the 8 unaffected were negative, suggesting its causative role in erythrocytosis (presented at this mtg, *Blood*. 2013;122,950).

Other authors (NEJM 2018; 378:924) reported a variant of autosomal dominant familial erythrocytosis with a different *EPO* mutation: a single-nucleotide deletion (c.32delG) in exon 2 of the *EPO* gene causing a frameshift and alternative *EPO* mRNA transcripts, leading to increased production of functional EPO protein with shortened signal peptide and a novel N-terminus as cause of their familial erythrocytosis.

In order to characterize function of our 5`UTR *EPO* variant, we introduced it into the EPO producing human hepatoma cell line Hep3B using CRISPR/Cas9 editing system by homologous recombination with single-stranded donor oligonucleotides. The targeted cells were sorted in 96 well plates (20 cells per well) and then each well tested for presence of -136 G>A variant by allele-specific PCR. We identified 3 heterozygous Hep3B for *EPO*$^{-136\ G>A}$; the second round of targeting generated homozygous Hep3B clones. The *EPO* mRNA of *homozygous* recombinants was greatly increased and detected even in normoxia, unlike non-edited Hep3B cells. No alternative *EPO* mRNA transcripts were detected in the engineered and non-edited Hep3B cells. To emulate human phenotype, the supernatants of cultured three *heterozygous EPO*$^{-136\ G>A}$ Hep3B single-cell derived clones and controls in normoxic and hypoxic conditions were used to detect production of EPO. The hypoxic treatment increased ~2x the production of EPO from edited clones compared to non-edited Hep3B cells. The secreted EPO from *heterozygous EPO*$^{-136\ G>A}$ Hep3B clones supported growth of EPO-dependent BaF3-EPOR cells more than supernatants from non--edited Hep3B cells. We then measured *EPO* transcript levels in Hep3B with *EPO*$^{-136\ G>A}$ and parental Hep3B cell lines in normoxia and hypoxia. The hypoxia increased the relative expression of *EPO*$^{-136\ G>A}$ allele in all three targeted *heterozygous EPO*$^{-136\ G>A}$ Hep3B cell lines. The *EPO* gene promoter was largely unmethylated in both wild and mutated clones. To evaluate activity of this mutant *EPO* promoter, we sub-cloned wild and mutated 5`UTR *EPO* sequence upstream of luciferase reporter gene and transfected them into two EPO producing cell lines – Hep3B, Hep2G. The mutant significantly increased activity of the reporter. To study the interaction of *EPO*$^{-136\ G>A}$ mutant promoter with HIF2 (principal transcription factor regulating *EPO*), we co-transfected *EPO*-luc reporter with HIF2-A expression plasmid. The activity of reporter with mutated *EPO*$^{-136\ G>A}$ was further increased in these cells with augmented HIF2 levels. Indeed, alignment tools predicted the *EPO*$^{-136\ G>A}$ genomic region as putative HIF2 binding site. This suggests that mutated 5`UTR of *EPO* augments interaction with HIF2, leading to increase production of EPO. Chromatin immunoprecipitation experiments are ongoing to model the transcriptional regulatory network accounting for augmented transcriptional regulation of this 5`UTR *EPO* gene variant.

Here we report a novel mechanism of inherited erythrocytosis caused by *increased transcription* of mutated 5`UTR of *EPO*.

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PRIMARY IMMUNE THROMBOCYTOPENIA (ITP): UPDATE ON GENETICS AND MANAGEMENT

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Primary ITP is a complex disorder affecting both adults and children. In common with the other 80 or 90 known autoimmune diseases, ITP involves a variety of factors including genetic, environmental, and others. There has been a lack of basic and genetic research in ITP to determine the underlying process is but there appears to be broadly two groups, those involving a B cell type response and those involving a T cell response. There will doubtless be many other subtypes of ITP which we have not yet elucidated.

The genetics of ITP is not Mendelian and it is likely that there are many genes involved in the pathogenesis. These genes are likely to involve components of the immune system, cytokines, receptors and many other as yet unidentified molecules which collectively predisposed to ITP. We have good evidence for a genetic component in autoimmune diseases from other diseases such as thyroid disease and the fact that autoimmune disease often runs in families which is likely to imply a genetic link. However, the concordance between monozygotic twins is not 100% so this means that genetics is not the sole driver of autoimmunity. Single nucleotide polymorphism (SNP) studies in adult and paediatric ITP to date have been disappointing but have provided some useful information.

In my lecture I will discuss a number of factors which influence ITP including environmental factors as well as genetics. Having a better understanding of the genetics of any disorder may allow the development of specific targeted therapies. For ITP at present we know little about the role of genetics in the pathogenesis.

In terms of management of ITP, there has been a major shift away from immune suppression and the use of non-evidence based treatments with a huge increase in the use of thrombopoietin receptor agonists (TPO-RAs). This has been driven largely by the COVID-19 pandemic and avoidance of immune suppression. Data from studies show that the quality of life for patients is much improved when TPO-RAs are used compared to the other immune suppressing treatments.

THE ROLE OF T CELLS IN THE PATHOGENESIS OF CHILDHOOD IMMUNE THROMBOCYTOPENIA

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Immune thrombocytopenia (ITP) is the most common autoimmune bleeding disorder in children. Its prognosis is mostly benign; however, severe refractory disease remains diagnostic and therapeutic challenge. The low counts of platelets (platelet count < 100×10^9/L) are associated with their removal by two parallel platelet clearance mechanisms –

antibody mediated platelet clearance and lectin-carbohydrate mediated platelet clearance. The later pathway is initiated by platelet desialylation, which is the process where terminal sialic acids are cleaved from glycoproteins (GPs) on the platelet surface. The loss of sialic acids causes exposure of the penultimate β-galactose residues on GP glycans to hepatocytes-expressed asialoglycoprotein receptors (ASGPRs), also called Ashwell-Morell receptor. Thus desialylated platelets are captured and phagocytosed by hepatocytes. Over the past decades, platelet desialylation has been shown to be responsible for platelet clearance in conditions such as infection-related thrombocytopenia, and the clearance of senescent platelets. The aim of our project is to determine the platelet desialylation in individual stages of children ITP and relative to ITP therapy. We correlate the level of platelet desialylation with the individual subpopulations of T cells.

Our data show, that the ITP progression does not correlate with platelet desialylation, nevertheless the level of desilalylation correlates with the rate of surface bound IgM and IgA immunoglobulins. The platelet desialylation is not stable over the time, the long term observation of individual patients show changes in the rate of platelet desialylation. Further, the rate of platelets desialylation correlates with the blood level of cytotoxic T cells. The amount of Treg cells does not differ among individual ITP stages, however patients with high Tregs (> 12% of CD3 + CD4 + cells) exhibited lower platelets counts (< 30×10^9/L).

Better understanding of immune-mediated processes involved in ITP pathogenesis may reduce adverse effects of immunosuppressive therapy and considerably improve quality of life in patients with ITP.

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GENETIC DIAGNOSTICS IN CHILDHOOD IMMUNE THROMBOCYTOPENIA

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Inherited thrombocytopenia (ITP) includes a group of inherited disorders often associated with mutations in genes implicated in megakaryocyte differentiation and/or platelet formation and clearance. Despite advances of next-generation sequencing (NGS) diagnostics (targeted panels, whole exome and whole genome sequencing), the molecular mechanism underlying ITP in some patients remains unexplained. This may be due to the type and size of the structural variant(s), which are difficult to detect by traditional NGS methods. Here, we review the advantages of novel genetic approaches, such as whole-genome optical mapping and long-read NGS, for the diagnosis of ITP. As an example, we present a case report of an 8 years old girl diagnosed with ITP, in whom optical mapping revealed a 14.5 kbp deletion involving the IL4R gene previously associated with ITP (Takahashi N. et al., 2017). In this patient, additional 35 deletions (size range 0.5–132.2 kbp), 19 insertions (0.7–48.5 kbp) and one inversion (16.1 kbp) were found that did not involve genes previously associated with ITP. Our data highlights the use of novel genetic approaches based on the analysis of long weight DNA to complement NGS results for the genetic diagnosis of ITP.

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